Phytochemistry, Vol. 22, No. 11, pp. 2451–2453, 1983.
Printed in Great Britain.

0031-9422/83 $3.00+0.00

INDUCTION OF SHIKONIN FORMATION BY AGAR IN *LITHOSPERMUM ERYTHRORHIZON* CELL SUSPENSION CULTURES

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(*Received* 14 *January* 1983)

Key Word Index—*Lithospermum erythrorhizon*; Boraginaceae; cell suspension cultures; biosynthesis; induction of biosynthesis; agar; agaropectin; acidic polysaccharides; naphthoquinone pigments; shikonin.

Abstract—Cultured cells of *Lithospermum erythrorhizon* capable of producing red naphthoquinone (shikonin) derivatives on Linsmaier–Skoog agar medium ceased synthesizing these quinones whenever they were grown in the same liquid medium in the absence of agar. However, when they were cultured in the liquid medium supplemented with a small amount of agar powder, they produced the same shikonin derivatives as those found in the callus cultures or the root of the original plant. The active component of agar was found to be agaropectin, an acidic polysaccharide. The pigment-producing variant clones selected on the medium solidified by agarose were shown to produce shikonin derivatives in the liquid medium without agar during long-term subculturings. These results suggest that the biosynthesis of a certain acidic polysaccharide is necessary for the *Lithospermum* cells to initiate shikonin biosynthesis.

INTRODUCTION

Although the two culture strains, M-18 and C-119, of *Lithospermum erythrorhizon* grown on Linsmaier-Skoog agar medium [1] containing 10^{-6} M IAA and 10^{-5} M kinetin are capable of producing large amounts of the same naphthoquinone pigments (shikonin derivatives) as those found in the root bark of the original plant [2–5], they ceased producing the pigments when grown as a cell suspension culture using the same liquid medium without agar. The present paper reports our new finding that the cell suspension cultures resume synthesizing shikonin derivatives if a small amount of agar powder is added to the liquid medium. This paper also deals with investigation on the active component of agar and the selection of variant clones capable of producing shikonin derivatives in the liquid medium without the addition of agar.

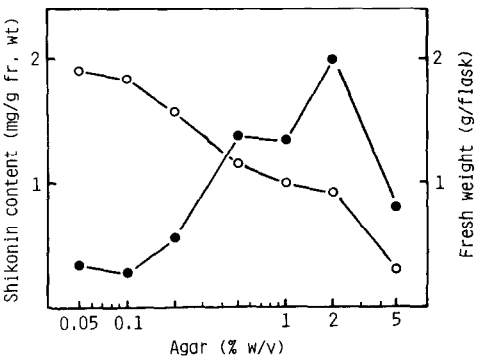

Fig. 1. Effects of agar on shikonin formation (●——●) and cell growth (○——○) in *Lithospermum* cell suspension cultures.

RESULTS AND DISCUSSION

Effect of agar on pigment formation

A high pigment-producing callus culture strain M-18 of *L. erythrorhizon*, which fails to produce shikonin pigments in Linsmaier–Skoog liquid medium without agar, was cultured in the same liquid medium supplemented with various amounts of agar powder. As shown in Fig. 1, pigment formation was induced even by the addition of a small amount of agar powder (0.05% w/v) and the pigment yield increased roughly in proportion to the amount of agar within the range 0.1–2%, although cell growth was inhibited as the amount was increased. TLC analysis indicated that these pigments consist of the same shikonin derivatives as those of the original callus cultures grown on the solid agar medium [2–5]. Furthermore, the shikonin content (2 mg/g fr. wt) of the cell suspension culture grown in liquid medium containing 2% agar was as high as that of the callus culture [2–5]. Similar results were obtained with cell suspension cultures of another high pigment-producing culture strain, C-119.

In a search for the active fraction of agar, a mixture of agar powder (1.5 g) and the liquid medium (30 ml) without cells, which had been shaken for 24 hr under the same condition as the cell suspension culture, was centrifuged at 10000 *g* for 20 min and the supernatant dialysed overnight against distilled water. Both the supernatant and the non-diffusate were found to be active in inducing pigment formation in cell suspension cultures. These results suggested that pigment formation was induced by a water-soluble polysaccharide.

Since agar consists of 70–80% agarose (a neutral polysaccharide) and 20–30% agaropectin (an acidic poly-saccharide) [6, 7], the agar (Wako Pure Chemical

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Industries Ltd., Japan) used in the present study was fractionated into agarose and agaropectin according to the method of Hjertén [8]. Culture experiments showed that pigment production could be induced by agaropectin, but not by agarose isolated from the agar. Similar results were obtained from experiments using a commercially available agarose (Sigma) and a sample of agaropectin provided by Dr. Fuse. Figure 2 shows that the optimal concentration of agaropectin for pigment synthesis was 0.2%, whereas the addition of 0.5% agaropectin reduced the cell growth as well as the pigment production.

