Single polyprenol and dolichol isolation by semipreparative high-performance liquid chromatography technique

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Abstract A new method of separation of single polyprenols (or dolichols) from a mixture of isoprenoid alcohols is described. Application of a high-performance liquid chromatography (HPLC) apparatus equipped with a semipreparative ODS column resulted in preparation of long-chain (dihydro)polyprenols of high purity (>95%). This approach substantially decreases the time scale of the conventional chromatographical preparative procedure. The method can be widely used in chemical and biochemical projects, where single polyprenols or dolichols are required.—Carlson, T., K. Skorupinska-Tudek, J. Hertel, T. Chojnacki, J. M. Olsson, and E. Swiezewska. **Single polyprenol and dolichol isolation by semipreparative high-performance liquid chromatography technique. *J. Lipid Res.* 2000.** 41: **1177–1180.**

Supplementary key words polyprenol • dolichol • preparative HPLC

Long-chain isoprenoid alcohols are common constituents of biological membranes (1). Dolichols (α-saturated isoprenoid alcohols) are found in all animal cells and tissues, for example, rat and human organs, also in yeast cells. Polyprenols (α-unsaturated isoprenoid alcohols) occur in green tissues of many plants and have also been found in bacteria. Studies of the structure of isoprenoid alcohols revealed that they always occur as a mixture ("family") of compounds differing in the number of isoprene units per molecule. The polyprenol and/or dolichol pattern can be considered as a chemotaxonomic criterion. The mixture of dolichols isolated from animal cells consists of seven or eight compounds irrespective of the organism. Dolichol-16, -18, and -19 have been found as the predominating prenologues in yeast, rat, and human, respectively (2). In contrast, plant polyprenols represent a huge diversity of chain length (3). In green tissue of some plants the family of polyprenols has four or five members (*Rhus typhina*, dominating prenol-11) in contrast to the polyprenol mixture ranging from prenol-14 to prenol-110 from *Lumnitzera racemosa* (4). However, the chain length of polyprenols isolated from more than 2,000 plants studied so far was found to be shorter. The search for plants accumulating polyprenols with chains longer than 120 isoprenoid units, to fill the gap up to natural rubber polymers (more than 160 isoprene units found in some plant species and mushrooms) (5), continues. The formation of such complicated mixtures has not been explained yet. The knowledge of the properties and regulation of *cis*-prenyltransferase activity (the enzyme responsible for the formation of polyprenols) in the plant system is far from complete (reviewed in ref. 6). More is known about the mammalian enzyme (7). The gene for the first bacterial *cis*-prenyltransferase from *Micrococcus luteus* has been cloned (8).

Studies of the biological role of dolichols in mammalian tissues (liver, brain, kidney) and polyprenols in plant tissues revealed that only a portion of these lipids accumulated in the form of (mono/di-)phosphates is active in the cell as cofactors of glycoprotein biosynthesis (9, 10). However, phosphorylated, and sometimes also glycosylated, forms are predominant in dividing cells and *Saccharomyces cerevisiae* (11, 12). Their putative role in protein prenylation has also been postulated (13). The portion of polyprenols and dolichols in the cell found as free alcohols or esters with carboxylic acids was considered as a structural component of the membranes. Biophysical studies showed that isoprenoids modulate the properties of model membranes. These compounds were found to increase the fluidity and permeability of the membranes, possibly resulting in enhanced fusion of membranes (14). Both polyprenol and dolichol contents show progressive increase during aging. In plants, for example, in 6-year-old needles a 20-fold increase in the amount of accumulated polyprenols was observed (15). In human tissues the extent of increase was different (e.g., 7-fold in lungs, and 150-fold in pancreas)

Abbreviations: HPLC, high-performance liquid chromatography; Pren-16, prenol-16, prenol composed of 16 isoprone residues.

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while neonatal and 81 year of age levels were compared (16). The levels of phosphorylated derivatives increased to a more limited extent, that is, 2- to 6-fold. The elevation of dolichol contents of rat tissues ranged from 20- to 30-fold, and dolichyl phosphate was almost not changed. In all of these cases, the pattern of polyisoprenoid alcohols was not changed. Under pathologic and experimental conditions (carcinogenesis, treatment with inducers of the endoplasmic reticulum or peroxisomes, feeding with specific diets) both the content and pattern of dolichol and dolichyl esters was found to be changed in contrast with the little or no change of dolichyl phosphate level (reviewed in ref. 14). For example, the content of dolichol was increased and simultaneously the relative amounts of longer polyisoprenoid alcohols were increased in preneoplastic rat liver cells (17). On the other hand, a decrease in dolichol level was noted in hepatocarcinoma of rat (J. Olsson, unpublished results, 1999).

