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Note

High-performance liquid chromatography of isoflavonoid phytoalexins from soybean (*Glycine max*, L. Harosoy 63)

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The fungitoxic phytoalexins are often synthesized during an incompatible host–pathogen interaction[1] and they are believed to play a role in disease resistance[2,3]. Typical phytoalexins in soybenas are the three glyceollin isomers shown in Fig. 1, the structures of which were elucidated by Lyne *et al.*[4].

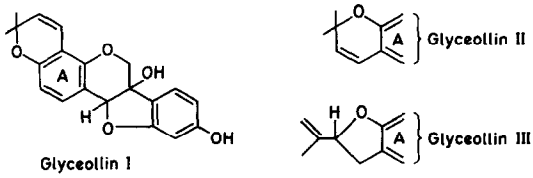

Fig. 1. Structures of the three glyceollin isomers of soybean.

Accumulation of phytoalexins can be induced by pathogenic fungi such as *Phytophthora megasperma* f. sp. *glycinea* (Pmg)[5] or by specific glucan elicitors which are components of mycelial cell walls of the pathogen[6–8]. Abiotic elicitors such as heavy metal ions can also induce the normal pterocarpan pattern in soybeans[9]. The glyceollin isomers together with their precursors can be isolated from hypocotyls and cotyledons of elicitor-treated soybean plants. Generally they are extracted according to Ebel *et al.*[10] and determined by measuring the absorbance of the total extract at 280 nm[11,12]. Further, the glyceollins can be isolated by thin-layer chromatography[13] or by high-performance liquid chromatography (HPLC) using silica columns[14,15]. In this paper the rapid separation of glyceollins by HPLC on different reversed-phase columns is described.

EXPERIMENTAL

Apparatus

An HPLC gradient system from Beckman (Munich, F.R.G.), consisting of a two-wavelength detector (Type 165), two pumps (Type 112), Organizer (Type 340), Controller (Type 420) and printer (Type C-R2AX), was used. Four different analytical reversed-phase columns were compared: Ultrasphere-ODS (5 μm), 250 × 4.6,

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250 × 2.0 and 150 × 2.0 mm I.D., and Ultrasphere-ODS (3 μm), 75 × 4.6 mm I.D. The glyceollin isomers were monitored at 280 nm. A Kontron Uvikon 810 spectrophotometer was used for measuring the absorption spectra of the different pterocarpans.

Materials

Soybean seeds (*Glycine max* L. Harosoy 63) were obtained from Prof. J. D. Paxton, University of Illinois, U.S.A. Seedlings were grown on autoclaved hydroponic stones under continaous light (8000 lux) in a growth chamber at 26°C and 40% relative humidity. Six-day-old cotyledons were sterilized with hypochlorite solution (0.75%) and sliced from the underside with a sterile razor blade. A 70-μl droplet of elicitor solution (equivalent to *ca.* 0.3 μg of glucose) dissolved in 10 m*M* phosphate buffer (pH 7.2) was placed on the cut surface and incubated in a growth chamber (26°C, 98% relative humidity) for 22 h. All preparations were carried out under sterile conditions. Twenty cotyledons were used for each experiment. Gentamycin sulphate (100 μg/ml) as an antibiotic was present in all solutions applied to cotyledons. The extraction of glyceollin isomers was carried out as described by Ebel *et al.*[10]. Before HPLC analysis, all extracts were subjected to membrane filtration (pore size 0.2 μm).

Pmg elicitor was isolated from mycelial wall fractions of the fungus *Phytophthora megasperma* f. sp. *glycinea* race 1 as described by Keen *et al.*[16], in cooperation with Dr. E. Ziegler, Aachen, F.R.G.

The standards of the three glyceollin isomers were a gift from Prof. J. Ebel, University of Freiburg, F.R.G. The trihydroxypterocarpan (THP) standard was prepared according to Zähringer[17].

Methanol (HPLC grade) was purchased from Promochem, Wesel, F.R.G. Methanol and water were degassed and subjected to membrane filtration (pore size 0.2 μm) before use.