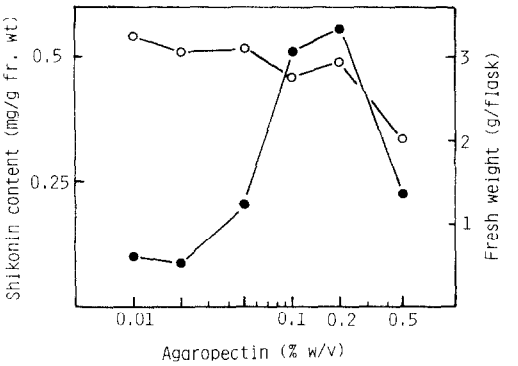

Fig. 2. Effects of agaropectin on shikonin formation (●——●) and cell growth (○——○) in *Lithospermum* cell suspension cultures.

It is known that the molecule of agaropectin is composed of D-galactose, 3,6-anhydro-L-galactose and small amounts of sulphate (1–5%), uronate (4–7%) and pyruvate (< 1%), whereas agarose is composed mainly of D-galactose and 3,6-anhydro-L-galactose [6, 7]. In view of these structural differences, as well as the fact that none of the chemical components of agaropectin is capable of inducing pigment production, it may be assumed that a polysaccharide having acidic functional groups in the molecule plays an important role in pigment formation by the cells. The possibility that the changes in shikonin production were due to a change in the viscosity of the medium seems unlikely, because the administration of agarose (0.01–0.5% w/v), which gives more viscosity to the medium than agaropectin of the same concentration, failed to induce pigment production in the cells. Therefore, several kinds of acidic polysaccharides were tested for their physiological activity. Sodium pectate was capable of inducing the production of shikonin derivatives as shown in Fig. 3. However, pectin was only slightly active and sodium chondroitin sulfate and sodium alginate were entirely inactive. Although the acidic polysaccharides are expected to have a cation-exchange function, the addition of such water-insoluble cation-exchange resins as CM-Sephadex, CM-Sepharose and SP-Sephadex to the liquid medium failed to induce pigment formation, indicating that the possible cation-exchange function of agaropectin or pectic acid was not critical.

Although nothing is known about the mechanism of action of agaropectin or pectic acid in connection with pigment formation in *Lithospermum* cells, these substances might combine with some proteins of the proto-

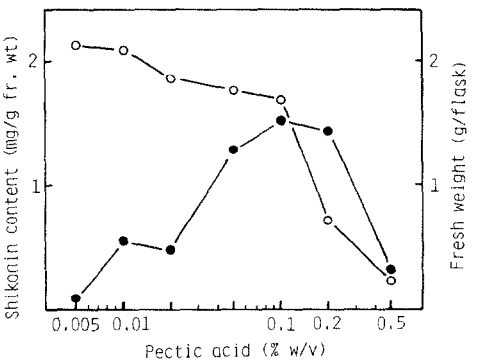

Fig. 3. Effects of pectic acid on shikonin formation (●——●) and cell growth (○——○) in *Lithospermum* cell suspension cultures.

plasmic membrane to bring about a drastic change in cell physiology. It is possible that the high pigment-producing culture strains used in the present study lack the ability to synthesize an acidic polysaccharide that is necessary, directly or indirectly, for the initiation of shikonin biosynthesis. In any case, it is interesting that the secondary metabolism in the cultured cells of *Lithospermum* is greatly influenced by agar, which has hitherto been considered a physiologically inactive substance.

Selection of shikonin-producing cell lines in the liquid medium

In an attempt to obtain a variant cell line which would not require agar for pigment production in the liquid medium, a mixture of single cells and small aggregates of cells prepared from a cell suspension culture of the strain M-18 were plated on a medium solidified with agarose in place of agar at a density of *ca* 5000 cells per 60 cm^2. Of a total of 8000 colonies formed, *ca* 200 were found to produce red pigments in the absence of agaropectin. These red colonies were separately transferred to the agarose medium in test-tubes. After 1 month of culture in the dark, 60 clones showing high pigmentation and good growth in the test-tubes were transferred to the liquid medium without agar to establish 47 cell suspension culture lines which were found to be capable of producing red pigments. The red pigments thus obtained were hydrolysed with 1 M KOH and each hydrolysate was shown to contain shikonin (TLC, UV-visible, mp and IR with an authentic sample).

A long-term testing on the stability of shikonin-producing capability in three selected cell lines (MA-21, MA-29 and MA-42) (Fig. 4) showed that their shikonin productivity was not lost during a 1-year period of subculturing, but that it increased gradually with time in two of the lines. The pattern of shikonin derivatives, as well as the shikonin contents, was comparable to those of the corresponding callus cultures. It seems likely that the selected cell lines possess the ability to synthesize a substance having the physiological activity analogous to agaropectin or pectic acid.

