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Enhancement of Peroxidase Production and Excretion from Horseradish Hairy Roots by Light, NaCl and Peroxidase-Adsorption *in Situ*

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Effects of light, NaCl and adsorption *in situ* on production and recovery of peroxidase from horseradish hairy root cell are reported in this paper. Illumination with fluorescent white light increased peroxidase content in the cell 2-fold. The treatment with NaCl stimulated excretion of peroxidase from the cell, and it was also subject to alteration in morphology without serious influence on proliferation. Adsorption of peroxidase from the extracellular medium with hydrophobic resin greatly enhanced the release of peroxidase. For a 43-day culture period, 70 U/ml of peroxidase was produced compared to 5 U/ml without special treatments. These results suggest that production and product recovery from plant cells can be greatly improved by application of the combined treatments.

Introduction

A major limitation in continuous production of proteins and secondary metabolites from plant cells is that desirable products are stored intracellularly. Several methods have been tested to overcome this limitation and to excrete products in the medium. These methods include temperature shift[1] and the use of agents for permeablizing[2,3]. Even if such strategies succeed in excreting products, they are usually detrimental to cell viability and overall productivity. Other strategies are required to improve overall yield productivity. Kim *et al*[4]. has reported that light plays an important role in product formation, but unfortunately light suppressed the secretion of secondary metabolites from plant cells.

To develop an effective production system, we have studied alternative means of stimulating production and excretion of a useful enzyme from horseradish (*Armoracia rusticana*). Peroxidase (EC 1. 11. 1. 7.) is present in higher plants, and widely used in the colorimetric analysis of biological materials. Horseradish peroxidase consists of more than 30 multiforms determined by isoelectric points[5,6]. Shannon *et al.*[7] isolated seven peroxidases among many isozymes from horseradish and characterized their properties. A study of peroxidase localization in the onion root indicated that this enzyme seemed to be present in the cell wall, in the plasmalemma, in the the Golgi apparatus cisternae and vesicles, in the endoplasmic reticulum and so on[8]. Despite many investigations of

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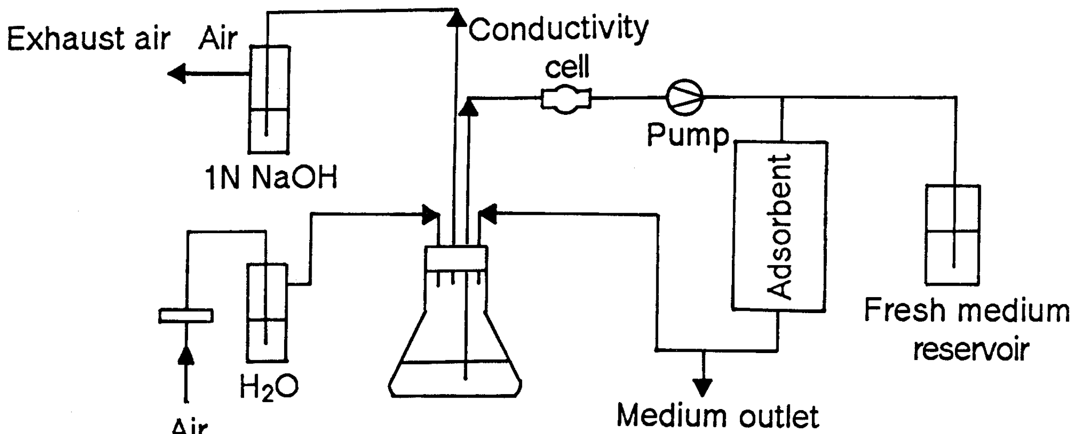

Fig. 1 Experimental set-up for operation of peroxidase adsorption.

peroxidase, the localization of the peroxidase isozymes is not completely assigned.

A previous study reported that small amounts of extracellular peroxidase from the hairy root are found in the medium and that low levels of peroxidase are excreted from the cells when the medium is supplemented with polypeptone[9].

In this paper, we have tested whether illumination with fluorescent white light, the addition of NaCl and product adsorption *in situ* would lead to a significant increase in extracellular peroxidase production, and we have examined the feasibility of using the technique to facilitate peroxidase recovery from horseradish hairy roots.