RESULTS AND DISCUSSION

HPLC analysis of the pterocarpan extract on different reversed-phase columns

A typical elution pattern for an elicitor-induced pterocarpan extract with a 5-μm Ultrasphere-ODS column (250 × 4.6 mm I.D.) is shown in Fig. 2. A water–methanol gradient was used as the eluent. The separation was finished after 31 min. The conditions used gave a complete separation of THP and the three glyceollin isomers.

The separation can be accelerated by using an Ultrasphere-ODS column of I.D. 2 mm (Fig. 3). The necessary low flow-rate of only 0.3 ml/min is a further advantage of this type of column.

As shown in Fig. 4, the separation can be further accelerated by using a 5-μm Ultrasphere-ODS column of I.D. 2 mm and a length of only 150 mm. The whole separation is finished within 16 min. The use of a 3-μm Ultrasphere-ODS column of length 75 mm and I.D. 4.6 mm is most appropriate (Fig. 5) when the flow-rate is increased to 1.2 ml/min.

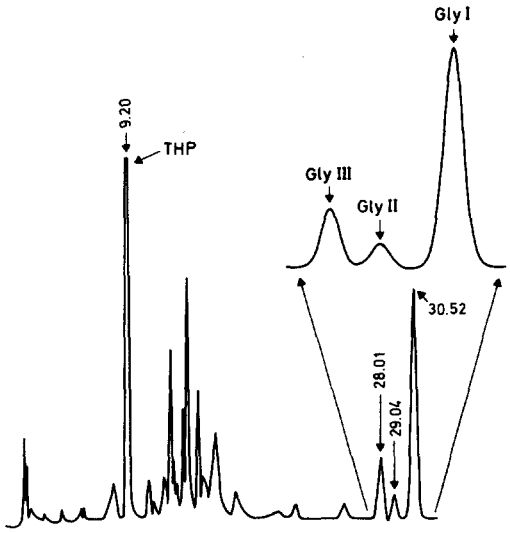

Fig. 2. Gradient elution profile of a pterocarpan extract from soybean using a 5-μm Ultrasphere-ODS, column (250 × 4.6 mm I.D.). Flow-rate, 1.0 ml/min. Eluent A, water; eluent B, methanol. Gradient: 0–3.5 min, 28% B; 3.5–10.5 min, 28–56% B. Numbers at peaks indicate retention times in min.

UV spectra of glyceollin isomers and trihydroxypterocarpan (THP) after HPLC separation

The three glyceollin isomers and THP were colected during different HPLC runs with a reversed-phase 5-μm Ultrasphere-ODS column (250 × 4.6 mm I.D.). After concentration of the effluents the spectra were recorded with a Kontron Uvikon 810 spectrophotometer. The peak maxima of all the glyceollins are located at 285 and 286 nm. However, glyceollin III and glyceollin II show further maxima at 291 and 318 nm. THP exhibits two typical maxima at 285 and at 280 nm (Fig. 6).

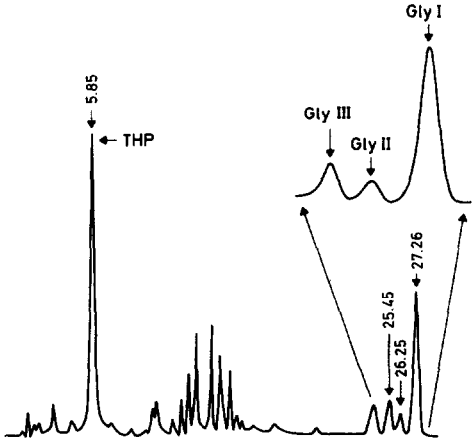

Fig. 3. Gradient elution profile of a pterocarpan extract from soybean using a 5-μm Ultrasphere-ODS column (250 × 2.0 mm I.D.). Flow-rate, 0.3 ml/min. Eluent A, water; eluent B, methanol. Gradient: 0–3.5 min, 30% B: 3.5–8.5 min, 30–56% B.

