특강 II - 1

Special Lecture II - 1

Lichen-derived Culture and Its Application

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Abstract

Methods of lichen-derived culture were spore- and thallus-derived cultures. Spore-derived culture was modified and thallus-derived culture was established for our two decades. We maintained about 500 lichen-derived cultures by using both methods. In recent several years, we have investigated to screen various pharmacological activities in lichen-derived cultures for the first time and found some activities in our cultures.

Introduction

Lichens are super organisms composed of fungal (mycobiont) and algal (photobiont) partners and have been used as medicines, dyes, perfumes, foods and drink stuffs since ancient times all over the world. Within the last two decades many pharmacologically active compounds have been isolated from lichens. However, mass harvesting of lichens as an industrial resource may lead to extinction of species. Therefore, if lichens are to be used in industrial applications, they must be cultured *in vitro*. In our laboratory, we succeeded in isolating, culturing and maintaining mycobionts as well as photobionts of about 500 culturesof lichens by using both methods of spore culture and thallus fragment culture (tissue culture, Yamamoto *et al.* 1985).

Lichensproduce characteristic secondary metabolites and have been used as crude drugs; recent works on their pharmacological activities were reviewed by Yamamoto *et al.* (1998a). A variety of products isolated from lichens shows a wide range of potentially useful biological activities. We believe that lichens have an original potentiality for novel biological activities, besides we have accumulated cultures of lichen symbionts that have even greater potential by genetic and biochemical manipulation. Therefore, we have been screening cultures derived from lichens for several kinds of biological activities.

Materials and Methods

Lichen-derived culture

A standard method for obtaining cultures of lichen mycobionts is to initiate them from their spores (Yoshimura *et al.* 2001). Considerable work has been undertaken since 1880 on the discharge and germination of lichen ascospores. Spore-derived culture established by Ahmadjian was modified by us and its procedure was described in the previous paper (Yamamoto *et al.* 1998b). Thallus-derived culture method for using thallus fragments, the lichen tissue culture method ("Yamamoto method") was described in previous paper (Yamamoto *et al.* 2001).

Pharmacological activities in lichen-derived cultures

Extracts of randomly selected lichen-derived cultures were prepared for tests of several pharmacological activities. For screening tests of growth inhibition, 15 animal-diseased bacteria (*Actinomyces pyrogenes*, *Bacillus subtilis*, *Bifidobacterium pseudolongum*, *Clostridium perfringens*, *Erysipelothrix shusiopathiae*, *Escherichia coli*, *Lactobacillus acidophilus*, *Micrococcus luteus*, *Pasteurella multocida*, *Propionibacterium acnes*, *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus faecalis*, *S. mutans* and *S. pyogenes*), five plant-diseased fungi (*Penicillium italicum*, *P. digitatum*, *Trichothecium roseum*, *Botrytis cinerea* and *Curvularia* sp.), and two wood decaying fungi (*Trametes versicolor* and *Fomitopsis palustris*) were used. The antibacterial test was carried out by using paper disk method (Yamamoto *et al.* 1993) and antifungal test was done by the co-culture method (Yamamoto *et al.* 1993 and Yamamoto *et al.* 2002). Epstein-Barr virus (EBV) was used for the screening of virus activation inhibition in latently-infected human B-lymphoblastoid cells (Raji cells). Cells were cultured with teleocidin B-4 as an activator and EBV was activated and produced early antigens. The Raji cells having early antigens were detected indirectly by fluorescence-antibody analysis (Yamamoto *et al.* 1994). For the screening of enzyme inhibition, mushroom tyrosinase (Wako Chemical Co., Ltd.) and monoamine oxidase prepared from mouse liver (Yamamoto *et al.* 2006) were used. For screening tests for anti-oxidant, SOD kits (Wako Chemical Co., Ltd.) and DPPH (Wako Chemical Co., Ltd.) were used.

Results and Discussion

Lichen-derived Cultures

Spore-derived culture - The effects of various new conditions on discharge and germination of ascospores were investigated (Yamamoto *et al.* 1998b). The spore discharge of 18 species collected from Japanese temperate areas in all seasons was tested and we found that the spore discharge of many lichens was influenced by seasons. All species stored for 3 or 6 months at -25 ℃ had the capability of spore discharge after collecting. Since Ahmadjian originally used MY medium to culture lichen mycobionts of *Cladonia*species, we often used this medium for many species. However, this medium is not suitable for mycobiont culture of cyanolichens. ***Thallus-derived culture (Lichen Tissue culture)*** - The spore culture method has several disadvantages. For example, apothecia may not discharge spores, spores do not always germinate *in vitro*, and not all species regularly produce apothecia. When we started to develop this method, we were concerned that our cultured thallus fragments would be contaminated. According to Ahmadjian's comment in the book "The Lichens", thallus-derived cultures are always contaminated by microorganisms present in the thallus. We found that we could reduce this problem by using carefully selected, very small thallus fragments (several hundred micrometers in size).

