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Ginkgolide Production in *Ginkgo biloba* Trees and Cultured Cells

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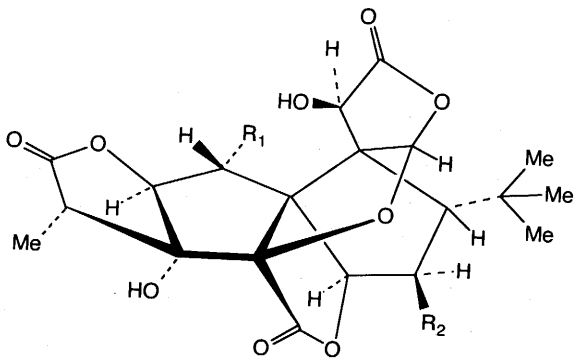
1. Introduction

Ginkgolides are unique diterpenoids which were isolated from the leaves and root bark of *Ginkgo biloba* L. by Nakanishi[1] and Okabe *et al.*[2] These compounds have pharmacological activity against platelet activating factor and are used for the treatment of septic shock and inflammatory disorders.[3]

Ginkgolides have a cage molecule structure with six five-membered rings and a *tert*-butyl group (Fig. 1).[4–7] Because of the complex structure, the chemical synthesis of these compounds is possible but difficult.[8] Therefore, the main commercial source of ginkgolides is the leaves of *G.biloba* tree. Several studies have been done using tissue culture of ginkgo to clarify the biosynthetic pathway and find out alternative methods for the production of ginkgolides.[9–11] Seasonal variation of the ginkgolide contents and the influence of growth and light level on the yields of ginkgolides in ginkgo tree were also reported.[12, 13]

In the present study, we investigated ginkgolide content in various parts of ginkgo trees to consider the behavior and function of ginkgolides in ginkgo plants.

Ginkgolide production in some ginkgo cultured cells were also studied.



Ginkgolide A $R_1=R_2=H$
Ginkgolide B $R_1=OH, R_2=H$
Ginkgolide C $R_1=R_2=OH$

Fig. 1. Chemical structures of ginkgolides.[4–7]

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2. Materials and Methods

Standards

Ginkgolides A, B and C were supplied by Dr. H. Schick, Department of Chemistry, Heidelberg University, Heidelberg, Germany.

Plant materials

Green leaves were collected from several ginkgo trees in August, and fallen leaves and seeds were gathered around a ginkgo tree as soon as they were detached from the tree in November, 1995. Inner and outer bark, and wood were collected from a four-year-old branch in the middle of April. These trees were planted at the Yayoi campus of the University of Tokyo. Roots were collected from two-year-old seedlings cultivated in a pot in October. These materials except seeds were immediately lyophilized and then pulverized mechanically with a coffee mill. The resultant powder was stored at −20℃ in an airtight vessel until used for the quantitative and qualitative analysis of ginkgolides. Seeds were stored at 3℃ until April, 1996. Then stony layers of the seeds were removed and albumens and embryos were separately collected. Albumens were treated as described above for ginkgolide analysis.

Cell cultures

Calli were induced from ginkgo embryos and petioles according to the method described elsewhere.[14] Linsmaier & Skoog's mineral salts[15] supplemented with 1.0 mg/*l* thiamine chloride, 100 mg/*l* *myo*-inositol and 30 g/*l* sucrose, solidified with 2 mg/*l* gellan gum were used as the basic medium. Plant growth regulators of 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), kinetin (K) and 6-benzyladenin (BA) were added in various combinations. Calli were induced from embryos on the media supplemented with 0.5 mg/*l* 2,4-D+0.4 mg/*l* K (DK), and 2.0 mg/*l* NAA+5.0 mg/*l* K (NK). Explants of petiole and cambium formed calli on the medium containing 1.0 mg/*l* NAA+0.2 mg/*l* BA (NB). These calli were maintained in darkness at 26.5℃, and under illumination with fluorescent light (about 1000 lux) at room temperature. They were subcultured on the same media every month. Cell suspension cultures were initiated from the callus derived from embryo on the DK medium. Pieces of the callus were inoculated into 200 m*l* and 500 m*l* Erlenmeyer flasks containing 40 m*l* and 100 m*l* of DK liquid medium, then cultivated for 3 weeks with shaking at 90 rpm under illumination with fluorescent light at room temperature, and at 120 rpm in darkness at 26.5℃, respectively. These cultures were subcultured every 3 weeks.

All cultured cells were harvested when subcultured, and the cells were immediately treated as described in plant materials.