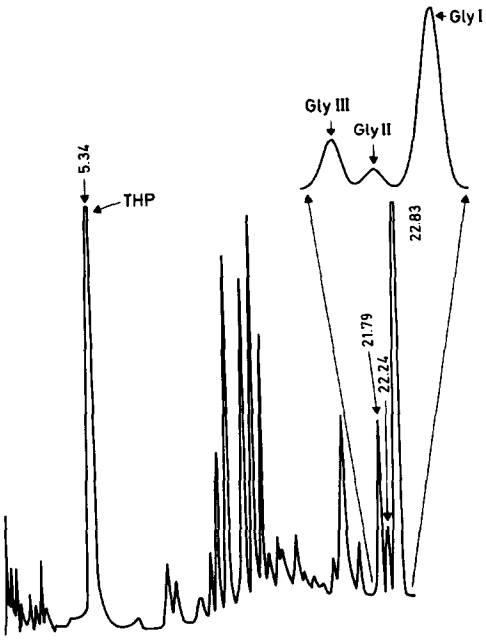

Fig. 4. Gradient elution profile of a pterocarpan extract from soybean using a 5-μm Ultrasphere-ODS column (150 × 2.0 mm I.D.). Flow-rate, 0.35 ml/min. Eluent A, water; eluent B, methanol. Gradient: 0–3.5 min, 25% B; 3.5–10.5 min, 25–56% B.

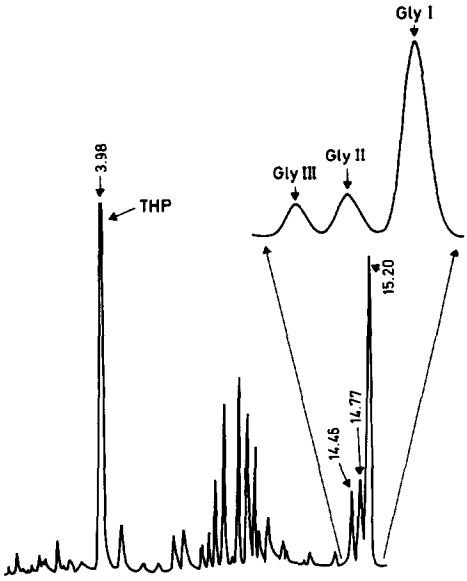

Fig. 5. Gradient elution profile of a pterocarpan extract from soybean using a 3-μm Ultrasphere-ODS column (75 × 4.6 mm). Flow-rate, 1.2 ml/min. Eluent A, water; eluent B, methanol. Gradient: 0–4 min, 26% B; 4–10 min, 26–55% B.

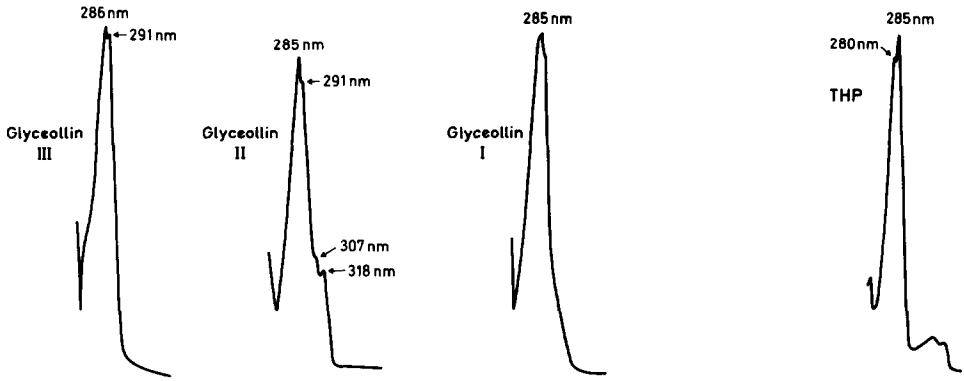

Fig. 6. Spectrum of THP and glyceollin I, II and III measured spectrophotometrically after HPLC separation.