Recently, Fujita *et al.* [9, 10] have found that when the cells of the cell line M-18 of *L. erythrorhizon* were transferred from Linsmaier-Skoog liquid medium to White liquid medium [11], they began to produce a large

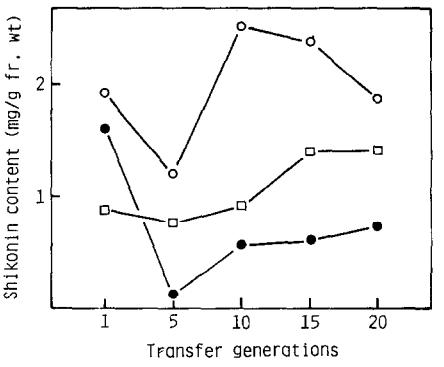

Fig. 4. Shikonin productivity of three selected cell lines (MA-21: ○——○, MA-29: □——□ and MA-42: ●——●) during successive subculturings in Linsmaier–Skoog liquid medium in the absence of agar.

quantity of shikonin derivatives in the absence of agar. The most important difference between the two basal media is the presence or absence of ammonium ion, which was found to repress the formation of shikonin derivatives in cell suspension cultures. Although the relationship between the effect of agaropectin and that of ammonium ion is not yet clear, the repression caused by ammonium ion could be removed by the addition of agaropectin through an unknown mechanism.

EXPERIMENTAL

Plant material and culture method. Callus cultures (M-18 and C-119) of *L. erythrorhizon* Sieb. et Zucc. derived in 1971 from the germinating seeds on Linsmaier–Skoog basal agar medium containing 10^{-6} M 2,4-D and 10^{-5} M kinetin were maintained on the same medium containing 10^{-6} M IAA and 10^{-5} M kinetin for 10 years at 25° in the dark. The cell suspension cultures were agitated on a reciprocal shaker at a speed of 100 strokes/min at 25° in the dark and were subcultured at 3-week intervals for more than 1 year.

Cell suspension cultures with various additions. Unless otherwise stated, the culture medium used in this study was Linsmaier–Skoog basal medium containing 10^{-6} M IAA and 10^{-5} M kinetin. In the expts for studying the effect of various additions on pigment formation in cell suspension cultures, agar and agaropectin were autoclaved before pouring into the sterilized liquid medium. Na pectate, Na chondroitin sulfate, pectin, Na alginate, CM-Sephadex, CM-Sepharose and SP-Sephadex were added to the medium before autoclaving. Na salts of glucuronic acid, galacturonic acid and pyruvic acid were added aseptically through a membrane filter (mesh size 0.45 μm). Each liquid medium was inoculated with cells and agitated under the same conditions as described above. The cells were harvested by filtration, dried and subjected to chemical analyses.

Analysis of red naphthoquinone pigments. The dried cells were extracted in a mortar with $CHCl_3$. The $CHCl_3$ extract was hydrolysed with 1 M KOH to yield a red residue, which was purified by repeated prep. TLC (silica gel, $CHCl_3$). The red compound isolated was identified as shikonin by comparison with an authentic sample. The quantitative estimation of the whole pigments in the cells was carried out spectrophotometrically according to the method described earlier [2, 3].

Preparation of agarose and agaropectin from agar. Agarose and agaropectin were prepared from commercial agar by the method described in ref. [8].

Acknowledgements—We are grateful to Dr. T. Fuse, Food Research Institute of Aichi Prefecture, for the generous gift of agaropectin. We also thank Miss A. Yuba for her excellent technical assistance. This work was supported in part by a Grant-in-Aid (No. 57101006) from the Ministry of Education, Science and Culture.

REFERENCES

1. Linsmaier, E. M. and Skoog, F. (1965) *Physiol. Plant.* **18**, 100.
2. Tabata, M., Mizukami, H., Hiraoak, N. and Konoshima, M. (1974) *Phytochemistry* **13**, 927.
3. Mizukami, H., Konoshima, M. and Tabata, M. (1977) *Phytochemistry* **16**, 1183.
4. Mizukami, H., Konoshima, M. and Tabata, M. (1978) *Phytochemistry* **17**, 95.
5. Tabata, M., Ogino, T., Yoshioka, K., Yoshikawa, N. and Hiraoka, N. (1978) in *Frontiers of Plant Tissue Culture* 1978 (Thorpe, T. A., ed.), p. 213. University of Calgary Press, Calgary.
6. Araki, C. (1956) *Bull. Chem. Soc. Jpn* **29**, 543.
7. Araki, C. (1958) in *Carbohydrate Chemistry of Substances of Biological Interest, 4th International Congress of Biochemistry Vienna*, Vol. 1, p. 15. Pergamon Press, London.
8. Hjertén, S. (1962) *Biochim. Biophys. Acta* **62**, 445.
9. Fujita, Y., Hara, Y., Suga, C. and Morimoto, T. (1981) *Plant Cell Rep.* **1**, 61.
10. Fujita, Y., Tabata, M., Nishi, A. and Yamada, Y. (1982) in *Plant Tissue Culture* 1982 (*Proceedings of the 5th International Congress of Plant Tissue and Cell Culture*) (Fujiwara, A., ed.), p. 399. Maruzen, Tokyo.
11. White, P. R. (1963) *The Cultivation of Animal and Plant Cells.* Ronald Press, New York.