Induction of berberine biosynthesis by cytokinins in *Thalictrum minus* cell suspension cultures

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ABSTRACT

Production of berberine could be induced by adding 6-benzylaminopurine (BAP) to *Thalictrum minus* cells, cultured in suspension in a medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), early in the growth cycle. In the presence of BAP, the precursor, L-tyrosine, was rapidly converted into berberine which was then released into the medium, whereas substantial amounts of the intermediates, tyramine and dopamine, accumulated in non-berberine-producing cells grown in the same 2,4-D-containing medium without BAP. These results suggest that BAP activated enzymatic reactions subsequent to the formation of the amines in the biosynthesis of berberine.

ABBREVIATIONS

2,4-D: 2,4-dichlorophenoxyacetic acid, BAP: 6-benzylaminopurine, NAA: 1-naphthaleneacetic acid, IAP: 6-isopentenylaminopurine, LS medium: Linsmaier-Skoog medium, "Growth medium": LS medium containing 10⁻⁶ M 2,4-D.

INTRODUCTION

Cell suspension cultures of *Thalictrum minus* var. *hypoleucum* grown in "production medium", i.e. Linsmaier-Skoog medium containing both 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP), are known to produce large amounts of berberine, most of which is released into the medium (Nakagawa et al. 1981; Nakagawa et al. 1984; Yamamoto et al. 1987). On the other hand, berberine production is strongly suppressed in "growth medium", i.e. LS medium containing 10⁻⁶ M 2,4-Dchlorophenoxyacetic acid (2,4-D) but not BAP, although culture growth is superior in growth medium. These observations suggested an important role of BAP as a promoter of berberine production. This paper describes the establishment of a model system for inducing berberine production in fast-growing cell cultures by the addition of BAP to the growth medium. The effect of BAP on the metabolism of the precursor, L-tyrosine, is also reported.

MATERIALS AND METHODS

Chemicals: NAA, 2,4-D, kinetin, and BAP were purchased from Sigma. 6-Isopentenylaminopurine (IAP) was from Wako Chemicals and L-[U-¹⁴C] tyrosine from Amersham.

Cell suspension culture: The stock culture of *Thalictrum minus* var. *hypoleucum* used in the present study was established in 1981 from a callus culture derived from a leaf segment of a wild plant collected in Nara, Japan, on LS agar medium containing 10⁻⁶ M 2,4-D and 10⁻⁶ M BAP (Nakagawa et al. 1984), and has been maintained in 100 ml of growth medium in 300 ml conical flasks at 25 °C in the dark (100 strokes / min) for ten years by subculturing every two weeks. The cultures were small cell aggregates which mostly consisted of 7 to 10 cells. In order to investigate the effect of cytokinins on berberine production, cells (0.27 g fresh weight) were transferred to growth medium (8 ml) supplemented with 10⁻⁷, 10⁻⁶, 10⁻⁵ or 10⁻⁴ M cytokinin (BAP, kinetin, or IAP) in 30 ml test tubes (17 x 180 mm). For determination of the optimum culture stage for induction of berberine production, 10⁻⁶ M BAP was added to 2,4-D BAP was added to *T. minus* cultures (8 ml medium), which had been precultured in growth medium (100 ml) for two weeks, on day 0, 2, 4 and 6 after inoculation, respectively. In time-course experiments for the formation of metabolic intermediates and alkaloids, 10⁻⁶ M BAP was administrated to *T. minus* cultures in 30 ml test tubes containing 8 ml of growth medium on day 2. The suspension cultures were agitated on a reciprocal shaker (100 strokes / min) at 25 °C in the dark.

Quantitative analysis of berberine: Berberine was separated and quantified by HPLC analysis as described previously (Nakagawa et al. 1984).

Administration of cytokinins: BAP, kinetin, and IAP were added to culture medium (8 ml) as aqueous solutions (0.2 ml) using membrane filter devices. An equivalent volume of
deionized water was added to control cultures.