Recording of UV spectra of glyceollin isomers and trihydroxypterocarpan (THP) during the HPLC run

During an HPLC run with a reversed-phase 5-μm Ultrasphere-ODS (250 × 4.6 mm I.D.) the three glyceollin isomers and their precursor THP were scanned without stop flow. The scan rate was 20 nm/sec. As shown in Fig. 7, all the spectra are very similar to those shown in Fig. 6. Owing to the high scan rate of 20 nm/sec the two maxima of THP, glyceollin II and III cannot be detected as different peaks.

Absorbance ratios of the different pterocarpans monitored during the HPLC run

With the aid of the two-wavelength detector it is possible to monitor the ratio of the absorbances at two different wavelengths simultaneously. This ratio can be used to obtain information on the purity of the substances. The two wavelengths were set at 280 and 293 nm for the three glyceollin isomers and at 280 and 290 nm

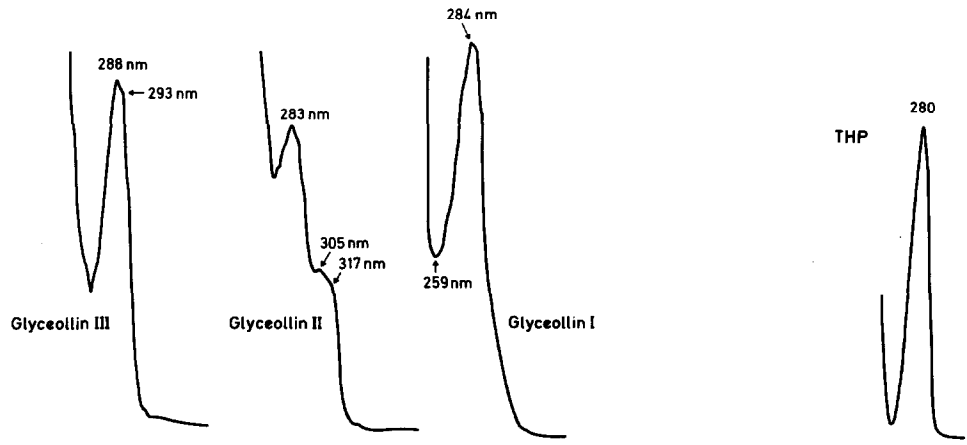

Fig. 7. Spectrum of THP and glyceollin I, II and III measured during the HPLC run with a two-wavelength detector without stop flow.

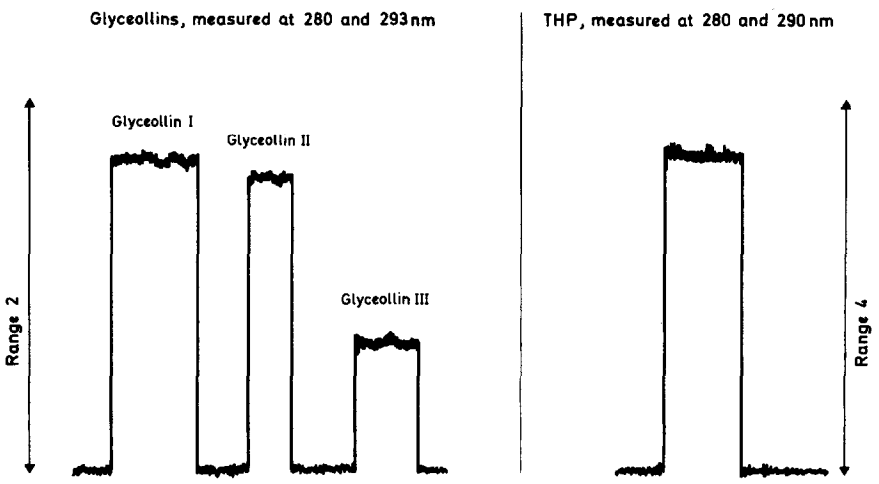

Fig. 8. Ratio of the glyceollin isomers and of THP during separation with a 5-μm Ultrasphere-ODS reversed-phase column (250 × 4.6 mm I.D.).

for THP. As shown in Fig. 8, a constant ratio was measured for THP and the glyceollin isomers, Which indicates that they are probably pure substances.

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