In many biochemical and biophysical studies the access to single prenologues is of great importance. The aim of this investigation was to develop an efficient and fast preparative method for isolation of individual isoprenoid alcohols.

MATERIALS AND METHODS

Reagents

Chemicals were purchased from the following companies: Sigma-Aldrich Chemie (St. Louis, MO), J. T. Baker (Phillipsburg, NJ), Merck (Darmstadt, Germany), and Polish Chemicals Company (POCh, Gliwice, Poland). All high-performance liquid chromatography (HPLC) solutions were sparged with helium during use.

The dolichol mixture was obtained from the Collection of Polyprenols (Institute of Biochemistry and Biophysics, Polish Academy of Sciences [IBB PAS], Warsaw, Poland).

Plant material

Leaves of *Ginkgo biloba*, *Sorbus intermedia*, and *Potentilla aurea* were collected during the fall season in the Botanical Garden, Polish Academy of Sciences, Powsin. Lipids were extracted with hexane–acetone 1:9 (v/v), and after alkaline hydrolysis polyprenol fractions were isolated by flush chromatography on silica gel column with a stepwise gradient of diethyl ether in hexane from 0 to 30%, as described previously (3). The resulting polyprenol (or dolichol) mixture is practically devoid of other isoprenoids (i.e., sterols, ubiquinone, etc.).

Apparatus

Reversed-phase HPLC was performed with a Waters (Milford, MA) system (two 510 Waters pumps). Analytical separations were performed on a 60 × 4.6 mm column packed with ODS-Hypersil (3 μm; Knauer, Berlin, Germany). Semipreparative separations were performed on a 100 × 10 mm column packed with ODS-Hypersil (5 μm; Chromtech, Stockholm, Sweden). Absorption of eluate was measured with a Waters UV detector set at 210 nm for analytical runs and at 215 nm for semipreparative runs.

High-performance liquid chromatography of isoprenoids

Semipreparative analysis. Polyisoprenoids were separated with a gradient starting with 90% solvent A (methanol–isopropanol–water, 60:40:5 v/v/v) and 10% solvent B (hexane–isopropanol, 7:3 v/v) at an initial flow rate of 1.5 mL/min. After 3 min of isocratic elution the flow was increased to 6 mL/min and the gradient was initiated as follows: 18 min, 15% B; 23 min, 16.5% B; 28 min, 18.5% B; 33 min, 21% B; 37 min, 24% B; 42 min, 28% B; 47 min, 33% B; 57 min, 48% B; 67 min, 68% B; and 72 min, 88% B. The amount of polyprenol mixture injected per run was up to 20 mg in 50 μL of chloroform–methanol 2:1 (v/v).

Analytical analysis

Polyisoprenoids were separated with a linear gradient starting from 90% A (methanol–isopropanol–water, 60:40:5 v/v/v) to 60% B (hexane–isopropanol, 7:3 v/v) in 25 min at a flow rate 1.5 mL/min.

RESULTS AND DISCUSSION

Lipids extracted from various plants as well as yeast, animal, and bacterial sources contain mixtures of isoprenoid alcohols. Simultaneously, single polyprenols and dolichols are required in biochemical and biophysical experiments, for example, in studies of glycoprotein biosynthesis, model membrane structure, and protein prenylation or apoptosis (18). Available data suggested that the specificity of certain enzymes [e.g., glucosyltransferases (19) or protein prenyltransferases (20)] was chain length dependent. The method of separation of polyprenol mixtures into individual polyprenols was based on classic chromatography on Lipidex-5000. Because this method was time and solvent consuming it was important to develop new preparative techniques for this purpose.

It should be noted that the biological material used as the source of polyisoprenoids must be selected carefully before performing the extraction, purification, and separation procedure. In addition to appropriate chain length of required alcohols, the chemical form (phosphorylated, glycosylated, or esterified with carboxylic acid or covalently protein bound) of polyisoprenoid alcohols in the tissue must also be taken into consideration. To simplify the initial steps and increase the yield of preparation, plant photosynthetic tissue (at the end of the vegetation season) for polyprenols and mammalian liver (originating from aged animal) for dolichols, are recommended for large-scale preparations. In these cases extensive extraction with organic solvents and alkaline hydrolysis are sufficient (3). Phosphorylated derivatives of isoprenoids are removed from the analyzed fraction during the purification step on silica gel; however, to diminish losses of biological material containing a high level of phosphorylated and glucosylated derivatives acid hydrolysis (21) and enzymatic digestion in the case of α-saturated isoprenoid phosphates are also required (22).