Materials and Methods

Plant cell culture and culture medium. Horseradish hairy root cells induced by the leaf-disc method[10] were used, and maintained by regular subculture in the dark at 3 week intervals, on hormone-free Murashige and Skoog (MS) medium supplemented with 2% (w/v) sucrose. For the culture experiments, the roots were grown for 14 days at 25°C and then were transferred aseptically into 100-ml Erlenmeyer flasks containing 40 ml aliquot of the required medium (2 g/l fresh weight of roots). Temperature during the cultivation was kept at 25°C. In the light condition, the period of illumination with fluorescent white light (ca. 3, 500 lux) was 14 hours a day. On-line estimation of cell concentration during the cultivation was based on conductometry as reported previously[11]. Dry cell mass was gravimetrically measured after drying the roots at 60°C for 24 hours.

Peroxidase assay. Peroxidase activity was determined at 25°C with *o*-aminophenol as a substrate[12]. For extraction of intracellular peroxidase, the hairy root cells were disrupted in 10 mM phosphate buffer (pH 6. 0) using a mortar with a pestle on ice. The supernatant was obtained by centrifugation (17, 000 × g, 10 min) at 4°C and used as a crude enzyme solution.

Configuration of adsorption system. The experimental reactor configuration is illustrated in **Fig. 1**. Cultivations were performed at 25°C in a flask with a working volume of 40 ml of MS medium containing 50 mM NaCl. These cultures were kept on a gyratory shaker (100 rpm) with the light condition of 14 hours light/10 hours dark cycle. Aeration was continuously supplied at a flow rate of 3 l/h. The hydrophobic adsorbent resin (DAIYAION HP-20, Mitsubishi Kasei Co., Ltd.) was parked in a column with a bed volume of 20 ml, and a working volume of 7 ml. The columns were equilibrated with 7 ml of MS medium supplemented with 2% sucrose prior to use. Adsorption operations were carried out by passing the reactor medium through the column, using a peristaltic

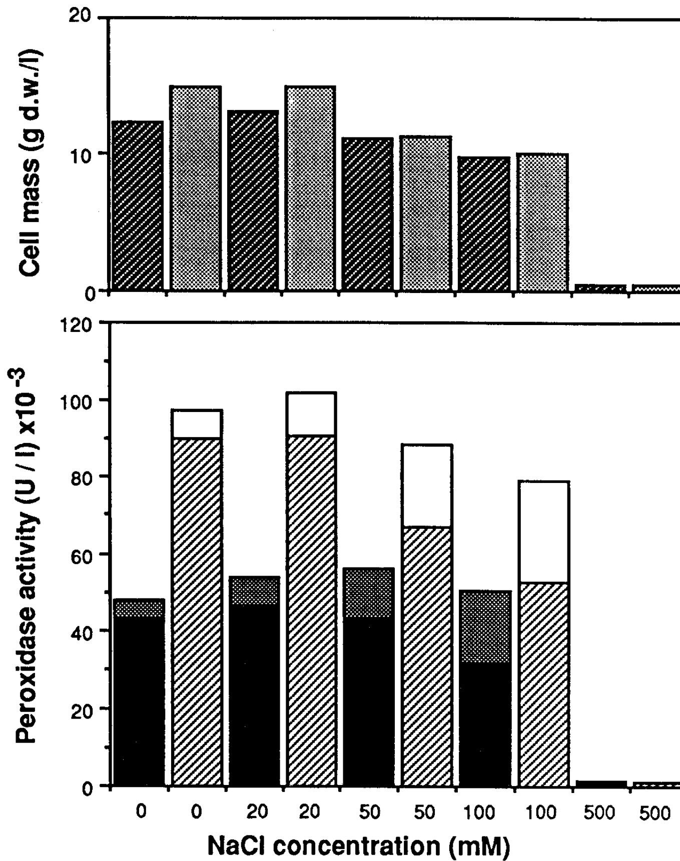

Fig. 2 Effect of NaCl and light on cell mass, peroxidase production and excretion from horseradish hairy roots at the 34th day. The upper half represents the cell mass grown in culture with various NaCl concentrations: ▒, in light; ▨ in dark. The lower half shows peroxidase activity of horseradish hairy roots: ▓, extracellular activity in dark-culture; ■, intracellular activity in dark-culture; □, extracellular activity in light-culture;▨, intracellular activity in light-culture.

pump. The initial 7 ml. composed mostly of the fresh medium, was drained out from this circuit and 40 ml of culture broth was recycled into the flask to reuse as medium . At the last circulation, the liquid in the column was flushed and substituted with fresh MS medium supplemented with 2% sucrose derived from the reservoir.