Pharmacological Activities in Lichen-derived Cultures

Growth inhibition of animal-diseased bacteria - The extract of a *Haematomma puniceum* mycobiont showed the highest activity of growth inhibition of many bacteria among 36 tested mycobionts. ***Growth inhibition of plant-diseased fungi*** - We tested the antifungal properties of lichen-derived cultures (Yamamoto *et al.* 1993). With the exception of *Thamnolia vermicularis*, which strongly inhibited the growth of *Botrytis cinerea* and *Curvularia* sp., none of tested mycobionts showed strong antifungal activity. ***Growth inhibition of wood decaying fungi*** - Many of 46 strains of lichen mycobionts growing on each of the three media had no apparent effect on the growth of the two wood decaying fungi (Yamamoto *et al.* 2002). Eight mycobionts, *Acarospora fuscata*, *Arthonia cinnabarina*, *Cladia*

aggregata, *Dibaeis absoluta*, *Haematomma puniceum*, *Ramalina exilis*, *Stereocaulon sorediiferum* and *Xanthoria elegans*, inhibited the growth of one or both of these two fungi. ***Tyrosinase inhibition*** - Tyrosinase, a kind of phenol oxidases, acts as a catalyst from tyrosine to dopaquinone via dopa in the biosynthetic pathway of melanin, therefore tyrosinase inhibitors such as ascorbate and arbutin have used as whitening agents in cosmetics. We screened inhibitory activity of tyrosinase in tissue cultures of lichens (Higuchi *et al.* 1993). The extract of *Hypogymnia physodes* tissue culture showed a highest inhibition activity (50 %) among them. ***Monoamine oxidase inhibition*** - Monoamine oxidase (MAO) is a key enzyme which plays an essential role in the turnover of biogenic amines. MAO inhibitors have been used for the treatment of depression, hypertension, etc. Screening test on MAO inhibition was performed for the extracts of 26 species of cultured mycobionts. The extract of a cultured mycobiont of *Graphis scripta* showed the strong inhibition. ***Inhibition of Epstein-Barr virus activation*** - Recently an assay for inhibition of tumor promotion has been developed that measures the inhibition of Epstein-Barr virus (EBV) activation. The higher relative index (Relative index = inhibition % of the extract/ inhibition % of the positive control, 3-oxoursolic acid) shows the stronger inhibitory effect of EBV activation. The *Nephromopsis ornata* culture showed the highest RI, 2.4 (Yamamoto *et al.* 1994). ***Superoxide dismutase-like activity*** - Speroxide anions protect the human body from attack by microorganisms, but excess superoxide anion injures tissues and causes tumors. Normally, the excess superoxide anion is scavenged by superoxide dismutase (SOD). The extract of *Bryoria furcellata* culture showed high scavenging activity for superoxide anion (SAS) (Yamamoto *et al.* 1993). ***Anti-oxidant activity with DPPH*** - DPPH is a radical compound, therefore if DPPH and radical scavenging substance are coexisted, DPPH would be changed to the non-radical compound. The *Graphis awaensis* and *Cladonia bellidiflora* cultures showed higher scavenging activity for DPPH radical than those of catechin and tocopherol.

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References

Higuchi M., Miura Y., Boohene J., Kinoshita Y., Yamamoto Y., Yoshimura I. & Yamada, Y. 1993. Inhibition of tyrosinase activity by cultured lichen tissues and bionts. Planta Medica 59: 253-255.

Kinoshita Y., Yamamoto Y., Yoshida E., Okuyama E. & Yamazaki M. 2006. Screening in natural thalli and cultured mycobionts of lichens for inhibitory activity of monoamine oxidase. Lichenology 5: 1-6.

Yamamoto Y., Hara K., Komine M., Doi S. & Takahashi K. 2002. Growth inhibition for wood decaying fungi by lichen mycobionts. Lichenology 1: 45-49.

Yamamoto Y., Kinoshita Y., Matsubara H., Kinoshita K., Koyama K., Takahashi K., Kurokawa T. & Yoshimura I. 1998a. Screening of biological activities and isolation of biological-active compounds from lichens. Recent Research Developments in Phytochemistry 2: 23-34.

Yamamoto Y., Kinoshita Y., Takahagi T., Kroken S., Kurokawa T. & Yoshimura I. 1998b. Factors affecting discharge and germination of lichen ascospores. J. Hattori Bot. Lab. (85): 267-278.

Yamamoto Y., Kinoshita Y. & Yoshimura I. 2001. Culture of thallus fragments and redifferentiation of lichens.. In Kranner I., Becknett R. & Varma A. (eds.) Methods in Lichenology: pp. 34-46. Springer-Verlag, Heiderberg.

Yamamoto Y., Miura Y., Kinoshita Y., Higuchi M., Yamada Y., Murakami A., Ohigashi H. & Koshimizu K. 1995. Screening tests of tissue cultures and thalli of lichens and some of their active constituents for inhibition of tumor promoter-induced Epstein-Barr virus activation. Chem. Pharm. Bull. 43: 1388-1390.

Yamamoto Y., Miura Y., Higuchi M., Kinoshita Y. & Yoshimura I. 1993. Using lichen tissue cultures in modern biology. Bryologist 96: 384-393.

Yamamoto Y., Mizuguchi R. & Yamada Y. 1985. Tissue cultures of *Usnea rubescens* and *Ramalina yasudae* and production of usnic acid in their cultures. Agric. Biol. Chem. 49: 3347-3348.

Yoshimura I., Yamamoto Y., Nakano T. & Finnie J. 2001. Isolation and culture of lichen photobionts and mycobionts. In Kranner I., Becknett R. & Varma A. (eds.) Methods in Lichenology: 3-33. Springer-Verlag, Heiderberg.

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