papers and notes on methodology

Separation of dolichol from dehydrodolichol by a simple two-plate thin-layer chromatography

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Abstract A novel thin-layer chromatographic procedure was devised to separate dolichol and dehydrodolichol from each other with the concomitant separation of each family with respect to the carbon chain length. This method involves development of the polyprenols successively on two different plates, a silica gel plate and a reversed-phase plate.—**Sagami, H., A. Kurisaki, K. Ogura, and T. Chojnacki.** Separation of dolichol from dehydrodolichol by a simple two-plate thin-layer chromatography. *J. Lipid Res.* 1992. **33:** 1857–1862.

Supplementary key words dolichol • polyprenol

Dolichols represent a family of polyisoprenoid lipids having 16–22 isoprene units, mostly in the *cis* configuration, with the α-isoprene unit saturated. The phosphorylated form, dolichyl phosphate, functions as a carrier of monosaccharide and oligosaccharide groups during *N*-linked glycoprotein biosynthesis (1) and formation of glycosylphosphatidyl inositol anchor (2, 3).

We have been particularly interested in studying the terminal reaction in dolichol biosynthesis, α-saturation of dehydrodolichyl products. In this study it is necessary to distinguish dolichol and dehydrodolichol compounds from each other. The possibility of distinguishing between dolichols and fully unsaturated polyprenols using a simple thin-layer chromatography (TLC) procedure would enable better characterization of natural mixtures of polyisoprenoid alcohols in which both polyprenols and dolichols are often present (4).

In previous studies, acid lability of dehydrodolichyl phosphates and its pyrophosphates was utilized for this purpose (5). When these compounds were treated with mild acid, the degradation products were identified as a mixture of tertiary alcohols and hydrocarbons. On the other hand, dolichyl phosphates and its pyrophosphates were not converted to phosphate-free products. However, this method was not applicable to free alcohols.

In order to separate the alcohols, normal-phase high performance liquid chromatography (HPLC) was used (6). But the separation was not satisfactory for our purposes. In particular, it was impossible to estimate exactly the small amount of reduced products when the reductase activity was very weak.

The complete separation of dolichols was carried out with the two-step HPLC system (7, 8). First, a mixture of dolichols and dehydrodolichols was separated by C_{18} reversed-phase HPLC according to their chain length. Then individual polyprenol pairs (dolichol and dehydrodolichol) were separated by silica gel HPLC from each other.

We recently developed a simple two-plate TLC system that enabled us to separate the dolichol family from that of dehydrodolichol.

In this communication we describe the details of this chromatographic system and its application to the analysis of natural mixtures of polyisoprenoid alcohols and the products of in vitro assay of their biosynthesis.

MATERIALS

Dehydrodolichol and dolichol chemically synthesized from the dehydrodolichol from *Ginkgo biloba* were kind gifts from Kuraray Corp. (Kurashiki, Japan). Dolichol from pig liver (grade I) was obtained from Sigma. Silica gel 60 thin-layer plates (cat. #11845) and reversed-phase LKC-18 thin-layer plates (cat. #4800-800) were purchased

Abbreviations: TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; Dol-OH, dolichol; Dedol-OH, dehydrodolichol.

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from Merck and Whatman, respectively. The magnetic bars were purchased locally. Rapeseed and maize were obtained locally. Male Sprague-Dawley rats were obtained from Funabashi Farm (Chiba, Japan). [1-^{14}C]isopentenyl-PP (sp act, 56 Ci/mol) was obtained from Amersham. *E,E*-Farnesyl-PP was generously given by Ajinomoto Corp. (Tokyo, Japan). *Z,E,E*-Geranylgeranyl-PP was prepared from *Z,E,E*-geranylgeraniol according to the method of Davisson, Woodside, and Poulter (9). RP-18 Sep-Pak cartridges (cat. #51910) were obtained from Waters. All other chemicals were of reagent grade.

METHODS

Separation of dehydrodolichol and dolichol by a two-plate TLC method

All procedures are depicted in **Fig. 1.**

(I) The first TLC was carried out on a silica gel glass plate (20 × 3 cm) developed in toluene–ethyl acetate 9:1. The amount of sample was less than 10 μg. The time of running was about 70 min. The dehydrodolichol family moved slightly faster than the corresponding dolichol family, but the separation was not sufficient.

(II) The glass plate was cut into three pieces with a diamond cutter so that one of the pieces contained the developed dehydrodolichol and dolichol. For estimating the migration of the sample, it was helpful to develop authentic dolichol and dehydrodolichol on both side lanes (20 × 1.6 cm). The side lanes were cut off, and the polyprenol standards on these lanes were visualized with iodine vapor. The sample lane was not exposed to iodine vapor.

