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Regulation of shikonin production by glutamine in *Lithospermum erythrorhizon* cell cultures

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ABSTRACT

Amino acid analysis has shown that Lithospermum erythrorhizon cell suspension cultures which are unable to produce shikonin derivatives in LS medium containing ammonium accumulate a large quantity of glutamine, as compared with shikonin-producing cells cultured in the production medium M9 containing nitrate as the sole nitrogen source. The addition of glutamine to M9 medium proved to be strongly inhibitory to shikonin production. Furthermore, culture experiments using an inhibitor of glutaminase suggested that shikonin synthesis is not inhibited by ammonium released from glutamine but by glutamine itself. These findings indicate that the repression of shikonin synthesis occurs in close association with an accumulation of glutamine in cultured cells grown in a medium containing ammonium.

INTRODUCTION

Fujita et al. (1981 a) have found that cell suspension cultures of Lithospermum erythrorhizon, which are incapable of synthesizing shikonin derivatives in Linsmaier-Skoog (LS) medium (1965), produce these naphthoquinone pigments when grown in White's medium (1963). The comparison of the chemical components between the two media suggested that the biosynthesis of shikonin derivatives is inhibited largely by NH_4^+ which is contained in LS medium in the form of NH_4NO_3, whereas it is not inhibited by NO_3^- which is contained in White's medium as the sole nitrogen source. Actually, the addition of NH_4^+ to White's medium caused an inhibition of shikonin production (Fujita et al. 1981 a). However, the biochemical mechanism of the inhibitory effect of NH_4^+ on shikonin biosynthesis remains to be clarified. This paper presents evidence to indicate that the biosynthesis of shikonin is repressed by an accumulation of glutamine in Lithospermum cells cultured in liquid medium containing NH_4^+.

MATERIALS AND METHODS

Culture method Callus cultures (strain M18) of L. erythrorhizon were originally derived from the germinating seed and subcultured on LS agar medium containing 1 μM 3-indoleacetic acid (IAA) and 10 μM kinetin (Tabata et al. 1974, Mizukami et al. 1978). Cell suspension cultures initiated from the callus were grown in the same medium without agar on a reciprocal shaker (100 strokes/min) at 25° in the dark and subcultured at intervals of 3 weeks. For preparation of shikonin-producing cells, pigment-free cells that had been precultured in LS liquid medium for 3 weeks were transferred to the production medium M9 (Fujita et al. 1981 b), which contains no NH_4^+ but NO_3^- as the nitrogen source.

An ammonium-free LS medium was prepared by substituting KNO_3 for NH_4NO_3 without altering the total amount of nitrogen. In addition, the concentration of $CuSO_4$ $5H_2O$ was increased from 0.1 μM to 1.2 μM to enhance shikonin production. In culture experiments testing for the effect of NH_4^+, NH_4Cl (0 - 3 μM) was added to M9 medium. Amino acids and DON (6-diazo-5-oxo-norleucine, Sigma Chemicals) added to M9 medium were sterilized by milipore filtration. All the cultures were carried out in 100 ml-Erlenmeyer flasks containing 30 ml medium and an inoculum of 1.2 g cells. Cells were harvested 2 weeks after inoculation.

Analysis of free amino acids Cell suspension cultures at the early stationary growth stage were filtered through Miracloth to collect cells (10 g), which were immediately homogenized by a Potter-Elvehjem homogenizer with 8-fold volume of 0.02 N HCl. After centrifugation (3000 rpm, 10 min), the supernatant was filtered through a membrane filter to provide a sample for the analysis of free amino acids by HITACHI Amino Acid Analyser 835.

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RESULTS AND DISCUSSION

The production medium M9 contains 6.7 mM KNO_3 as the nitrogen source in contrast to LS medium that contains 20.6 mM NH_4NO_3 and 18.8 mM KNO_3. Shikonin production in cultured cells of strain M18 was induced by the substitution of NO_3^- for NH_4^+ in LS medium which contained a higher amount of Cu^{2+} (1.2 μM) to enhance the pigment production (Fig. 1-A). On the other hand, the addition of NH_4^+ (1 - 3 mM) to M9 medium almost completely inhibited shikonin production in strain M18 (Fig. 1-B). These results suggested that shikonin biosynthesis might be strongly influenced by nitrogen metabolism or amino acid metabolism. The quantitative analysis of free amino acids extracted from cultured cells at the early stationary growth stage showed that the shikonin-free cells cultured in LS medium accumulated much larger quantities of glutamine (Gln), arginine (Arg), glutamic acid (Glu), aspartic acid (Asp), asparagine (Asn) and NH_4^+ than the shikonin-producing cells cultured in M9 medium (Table 1).