Scanning electron microscopy. For scanning electron microscopy, the cells were fixed with 2% glutaraldehyde in 10 mM phosphate buffer (pH 6. 0) for 2 h. After this, they were dehydrated with an increasing acetone series and then soaked in isoamyl acetate for 2 h. After critical-point drying and sputtering, micrographs were taken using a Hitachi S-570 scanning electron microscope.

Results

Effects of light and NaCl on cell growth and peroxidase production

Figure 2 provides growth data from horseradish hairy root in the dark and light conditions and also the effect of various concentrations of NaCl on cell mass and peroxidase production in the hairy root cultures. Illumination with the light enhanced final cell mass in the medium with low concentration of NaCl, while at higher NaCl concentrations (50-500 mM), the light effect on cell growth was not significant for the final cell mass. Light-grown culture increased peroxidase

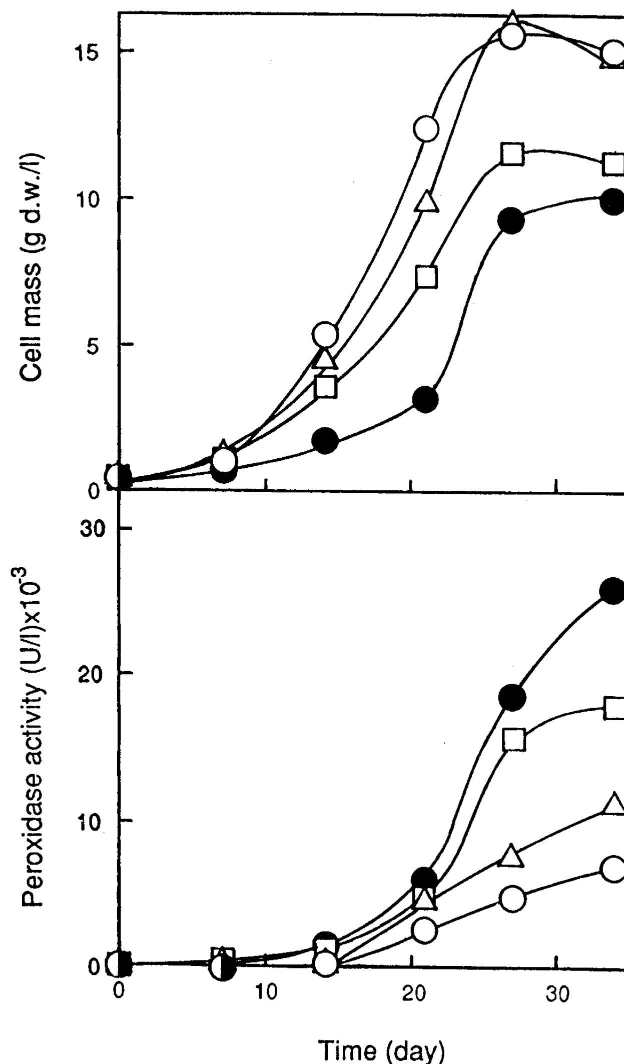

Fig. 3 Time course of cell growth and peroxidase production in culture medium under light condition. Horseradish hairy roots were grown in the medium supplemented with various NaCl concentrations: ○, 0 mM; △, 20 mM; □, 50 mM; ●, 100 mM

production approximately 2-fold compared with that of the dark-grown culture.

Maximum cell mass decreased with the addition of NaCl to the medium. The cell mass was almost the same in the presense of a low concentration of NaCl (20 mM) as without NaCl. On the other hand, the cell mass decreased when the NaCl concentration was above 50 mM and a severe growth inhibition was observed at higher NaCl concentration (500 mM). NaCl concentrations from 0 to 100 mM did not have much effect on the enhancement of peroxidase productivity. However, the addition of NaCl to the dark- and light-culture enhanced peroxidase excretion into the medium. In the light condition, peroxidase activity of the culture containing NaCl increased drastically compared with the culture without NaCl; approximately 2. 4-fold for the culture medium containing 50 mM NaCl and 3. 4-fold for the culture medium containing 100 mM NaCl at day 34.