(III) The removed glass plate (in II) was rotated by 90° and turned over.

(IV-V) The silical gel face on which dehydrodolichol and dolichol were located was bound to the gel face of the concentration zone of a reversed-phase LKC-18 glass plate (Whatman) by means of two strong magnetic bars (4.0 × 1.1 × 0.8 cm) such that the second chromatography was developed perpendicularly to the first chromatographic separation. The hatched bar in V represents the magnetic bars. The dehydrodolichol and dolichol were transferred to the starting position of the reversed-phase TLC plate through the concentration zone by developing the plate in acetone–methanol 19:1 in a chamber (24 × 15 × 31 cm). When the solvent reached the boundary between the concentration zone and the resolving zone, the plate was removed from the development chamber and air dried. This development takes about 5 min. The development of the plate was repeated until the transfer of the samples was completed. Two transfers are usually sufficient when the chain lengths of the prenols are longer than C_{50}. The completion of transfer was checked by exposing the silica gel section to iodine vapor.

(VI) After the silica gel glass section was removed, the transferred samples on the reversed-phase LKC-18 glass plate were developed vertically in acetone–methanol 19:1. The time of running was about 40 min. Total chromatography time was approximately 2.5 h.

(VII) The positions of separated dehydrodolichols and dolichols were visualized with iodine vapor. When the samples were radioactive, these families were detected by exposing the plate on Fuji RX X-ray film at −80°C for a week to 1 month.

Multiple samples can also be analyzed by this method (**Fig. 2**). For example, four samples can be applied on one silica gel glass plate (20 × 20 cm) at the same time. The width of the sample lane (a) and that of the side lane (b)

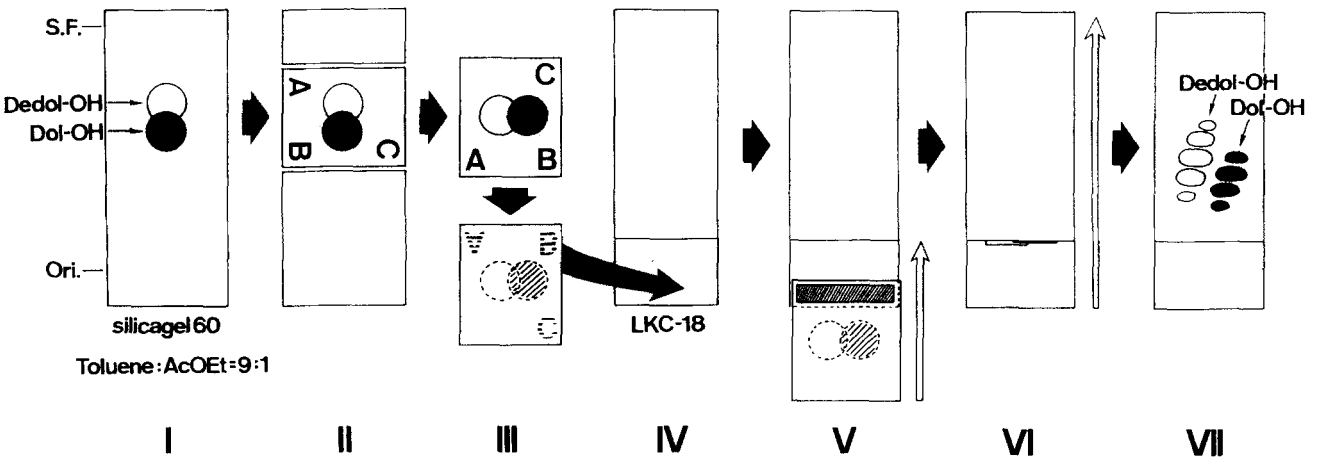

Fig. 1. New two-plate TLC for the separation of dehydrodolichol and dolichol. The detailed procedure is described in Methods; Dedol-OH, dehydrodolichol; Dol-OH, dolichol.