In the present investigation separation from three plant species of polyprenols differing in the chain length of the dominating prenol and also polyprenol range was achieved (**Fig. 1**). In our system it was possible to analyze as much as 20 mg of prenol mixture per run. This amount of lipid injected per run was dissolved in 50 μL of chloroform–methanol 2:1 (v/v), and to avoid a high-pressure jump the initial flow was 1.5 mL/min (see Materials and Methods). Each polyprenol was collected manually, at the threshold of 8 to 10 milliabsorbance units from the baseline value. In all cases the recovery at the HPLC step was

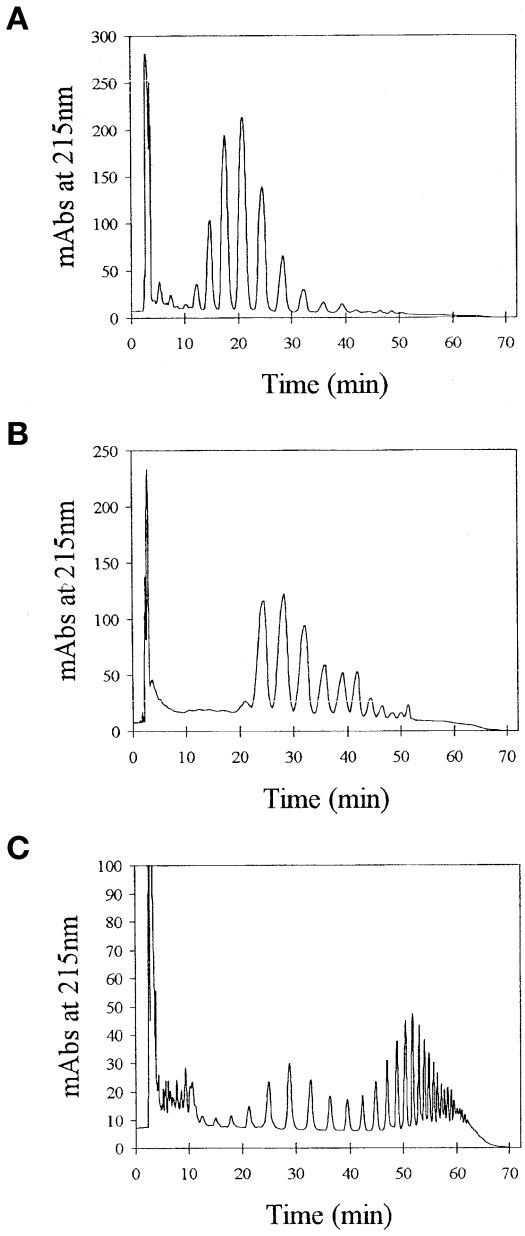

Fig. 1. Semipreparative HPLC separation of polyprenol mixture isolated from (A) *Ginkgo biloba*, (B) *Sorbus intermedia*, and (C) *Potentilla aurea*. For details of chromatographic conditions see Materials and Methods.

higher than 95%. The range up to 20 mg of isoprenoids injected per run was found to be optimal because larger amounts of polyprenols required prolonged times of analysis and consequently larger solvent consumption.

The reversed-phase chromatography system seemed to be much more efficient than straight-phase chromatography when isoprenoid prenologues were to be separated. The latter generally provides the possibility of loading greater amounts of lipids but reasonable separation of prenologues demands low flow rates and was also achieved only when limited amounts of lipids were injected per run (23).

Excellent baseline separation of a mixture of polyprenols from *G. biloba* allowed for complete separation of the whole family (with prenol-17 and -18 dominating) (Fig. 1A). The gradient described in Materials and Methods gave the best separation among several tested. *Sorbus intermedia* contained a prenol mixture ranging from prenol-15 up to prenol-50; however, the dominating prenols were composed of 18 to 29 isoprene units. Applied experimental conditions allowed for baseline separation of prenols in this predominant range (Fig. 1B), and a fraction containing longer polyprenols could be separated in a second run under modified chromatographic conditions. A similar result was obtained for a prenol mixture from *P. aurea* (Fig. 1C). In this two-family mixture of polyprenols the applied elution conditions allowed for separation of polyprenols from the first group (with prenol-19 dominating). Although separation of polyprenols with chains longer than 26 isoprene units was sufficient for analytical purposes the method requires some modifications for preparative applications. Using analytical HPLC, the purity of the polyprenols was found to be 95–98%. An analytical run of selected polyprenol isolated from a mixture originating from *Sorbus* is shown in **Fig. 2**.