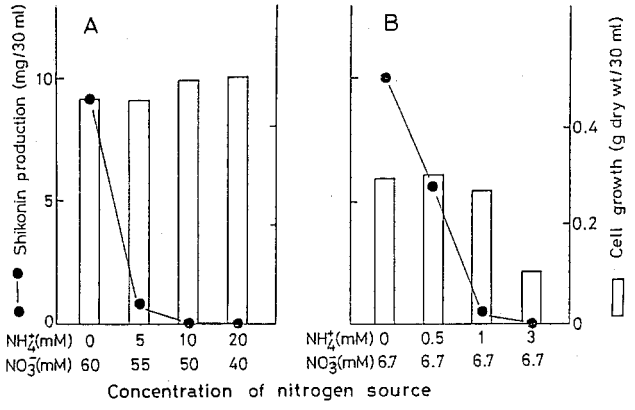

Fig. 1. Effects of NH_4^+ on cell growth and shikonin production in Lithospermum cell suspension cultures. A: The concentration of NH_4Cl was changed without altering the total nitrogen concentration in LS medium supplemented with 1.2 μM Cu^{2+}. Culture period: 3 weeks. B: NH_4Cl was added to M9 medium that originally contains 0.7 mM KNO_3 as the sole nitrogen source. Culture period: 2 weeks.

Fig. 2 shows changes in the contents of main amino acids, NH_4^+, and shikonin after the transfer of M18 cells from LS medium to M9 medium. Not only the level of NH_4^+ but also the contents of Gln, Arg and Glu in the cells rapidly decreased as the cells started synthesizing shikonin on the third day after inoculation. After 14 days of culture, the shikonin content increased to 4.2 % of dry wt, while the contents of the above amino acids decreased to less than 1/20 of the initial contents. Since this relationship seemed to suggest that certain amino acids might be inhibitory to shikonin synthesis in Lithospermum cells, various L-amino acids

Table 1. Contents of amino acids in Lithospermum cells grown in LS and M9 medium.

Amino acid	Shikonin-free cells in LS medium[1] (nmole/g fr. wt)	Shikonin-producing cells in M9 medium[2] (nmole/g fr. wt)
Gln	6530 (40.81)[3]	70 (9.61)
Arg	3300 (20.60)	53 (7.30)
Glu	2340 (14.62)	134 (18.30)
Asp	1110 (6.94)	23 (3.19)
Asn	1020 (6.37)	157 (21.46)
Ala	442 (2.76)	101 (13.79)
Ser	403 (2.52)	65 (8.89)
His	357 (2.23)	35 (4.83)
Gly	205 (1.28)	23 (3.15)
Lys	157 (0.98)	22 (3.06)
Val	117 (0.73)	20 (2.74)
Leu	30 (0.19)	-[4]
Cys	19 (0.12)	16 (2.12)
Tyr	16 (0.10)	11 (1.54)
Total amino acids (nmole/g fr. wt)	16000	731

[1] 3-week-culture, [2] 2-week-culture, [3] Molar ratio (%) of each amino acid, [4] Not detected

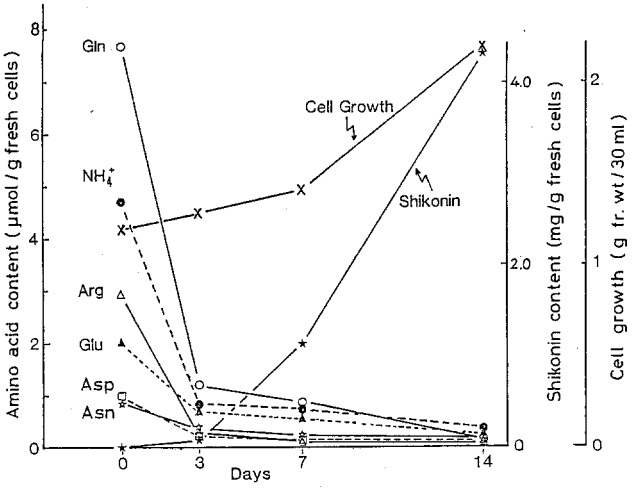

Fig. 2. Contents of amino acids and shikonin in Lithospermum cells (strain M18) transferred from LS medium to M9 medium and cultured in the dark. Gln: glutamine, Arg: arginine, Glu: glutamic acid, Asp: aspartic acid, Asn: asparagine.

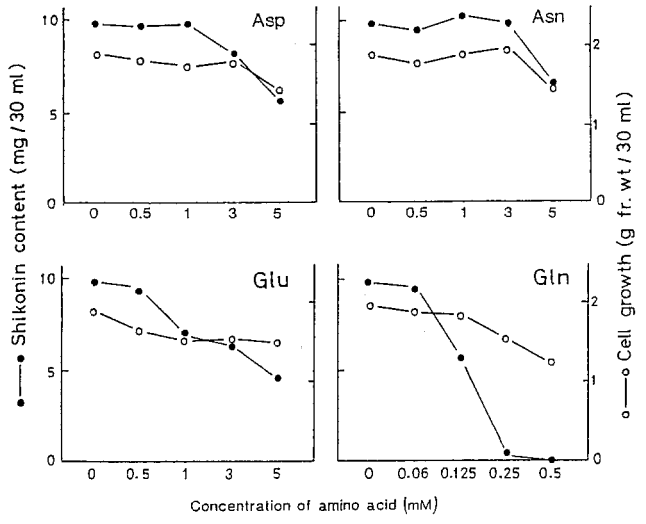

Fig. 3. Effects of amino acids on cell growth and shikonin production in Lithospermum cells grown in M9 medium for 2 weeks. Asp: aspartic acid, Asn: asparagine, Glu: glutamic acid, Gln: glutamine.