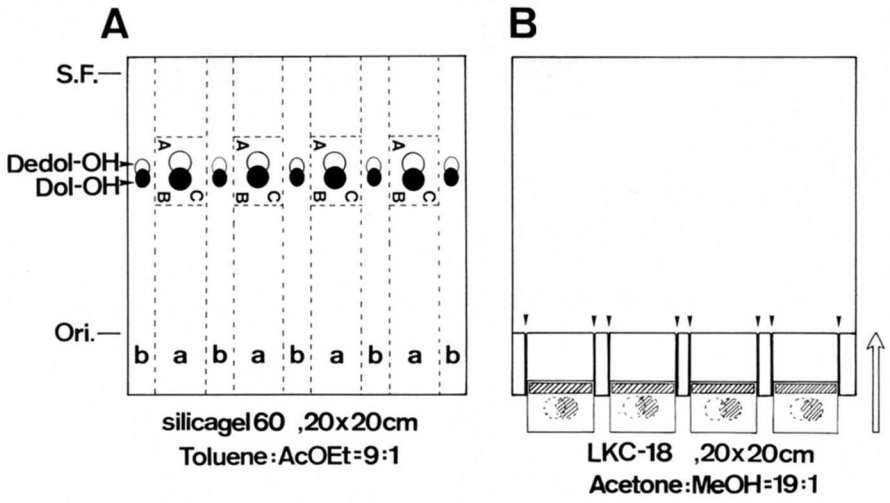

Fig. 2. New two-plate TLC for the separation of dehydrodolichol and dolichol in the case of multiple samples. A and B correspond to steps I and V, respectively, in Fig. 1. The sample lane and the side lane in A are a and b, respectively. The arrowheads in B show vertical grooves on the concentration zone.

for standard samples are 3 cm and 1.6 cm, respectively. The first dimension is developed out in the same way as shown in Fig. 2A. The silica gel plate is cut into nine pieces along the vertical dotted line as shown in Fig. 2A. After the positions of the developed samples were deduced from those of the standard samples detected by exposing the five pieces (b) to iodine vapor, the four pieces (a) were each cut into three sections along the horizontal dotted line as shown in Fig. 2A. The four pieces with the developed sample are bound to the concentration zone of one reversed-phase LKC-18 glass plate (20 × 20 cm). It is necessary to make vertical grooves on the concentration zone by scraping the gel as indicated by the small arrows in Fig. 2B in order to avoid irregular transfer of the samples. This method of two-plate TLC for multiple samples provides very reproducible results. By use of one chromatography chamber, eight samples on two plates can be analyzed at the same time.

Isolation of polyprenols from rapeseed, maize and rat testis

Procedures for extraction of tissue polyprenols were a modification of those described by Ravi, Rip, and Carroll (10) and Nyquist and Holt (11). Finely ground seeds or homogenates of the testis from a 6-month-old rat were saponified and extracted with diethyl ether. The extracts were washed with saturated NaCl and applied to a silica gel column equilibrated with hexane–ethyl acetate 10:1.