Figure 3 represents the time course of growth and peroxidase productivity in a light-grown culture at various NaCl concentrations. The duration of the growth phase and the resulting dry-cell weight appeared to be dependent on the NaCl concentration in the medium. At the 34th day, cell

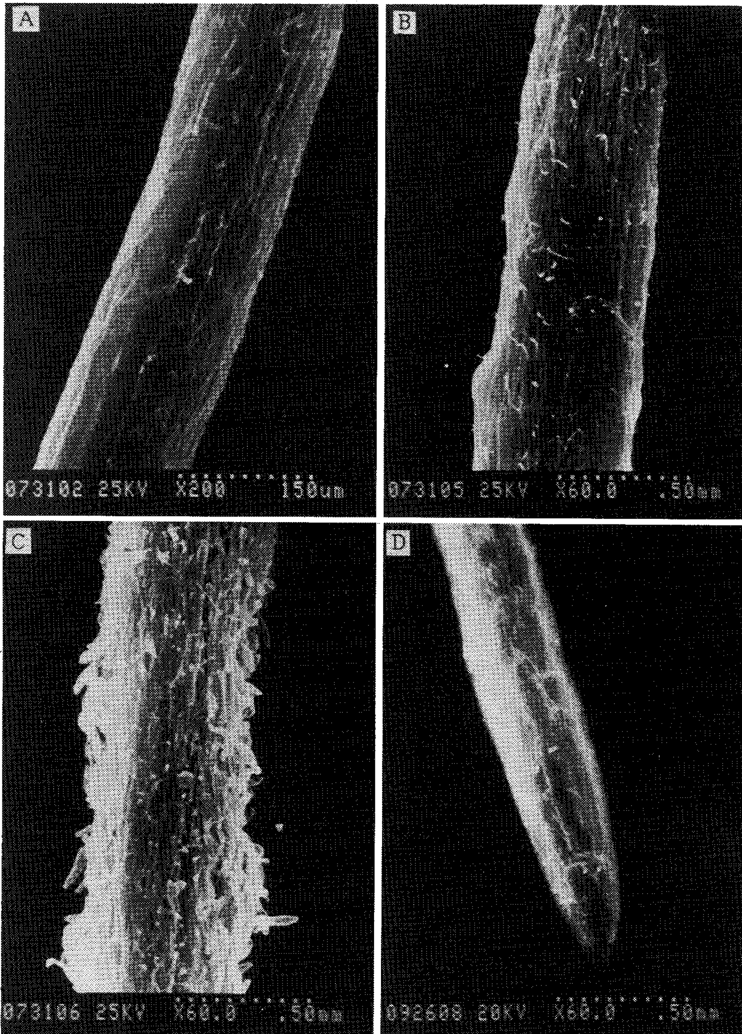

Fig. 4 Scanning electron micrographs of surface of the horseradish hairy roots grown for 30 days in various conditions. A, Dark-grown control hairy root; B, Light-grown hairy root; C and D, Light-grown hairy root treated with 50 mM NaCl.

concentrations in the light-grown culture without NaCl or with 20 mM NaCl were approximately 15 g dry-weight/l. At a high concentration of NaCl (100 mM), a lag phase was observed, and both growth rate and final cell mass decreased significantly.

The amount of peroxidase in the medium was almost the same during the first few days in the NaCl-treated root cultures and in the control culture. The amount of peroxidase in the medium without NaCl and with 20 mM NaCl increased constantly after 13 days, while the peroxidase productivity in the medium with 100 mM NaCl was accelerated. Peroxidase accumulation in the culture continued after the cells stopped growing.

Morphological analysis of hairy root

The surface of the hairy root at the last logarithmic growth phase was examined using a scanning electron microscope. **Figure 4A** shows hairy root in the dark condition. In this case, the surface appeared to be rather smooth. In the light condition, the root surface looked wavy and wrinkled (**Fig. 4B**). The diameter of the light-grown hairy root was approximately 3 times that of the