The method of separation described above may be used for separation of dolichols (α-saturated isoprenoid alcohols) isolated from mammalian tissues. The HPLC spectrum of a well-separated pig liver dolichol mixture is shown in **Fig. 3**. As the isoprenoid alcohol pattern is different in this case, the run could be shortened (e.g., 50 min).

Isoprenoid alcohols are difficult to separate both by conventional chromatography techniques and by analytical HPLC. Time-extended liquid chromatography (24) performed on a Lipidex column requires from 5 weeks to 5 months (for longer polyprenols). The longest polyprenol fraction separated in this way contained up to 43 isoprene units. In spite of prolonged chromatography time, overlapping of polyprenols in some fractions obtained from the Lipidex column resulted in a decrease in recovery (approximately 70%).

Special attention must be given for samples containing mixtures of polyprenols and dolichols (25). A single semipreparative HPLC run would result in an isolation of a mixture of polyprenol and dolichol of the same chain length in the same fraction. An additional polyprenol and

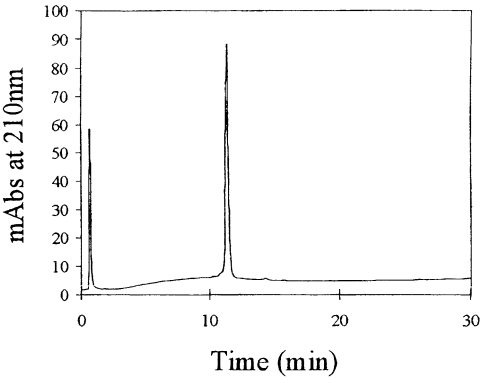

Fig. 2. Analytical HPLC run of prenol-19 isolated by semipreparative HPLC separation of a prenol mixture (Fig. 1B). For details of chromatographic conditions see Materials and Methods.

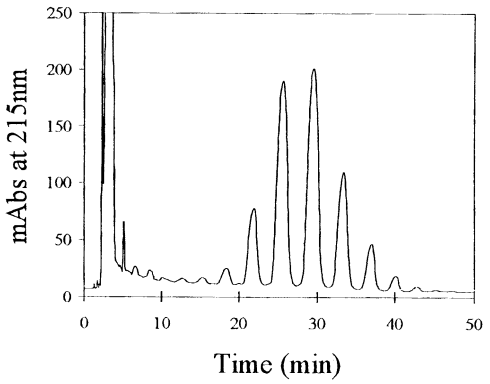

Fig. 3. Semipreparative HPLC separation of the dolichol mixture isolated from pig liver. For details of chromatographic conditions see Materials and Methods.

dolichol HPLC separation step should be performed by application of a straight-phase chromatographical system.

The amount of lipid injected per run makes it possible to obtain a single, pure polyprenol on a 100-mg scale over 2–3 days. This has never been possible with classic column chromatography, by which separation of shorter polyprenols must be performed for 1 month, and separation of longer polyprenols requires several months.

As can be seen in Figs. 1 and 3 the baseline separation achieved on a semipreparative column provides the opportunity of using this system for regular preparative applications. The purity of polyprenols prepared on this way is high. Because of the high reproducibility of retention time values observed during several sequential runs automatization of the procedure is also possible. The process of polyprenol separation can be fully automated by completing the apparatus described above with an automatic injector and fraction collector collecting the "windows" of higher absorbancy of polyprenols. This new method of separation of isoprenoid alcohols offers the possibility of preparing high amounts of pure polyprenols and dolichols for further experiments.

This work was supported by the Polish State Committee for Scientific Research project 6PO4A 046 12. Traveling grants from the Royal Swedish Academy of Sciences (awarded to To.C. and J.O.) are gratefully acknowledged. The authors are also grateful to Prof. Gustav Dallner (Stockholm University) for lending a HPLC column.

Manuscript received 20 January 2000 and in revised form 3 April 2000.

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