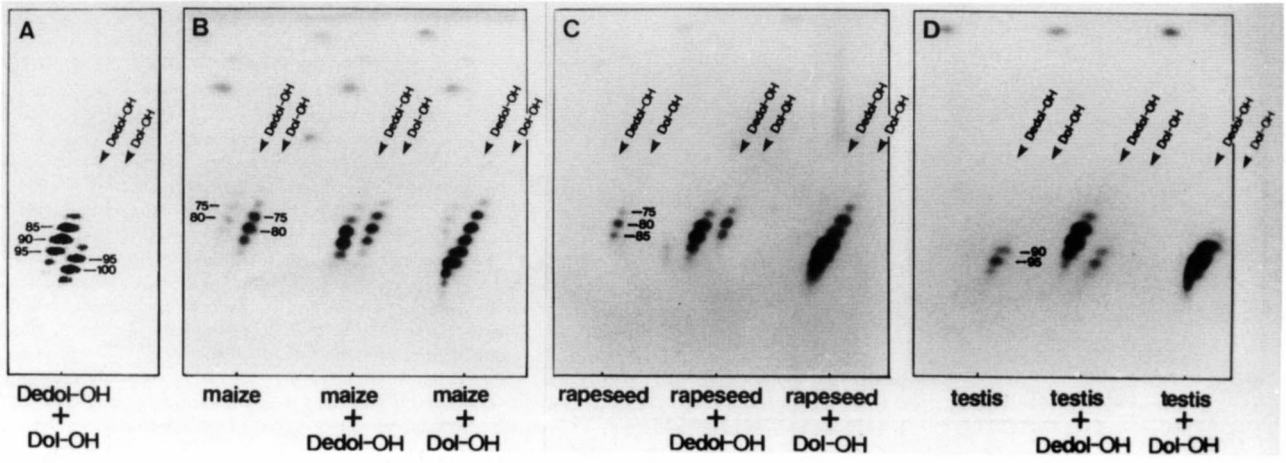

Fig. 3. Separation of polyprenols into dehydrodolichol and dolichol by two-plate TLC. A: Dehydrodolichol of *Ginkgo biloba* and dolichol of pig liver as references; B: polyprenol of maize (left), the polyprenol plus reference dehydrodolichol (middle), and the polyprenol plus reference dolichol (right); C: polyprenol of rapeseed (left), the polyprenol plus reference dehydrodolichol (middle), and the polyprenol plus reference dolichol (right); D: polyprenol of rat testis (left), the polyprenol plus reference dehydrodolichol (middle), and the polyprenol plus reference dolichol (right).

The fraction containing polyprenols was further purified by preparative silica gel TLC in a solvent system of toluene–ethyl acetate 9:1. The silica gel corresponding to polyprenols was scraped from the plate and extracted with diethyl ether. The purified polyprenols from maize (81 g), rapeseed (42 g), and testis (20 g) were 5.7 mg, 6.6 mg, and 1.3 mg, respectively.

Enzyme preparation

Male Sprague-Dawley rats were killed by decapitation and the livers were removed. The livers were cut into small pieces with scissors and homogenized in a twofold volume of phosphate-buffered saline (pH 7.2) containing 0.25 M sucrose, 1 mM dithiothreitol, and 10 mM EDTA. The homogenates were centrifuged at 1,000 *g* for 15 min and then at 10,000 *g* for 20 min. The supernatant was dialyzed against phosphate-buffered saline (pH 7.2) containing 0.25 M sucrose and 1 mM dithiothreitol. The dialyzed enzyme preparation was divided into multiple aliquots and stored at −80°C.

RESULTS AND DISCUSSION

Fig. 3A shows a thin-layer chromatogram of a mixture of the dehydrodolichol of *Ginkgo biloba* and the dolichol of pig liver. Both prenols contain two internal *trans* double bonds but differ with respect to the saturation of α-isoprene unit (12). Comparison of the mobilities of dehydrodolichol and dolichol with the same carbon chain length revealed that the dolichols had a smaller mobility than dehydrodolichols but a greater mobility than a dehydrodolichol with a chain length longer by one isoprene unit. We also tested the separation between stereochemically different dehydrodolichols: *Ginkgo biloba* dehydrodolichol with two internal *trans* double bonds and dehydrodolichol with three internal *trans* double bonds formed by acid phosphatase treatment of the products derived from *E,E,E*-geranylgeranyl-PP and [1-^{14}C]isopentenyl-PP with rat liver microsomal dehydrodolichyl diphosphate synthase. However, the two-plate TLC method did not resolve the radioactive dehydrodolichol with three internal *trans* double bonds and the stereoisomeric dehydrodolichol with two internal *trans* double bonds (data not shown).

Next we applied two kinds of natural polyprenol samples to the two-plate TLC. The samples were prepared as described in Methods from monocotyledonous maize containing both dehydrodolichol and dolichol and from dicotyledonous rapeseed containing only dolichols (10). As shown in Fig. 3B and 3C, the polyprenols from maize were indeed separated into two families corresponding to dehydrodolichol and dolichol but the polyprenols from rapeseed consisted of only dolichol. We also examined a polyprenol sample from the testis of an aged rat to see whether the accumulation of dolichol by aging (11) is accompanied by that of dehydrodolichol (Fig. 3D). It was shown that the only polyprenol from the testis was dolichol.

Next we used the two-plate TLC to establish a simple in vitro assay system to detect a reductase activity. Ekström et al. (8) have found the enzyme activity for dolichol biosynthesis in rat liver homogenates in experiments using [^{3}H]mevalonate as a precursor. In our assay we substituted [1-^{14}C]isopentenyl-PP (56 mCi/mmol) and