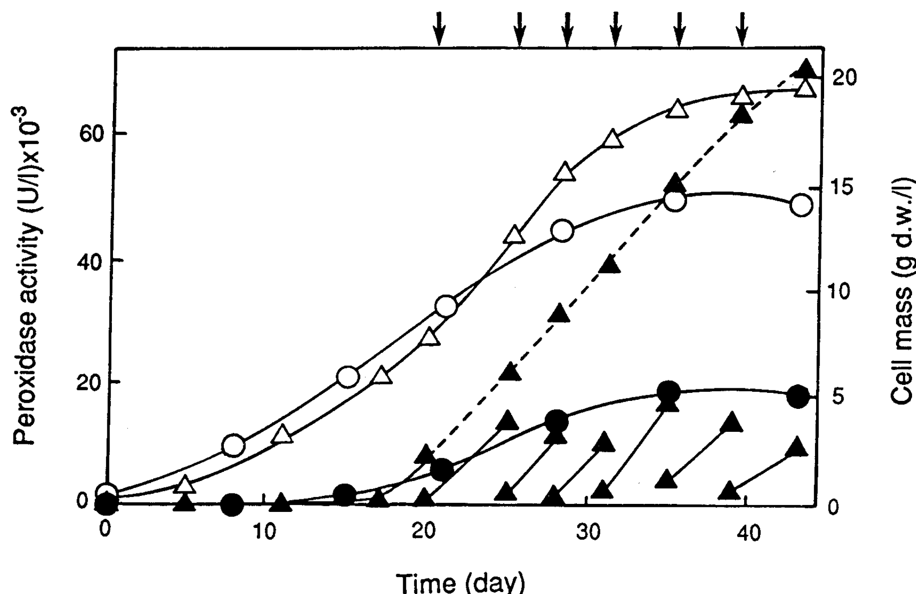

Fig. 5 Enhancement of extracellular peroxidase production on hairy root cultivation in combination with the resin adsorption. Peroxidase activities in the medium supplemented with 50 mM NaCl and without NaCl addition are indicated by closed triangles and circles, respectively. The total peroxidase activity estimated from each adsorption recovery is shown by a dotted line. Cell mass are denoted by open triangles (addition of 50 mM NaCl) and open circles (control). The adsorption operations are indicated by arrows.

dark-grown root. However, the light-grown and the dark-grown cell masses were comparable. Therefore the effect of light was likely to expand the cells or to expand the interspace between the cells. **Fig. 4C** shows the morphology of the hairy root grown in the light condition with 50 mM NaCl. Needle-like tissues were detached from the root surface. These detachments seem to contribute to extensive peroxidase excretion. Microscopic examination of the samples from different parts of the hairy root showed different microscopic morphologies. The detachments were not observed at the apical meristem on NaCl-treated roots (**Fig. 4D**). The older cells apart from the apical meristem tended to be more susceptible to NaCl stress.

Cultivation in combination with adsorption

Preliminary studies have shown that repeated batch cultivation improved growth rate and peroxidase productivity compared with batch cultivation (data not shown). To prevent peroxidase degradation during the culture, excreted peroxidase should be recovered from the medium periodically. Recovering peroxidase from the medium will also enhance the enzyme excretion by maintaining its concentration in the medium at a low level. As a consequence, we tested the cultivation in combination with the resin adsorption. The adsorption operations were carried out on each of several days. The results are shown in **Fig. 5**. The culture was performed with MS medium supplemented with 2% sucrose and 50 mM NaCl in the light condition. When peroxidase activity in the medium was kept below the maximum level of the batch culture, the productivity after 20 days retained above 2. 8 U/ml-day. Thus, the stepwise adsorption gave rise to a significant increase in the total amount of peroxidase produced. In addition, the adsorption caused slightly higher final cell mass. Final total peroxidase activity reached approximately the 70 U/ml level.

Discussion

The results in the present paper clearly show that the combined use of illumination with fluorescent white light, NaCl addition and the adsorption of excreted peroxidase leads to peroxidase overproduction. To release peroxidase into the medium, it is necessary to alter cell membrane and cell wall properties. In the previous report[9], we observed that polypeptone enhanced intracellular peroxidase activity, but unfortunately was not effective for excretion. Taking the polypeptone content into consideration, the enhanced production was attributed to NaCl present in the polypeptone. In this study, NaCl was found to induce peroxidase excretion into the medium, rather than to activate peroxidase synthesis by expression of peroxidase gene (Fig. 2). In contrast, light enhanced peroxidase content in the cell, but not peroxidase excretion.

Excretion of desirable products is sometimes difficult to achieve without damaging cells. Microscopic observations of the light-grown cultures supplemented with NaCl revealed that the hairy root surface in the late stage of the cultivation was altered (Fig. 4). This alteration, the tissue detachment on cell surface, seemed to be correlated with decreased cell viability, which results in leakage of intracellular peroxidase into the medium.