Table 2. Contents of shikonin, NH_4^+, Gln and total amino acids in Lithospermum cells grown in different culture media

Medium	Growth Increment	Shikonin Production	NH_4^+	Gln	Total Amino Acids
	(g fr. wt)	(μmol/g dry wt)	(μmol/g fr. cells)		
LS	5.1[1)]	0	4.75	7.72	16.00
LS + Cu^{2+}	5.5[1)]	0	9.49	9.79	19.30
LS + Cu^{2+} - NH_4^+ + NO_3^-	6.9[1)]	11.8	0.45	0.16	2.00
LS (agar medium)	5.5[1)]	28.8	11.93	0.61	7.07
M9	2.1[2)]	149.5	0.33	0.05	0.75

1) Culture period: 3 weeks, 2) Culture period: 2 weeks.

were administered to the cells cultured in M9 medium in order to observe their effects on shikonin production. Fig. 3 showed that only Gln strongly inhibited shikonin production at a much lower concentration than the other amino acids tested, i.e. Ala, Arg, Asp, Asn and Glu. It is interesting that Gln administered at a low concentration of 0.5 mM completely inhibited shikonin production, whereas Glu inhibited it only to 50 % of the control even at a high concentration of 5 mM.

Table 2 shows the relationship between the shikonin production and the endogenous levels of NH_4^+ and Gln under different culture conditions. In shikonin-free cells cultured in LS medium or in the same medium supplemented with a higher amount of Cu^{2+} (1.2 μM), the contents of NH_4^+ and amino acids, especially Gln, were very high. However, when NH_4^+ of the latter medium was substituted by the same mole of NO_3^- to induce shikonin synthesis, the contents of NH_4^+ and free amino acids including Gln decreased sharply without affecting cell growth. Similarly, Lithospermum callus tissues cultured on LS solid medium, on which cells can produce shikonin derivatives in the presence of agar (Fukui et al., 1983), the content of Gln was found to be very low (0.61 μM) in spite of a high NH_4^+ content (11.9 μM) in the cells.

To examine the possibility that the inhibitory effect of Gln on shikonin production might be due to NH_3 released from Gln by glutaminase, DON (6-diazo-5-oxo-norleucine), a specific and irreversible inhibitor of glutaminase (Hartman, 1968), was administered to M9 medium together with Gln (0.2 mM). However, DON at a concentration of 10^{-6} or 10^{-5} M in combination with Gln was more inhibitory to shikonin production than the single administration of Gln, without affecting cell growth (Fig. 4). These results suggest that shikonin synthesis is not inhibited by NH_3 released from Gln but by Gln itself.

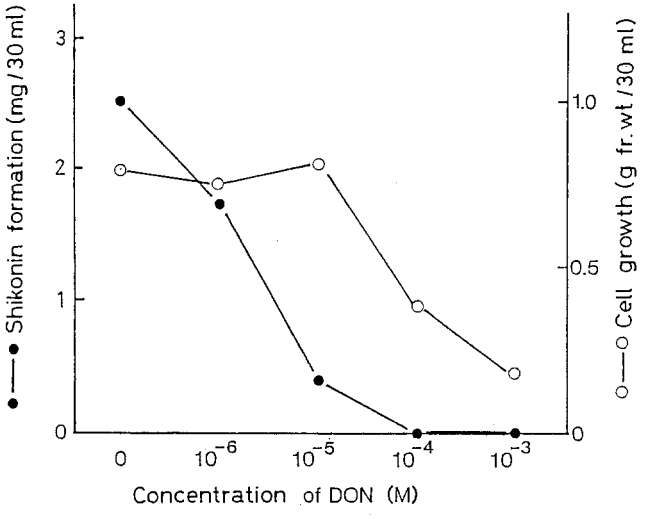

Fig. 4. Effects of DON on cell growth and shikonin formation in Lithospermum cells grown for 2 weeks in M9 medium containing 0.2 mM glutamine. Shikonin yield of the control (glutamine-free) culture was 9.3 mg/30 ml medium.

The present studies have indicated that the repression of shikonin production in Lithospermum cells cultured in a medium containing ammonium is closely related to an accumulation of glutamine at a high level. It is of special interest that the production of shikonin is under the strong influence of glutamine which is a remote primary metabolite. It is expected that further studies on the action mechanism of glutamine as regulatory factor of shikonin production would lead to a better understanding of the interrelationship between the primary and secondary metabolism.

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