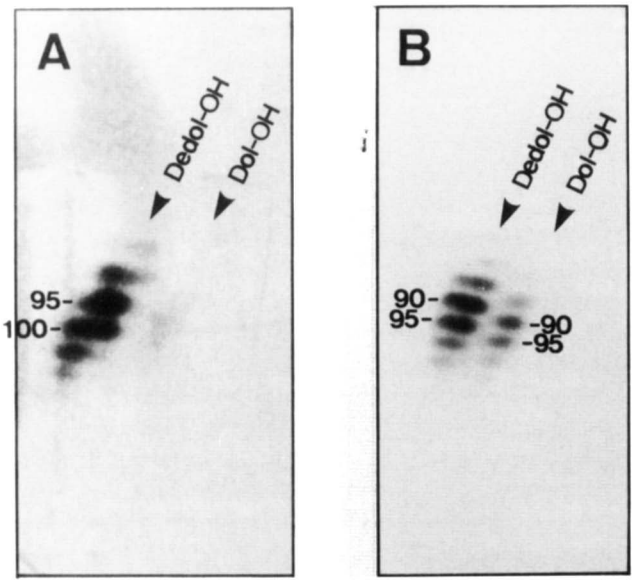

Fig. 4. In vitro formation of dolichyl compound. A: The assay was carried out according to the method of Ekström et al. (8). The mixture contained, in a final volume of 1.0 ml, 0.1 M potassium phosphate buffer (pH 7.5), 10 mM phosphoenol pyruvate, 5 mM ATP, 15 units of pyruvate kinase, 5 mM $MgCl_2$, 5 mM NADH, 20 μM *Z,E,E*-geranylgeranyl diphosphate, 5 μM [1-^{14}C]isopentenyl diphosphate, and 60 mg protein of 10,000 *g* supernatant of rat liver homogenate. After 17 h incubation at 30°C, the mixture was saponified at 85°C for 45 min after the addition of 1 ml of 0.25% pyrogallol in methanol and 1 ml of 60% KOH. The mixture was extracted with butanol and the butanol extracts were treated with acid phosphatase by the method of Fujii, Koyama, and Ogura (13) and extracted with hexane. The hexane extract was filtered through RP-18 Sep-Pak equilibrated with methanol and eluted with hexane. The radioactive prenols were analyzed by two-plate TLC. The exposure time for autoradiography was 2 weeks. The amounts of dehydrodolichol and dolichol were determined to be 293 dpm and 40 dpm, respectively, by scraping out the chromatographic sections of these prenols followed by liquid scintillation counting. B: The standard assay mixture contained, in a final volume of 1.0 ml, 50 mM Tris-HCl buffer (pH 8.0) 1 mM dithiothreitol, 50 mM KF, 20% (v/v) glycerol, 1 mM $MgCl_2$, 10 mM NADPH, 40 μM *Z,E,E*-geranylgeranyl-PP, 5 μM [1-^{14}C]isopentenyl-PP, and ca. 10 mg/ml of dialyzed enzyme. The mixture was incubated at 37°C for 18 h and treated as described in A. The exposure time for autoradiography was 4 days. The amounts of dehydrodolichol and dolichol were 3,437 dpm and 1,092 dpm, respectively.

Z,E,E-geranylgeranyl-PP for [^{3}H]mevalonate (24 Ci/mmol) in the in vitro incubation mixture. As a result, little if any of the dolichol family was detected, although members of the dehydrodolichyl family were formed (**Fig. 4A**). Next, we examined the effects of various factors on the formation of dolichol compounds, including temperature, pH, enzyme concentration, metal ions (Mn^{2+}, Mg^{2+}), coenzymes (FMN, FAD, NADH, NADPH, NAD, NADP), ATP, and their related compounds (GTP, CTP). Fig. 4B shows that three or four dolichol family members are formed under the best incubation conditions.

We have developed a new two-plate TLC method that is surprisingly effective in separating dolichols from dehydrodolichols. Although we also attempted the separation of these polyprenols with commercially available two-dimensional TLC plates (Whatman SC5 multi-K K5F/KC18F), the separation was not sufficient. The crucial point of our method is the transfer of the sample on a silica gel plate to a reversed-phase LKC-18 plate through the concentration zone. In our research for the reductase, we used this method hundreds of times and obtained good separation and reproducibility. This method seems to be superior to the widely used HPLC method in that the distribution of dolichol and dehydrodolichol families can be detected on the final TLC plate and multiple samples can be quickly and conveniently analyzed at the same time. Also, this method enables us to detect small amounts of radioactive dehydrodolichol and dolichol as described in the legend to Fig. 4. Though the two-plate TLC was found to be effective for a wide range of polyprenols and dolichols with respect to their chain length, some modifications of the developing solvents might be necessary to study more complex mixtures of polyisoprenoid alcohols, e.g., glycinoprenols (14) and hexahydroprenols (15, 16). The principle of the method, i.e., the use of adsorption chromatography and reversed-phase chromatography in sequence, should remain unchanged. We would expect that with the use of a reliable two-dimensional scanner (especially in the far UV regions, e.g., Chromoscanner) at least semiquantitative measurements of individual polyisoprenoid alcohols can be made by our method.

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