Analysis of ginkgolides

The aliquots of the pulverized materials were weighed (about 500 mg) and then extracted and fractionated according to the method of Huh and Staba.[10] The purified extracts were dissolved in appropriate amount of methanol and successively analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). For the analysis by GC, an appropriate amount of purified methanolic fraction was evaporated to dryness and trimethylsilylated by adding 50 μl of a silylating agent (Tri-Sil BSA formula D, Pierce Chemical Co., USA). This mixture was vortexed and heated for 1 hour at 80℃. Analyses by GC-flame ionization detection were performed with a Shimadzu GC-14A (Shimadzu, Japan) equipped with a 30 m×0.25 mm TC-1 capillary column (GL Sciences Inc.,

Japan). The temperatures of the column, injector and detector was maintained at 285℃, 300℃ and 300℃, respectively. N_2 was used as the carrier gas at a flow rate of 2.6 *ml*/min. For quantitative determination of ginkgolides, standard curves were prepared with known amounts of authentic ginkgolides A, B and C after silylation. The concentration of each ginkgolide was determined using the standard curves. The individual retention times of ginkgolides were compared with that of standards. Each ginkgolide peak was also identified by comparing the fragmentation pattern of each peak with that of authentic ginkgolides in GC-MS (DX-303, JEOL, Japan).

3. Results and Discussion

Ginkgolide contents in the various parts of ginkgo trees, and in several cultured cells are shown in Table 1 and Table 2, respectively.

As shown in Table 1, ginkgolides were detected in all the parts of ginkgo trees examined. In green leaves, the contents of ginkgolide A (G-A) and ginkgolide B (G-B) were almost the same, while the content of ginkgolide C (G-C) was about one fifth of those of G-A and G-B. In fallen leaves, the amount of total ginkgolides was rather less than green leaves. The rate of decrease in G-A was higher than G-B and G-C. These results suggest that during leaf senescence G-A is first degraded or hydroxylated to G-B or G-C. Then these ginkgolides might be translocated or catabolized.

Inner bark collected in April, when new leaves first appeared, contained substantial amount of ginkgolides. Considering that young leaves are known to accumulate lower amount of ginkgolides than mature leaves[12], these ginkgolides are likely to be biosynthesized in phloem or cambium, not translocated from the young leaves. There is also a possibility that ginkgolides were biosynthesized in other parts last year and then translocated and accumulated in inner bark. The amount of total ginkgolides in outer bark was a little less than a half of that in inner bark, while the ratio of G-A, G-B and G-C was similar in each sample. These facts suggest that ginkgolides located in inner bark are not intensively catabolized or translocated during cell death in phloem to outer bark transition. In contrast, wood contained far less amount of ginkgolides than inner bark. This might

Table 1. Ginkgolide contents (μg/g dry weight) in various parts of ginkgo trees

Sample	Content of ginkgolides (μg/g dry weight)		
	G-A	G-B	G-C*1
green leaves	318	322	64.5
fallen leaves	27.2	75.5	17.3
inner bark	168	120	129
outer bark	80.2	47.3	56.9
wood	4.29	10.4	—*2
roots	1,100	1,060	135
albumens	8.10	123	16.8

*1 G-A, G-B, G-C=ginkgolide A, B and C, respectively.
*2 —: trace amount.

Table 2. Ginkgolide B contents (μg/g dry weight) in cultured cells of *Ginkgo biloba*

Sample	Content of ginkgolide B (μg/g dry weight)
Callus	
embryo/DK/light*1	2.23
embryo/DK/dark	2.83
embryo/NK/light	—*2
petiole/NB/light	1.87
petiole/NB/dark	—
cambium/NB/dark	—
Suspension culture	
embryo/DK/light	2.53
embryo/DK/dark	—

*1 origin/phytohormones/light condition.
*2 —: trace amount.

mean that ginkgolides are hardly retained during xylem differentiation, or that cambium cells originally contain little ginkgolides.

In the roots of young trees collected in October, the amount of total ginkgolides was about three times as high as that of green leaves. The ratio of each ginkgolide was similar to that of green leaves. It is unclear whether these ginkgolides are mainly biosynthesized in the roots or translocated from the other parts and accumulated. In October the leaves must be in the process of senescence, and ginkgolides which have existed in green leaves in summer might be translocated to the other parts and/or catabolized. If most of the ginkgolides are translocated, the amount of total ginkgolides in the other parts of the tree than leaves should increase in autumn while total ginkgolide contents in the leaves decrease. If ginkgolides contained in green leaves are mainly catabolized in autumn, ginkgolides should be independently biosynthesized in roots and inner bark. To determine which notion is adequate, ginkgolide contents in leaves and the other parts of the tree are to be scrutinized at regular intervals throughout a year.

In albumens, G-B was the principal ingredient, and G-A and G-C were much less. As albumens are unlikely to produce ginkgolides by themselves, these ginkgolides should have been translocated from the other parts. Considering that G-B is the most abundant of ginkgolides in albumens, G-B might be an intermediate in ginkgolide translocation.