Some of the peroxidases are known to be associated with the cell wall[13]. Only specific proteins were excreated as the cells grew, which was measured by sodium dodecylsulfate-polyacrylamide gel electrophoresis (data not shown). Assuming that there was no extensive leakage of intracellular protein after the late growth phase, the detachments would make a large contribution to the cell-wall associated peroxidase excretion, since the amount of excreted peroxidase depended on the amount of cell surface area in contact with culture medium.

On the other hand, the morphology at the apical meristem of the roots looked normal, as shown in Fig. 4D, which indicates that the roots possessed good viability. It is unclear whether the cause of increased peroxidase activity in the medium was due to ionic strength or specific ions. Further work is now in progress to clarify the excretion mechanism.

Enhanced enzyme production was observed during cultivation in combination with the resin adsorption of peroxidase (Fig. 5). The adsorption seems to be useful to retain peroxidase production and to prevent peroxidase degradation. Metabolic by-products which inhibit the growth of root cells may also be adsorbed, since the final cell mass with the adsorption operations was 1.3-fold higher than that in the batch culture (Fig. 5). Nevertheless, the substrates required for the cell growth were not removed by the adsorption operation, which was checked by another experiment without the root cells. Regulation of peroxidase concentration at a low level in the medium by the adsorption led to the stimulation of enzyme excretion, thereby providing a large concentration gradient of peroxidase across the cellular membrane.

We have attempted to determine favorable conditions to elute the peroxidase from the resin. The best product recovery was 85% when we used 80% cold acetone as elution agent (data not shown). For the effective production of peroxidase from plat cell cultivation, we must develop a more efficient elution agent.

Acknowledgments

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References

1) Hilton, M. G., M. J. C. Rhodes, 1990. Appl Microbiol. Biotechnol., **33**: 132-138.
2) Brodelius, P., K. Nilsson, 1983. Eur. J. Appl. Microbiol. Biotechnol., **17**: 275-280.
3) Pu, H. T., R. Y. K. Yang, F. L. Saus, 1989. Biotechnol. Lett., **11**: 83-86.
4) Kim, D. I., H. Pedevsen, C. K. Chin, 1988. Biotechnol. Lett., **10**: 709-712.
5) Hoyle, M. C., 1977. Plant Physiol., **60**: 787-793.
6) Fujiyama, K., H. Takemura, S. Shibayama, K. Kobayashi, J. K. Choi, A. Shinmyo, M. Takano, Y. Yamada, H. Okada, 1988. Eur. J. Biochem., **173**: 681-684.
7) Shannon, L. M., E. Kay, J. Y. Lew, 1966. J. Biol. Chem., **241**: 2166-2172.
8) Goff, C. W., 1975. Am. J. Bot., **62**: 280-291.
9) Taya, M., A. Yoyama, R. Nomura, O. Kondo, T. Matsui, T. Kobayashi, 1989. J. Ferment. Bioeng., **67**: 31-34.
10) Noda, T., N. Tanaka, Y. Mano, S. Nabeshima, H. Ohkawa, C. Matsui, 1987. Plant Cell Rep., **6**: 283-286.
11) Taya, M., A. Yoyama, O. Kondo, T. Kobayashi, 1989, J. Chem. Eng. Jpn.. **22**: 89-94.
12) Yamada, Y., S. Kobayashi, K. Watanabe, U. Hayashi, Y. Yajima, H. Inoue, 1987. J. Chem. Tech. Biotechnol., **38**: 31-39.
13) Varner, J. E., L. S. Lin, 1989. Cell, **56**: 231-239.

《和文要約》

西洋ワサビ毛状根におけるペルオキシダーゼ分泌生産
(光，NaCl およびペルオキシダーゼの回収操作による分泌生産促進)

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西洋ワサビ毛状根からペルオキシダーゼを効率的に生産させる培養条件を検討した．光照射はペルオキシダーゼ含有量を 2 倍に高める効果があり，培地中への NaCl 添加は分泌を促進した．更に培地に 50 mM NaCl を添加して，分泌されるペルオキシダーゼを疎水性担体カラムにより回収しつつ明所において培養を行ったところ，培養 43 日後に回分培養に比べて 4 倍の総生産量を得ることができた．