**Analysis of alkaloids and biosynthetic intermediates**

Fresh cells (1 g) were homogenized with a Potter homogenizer in 1.5 ml of a mixture of methanol and 0.1 N HCl (70 : 30). The homogenate was centrifuged at 10,000 g for 10 min and the supernatant was subjected to HPLC analysis on a TSK-GEL ODS 120A column (4.6 x 150 mm) using 20 mM tartaric acid - 4 mM sodium dodecyl sulfate (SDS) - CH₃CN - MeOH (250 : 100 : 25) as solvent system A and 50 mM tartaric acid - 10 mM SDS - CH₃CN - MeOH (100 : 100 : 25) as solvent system B. The UV detector was set at 280 nm and the flow rate was maintained at 1.5 ml/min. Essentially all of the intermediates and compounds related to berberine biosynthesis could be detected by these systems: DOPA, tyrosine, dopamine, tyramine, norlaudanosoline, coclaurine, reticuline and magnoflorine by solvent system A and scoulerine, tetrahydroberberine and berberine by solvent system B. The identification of peaks corresponding to tyramine, dopamine, magnoflorine, and berberine in culture extracts was accomplished by co-chromatography with authentic sample on TLC plates using the following systems: CHCl₃ - acetone - MeOH - AcOH - H₂O (10 : 4 : 2 : 3 : 1), n-BuOH - AcOH - H₂O (5 : 1 : 4), ethylacetate - benzene - n-propanol - MeOH - EtNH₂ (1 : 8 : 2 : 2 : 1.5). The identities of components separated by TLC and extracted from plates with methanol were confirmed by UV spectrophotometry and the use of spray reagents, namely Dragendorff's reagent and ninhydrin reagent for alkaloids and amines, respectively.

Administration of L-[^4]C-tyrosine:
In tracer experiments, cultured cells (inoculum size: 1 g FW) were incubated for 12 days in 30 ml of LS medium supplemented with 10⁻⁵ M BAP in addition to 10⁻⁶ M NAA or 10⁻⁶ M 2,4-D at 25 °C in the dark, and then fed with 1 μCi (1 μmol) of L-[U-¹⁴C] tyrosine. After 48 hr, cells were separated from the medium and extracted with acidic MeOH, as described earlier. A part of the medium was freeze-dried and the residue was dissolved in a small amount of distilled water. Alkaloids and intermediates extracted from the cells and the medium were separated by two dimensional TLC (Silica gel) using CHCl₃ - acetone - MeOH - AcOH - H₂O (10 : 4 : 2 : 3 : 1) and n-BuOH - AcOH - H₂O (5 : 1 : 4) as the solvent systems. The radioactivity of each compound was measured by a radiochromatoscanner (Aloka JTC/ACM 505), and the total radioactivity by a scintillation counter (Aloka LSC-900). Major radioactive compounds were identified by co-chromatography on TLC with authentic samples.

**RESULTS AND DISCUSSION**

**Effects of cytokinins on induction of berberine production**

Although *T. minus* cell suspension cultures produced little berberine in LS medium containing 2,4-D (10⁻⁴ M) or NAA (10⁻⁴ M) alone, addition of BAP (10⁻⁵ M) to the medium at the beginning of culture induced berberine synthesis on days 5 and 10 in the presence of 2,4-D and NAA, respectively. BAP (10⁻⁵ M) added to medium at day 0 was found to be more effective than either of two other cytokinins, kinetin and IAP, in increasing berberine yield in the presence of 10⁻⁴ M 2,4-D (Fig. 1). The administration of 10⁻⁵ M BAP to cultures in growth medium on day 2 gave a higher yield of berberine (470 mg/l) in 10 days of culture compared to its administration on day 0, 4, or 6 (Fig. 2). The successful induction of berberine production by addition of BAP to *T. minus* cultures at any point in the culture period should serve as an experimental system favorable for studying dynamic aspects of secondary metabolism related to the activation of berberine biosynthesis.

**Effect of BAP on accumulation of metabolic intermediates**

The influence of BAP on the accumulation of intermediate compounds involved in the biosynthesis of berberine was investigated by using *T. minus* cell cultures treated with 10⁻⁵ M BAP on day 2 and harvested after 6 days of incubation (Fig. 3). In the absence of BAP, *T. minus* cells accumulated dopamine and tyramine, which are known as precursors of berberine (Rueffer and Zenk 1987), and