As mentioned above, the main commercial source of ginkgolides is ginkgo green leaves. In this study, we found that substantial amount of ginkgolides were present in bark, root, seeds and even in fallen leaves. So pruned stems and fallen leaves, which are exclusively treated as trash, can be used as ginkgolide sources.

As shown in Table 2, G-B was detected in several cultured cells, but the concentration was far less than any part of mother plants. The other ginkgolides, G-A and G-C, were not clearly separated and determined. Embryo-derived calli or suspension cells generally produced G-B, while petiole- and cambium-derived calli showed poorer results. As petiole- and cambium-derived calli were able to grow only on NB medium, the effects of the other phytohormones were not examined. Ginkgolide content in embryo-derived cells cultured on DK medium was about one fifth of that observed in leaf-derived suspension cells reported by Jeon *et al.*[11]. Differences in light levels and medium components do not seem to have important influence on G-B production in several cultured cells examined in this study. Especially, all cells cultured under illumination are green in color, but this fact seems to have no connection with ginkgolide production. Other factors such as temperature, composition of mineral salts in the media, and time of harvest, and so on are to be examined to promote ginkgolide production in cultured cells.

Acknowledgement

We thank Dr. H. Schick, Heidelberg University, Heidelberg, Germany, for his generous gift of ginkgolides A, B and C.

Summary

Ginkgolide content in various parts of ginkgo trees and in some cultured cells were studied.

In all the parts of ginkgo trees examined, green and fallen leaves, wood, outer and inner barks, roots, and albumens, ginkgolides were detected. Fallen leaves had rather less ginkgolides than green leaves. Inner bark collected in April contained substantial amounts of ginkgolides, whereas outer bark had half the amount and wood had far less. In the roots of young trees collected in October, the amount of total ginkgolides was about three times as high as that of green leaves. In albumens, ginkgolide B was the main ingredient, and

ginkgolides A and C were far less.

Ginkgolide production was examined in cultured cells derived from embryo, petiole and cambium. Several cultured cells produced ginkgolide B, but the concentration was far less than any part of mother plants. Embryo-derived cultured cells generally produced ginkgolide B, while petiole- and cambium-derived calli showed poorer results. Greening of the cultured cells under illumination seemed to have no connection with ginkgolide production.

Key words: ginkgolide, *Ginkgo biloba*

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イチョウ植物体及び培養細胞におけるギンコライド生成

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要　　旨

イチョウ植物体の種々の部位と，数種の培養細胞におけるギンコライド含有量を調べた。

イチョウ植物体では，今回調べた緑葉，落葉，木部，外樹皮及び内樹皮，根，さらに胚乳の全てにおいてギンコライド類が検出された。落葉での含有量は緑葉と比較してかなり少なかった。4月に採取した内樹皮にはかなりの量が含まれていたが，外樹皮はその半分，木部ではさらに少量であった。10月に採取した若木の根には緑葉の約3倍の総ギンコライド量が検出された。胚乳ではギンコライドBが主成分で，ギンコライドA, Cははるかに少量であった。

胚，葉柄，形成層由来の培養細胞でのギンコライド生成を調べたところ，数種の培養細胞でギンコライドBの生成が確認されたが，その量は原植物における含有量と比較してはるかに少なかった。胚由来の培養細胞では総じてギンコライドB生成が認められたが，葉柄および形成層由来の細胞でのギンコライド含有量はより少量であった。明所培養の細胞は緑色を呈するが，このこととギンコライド生成との関連は認められなかった。

キーワード: ギンコライド，イチョウ

Shear Strength of Wood Obtained by Torsion Test

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In this paper, we tried to obtain the real shear strength of wood by torsion tests with considering plastic deformation.

Sitka spruce (*Picea sitchensis* Carr.), agathis (*Agathis* sp.), katsura (*Cercidiphyllum japonicum* Sieb. and Zucc.) and buna (Japanese beech, *Fagus crenata* Bl.) were used as specimens. These specimens were twisted around the longitudinal axis, and the shear stress-shear strain relationships of LR- (longitudinal-radial) planes and LT- (longitudinal-tangential) planes were obtained. These relationships were formulated by n-power functions, and the shear strengths were predicted by putting the shear strain at the occurrence of failure into the formula. On the other hand, the shear strength was independently calculated by the conventional method which is based on the hypothesis that the specimen keeps its elastic stress condition under the torsional loading. The strengths obtained by the different methods were compared with each other.

Although the shear stress-shear strain relationships varied with species, the values of the elastic-plastic strengths were about 70% of those obtained by the conventional method. There are several results of mechanical tests which show the coincidence between the testing data and theoretical analyses more precisely when the shear strength is evaluated as about 70% of that obtained by conventional solution

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