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FORMATION OF GALLOTANNINS IN CALLUS CULTURES FROM OAK (*QUERCUS ROBUR*)

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Key Word Index—*Quercus robur*; Fagaceae; oak; callus culture; high-pressure liquid chromatography; gallotannins; enzymes.

Abstract—Among a variety of callus cultures derived from shoots of oak seedlings, a few strains were found to contain gallotannins. As determined by HPLC on silica gel, the spectrum of these substances ranged from tri- to nonagalloylglucose, with a clear preference for tetra- to hexagalloylated derivatives. Closer analyses based on co-chromatography with authentic references, by means of reversed-phase HPLC, indicated the occurrence of minor amounts of 1,2,6- and 1,3,6-trigalloylglucose. Two of the major peaks were tentatively identified as 1,2,3,6-tetragalloylglucose (plus traces of the 1,2,4,6-isomer) and 1,2,3,4,6-pentagalloylglucose. A significant additional peak detected in one culture was ascribed to 1,6-digalloylglucose. In addition, enzymes catalysing the synthesis of β-glucogallin, the first intermediate in the biogenesis of gallotannins, could be isolated from the cultures.

INTRODUCTION

In the course of recent studies on the enzymology of gallotannin biosynthesis we were frequently confronted with serious problems resulting from the seasonal unavailability or unsuitability of the plant material, i.e. young oak (*Quercus robur*) leaves (*cf.* [1]). This prompted us to establish callus cultures of this species as a potential alternative enzyme source. Apart from this practical approach we became aware that, to our knowledge, almost no data on gallotannins from tissue cultures have been published, a fact which is in clear contrast to the many investigations dealing with intact plants (*cf.*, e.g. [2–5]). Here, we report that callus cultures from oak do have the potential of synthesizing various gallotannins and that, in spite of the high concentrations of phenolic compounds within these cells, enzymes reported to date to be involved in the biosynthesis of tannins [1, 6, 7] can be isolated from such tissues.

RESULTS AND DISCUSSION

The morphological appearance of the oak tissue cultures grown either on LS or 4x-medium was very heterogenous. On both media, it ranged from bright, transparent calli to dark-brown and occasionally almost black cultures. This increase in colouration was paralleled by drastically reduced growth-rates. A representative variety of these different calli was selected for analyses on the eventual formation of gallotannins. The screening experiments were done by normal-phase HPLC on silica gel. A particular advantage of this method was the fact that it was unnecessary to possess the whole spectrum of isomers required as reference compounds because the different galloylglucose derivatives elute sequentially in groups according to their degree of galloylation. Moreover, we observed that plots of the degree of galloylation vs the logarithm of the retention time resulted in a straight line which enabled us to predict the nature of unknown peaks.

By this method, we detected a few cultures that actually contained gallotannins, as determined by co-chromatography and application of the above hemi-log plot procedure. The spectrum of these substances showed some variation with respect to the individual extracts. In general, it ranged from tri- to nonagalloylglucose, with a clear preference for tetra- to hexagalloylated derivatives. A representative example is depicted in Fig. 1. We further observed that gallotannins were produced exclusively in those calli which had been grown on LS medium. Among these, only dark-brown cultures of low meristematic activities obviously containing high concentrations of phenolics had gallotannins; it should be noted, however, that many cultures of identical appearance were devoid of such substances.

In further experiments the gallotannin containing extracts were analysed in more detail by means of reversed-phase HPLC, a method allowing the separation of galloylglucose isomers with the same degree of galloylation. For a typical example see Fig. 2. By co-chromatography with the authentic references available to us, we have tentatively identified two minor peaks as β-1,2,6- and β-1,3,6-trigalloylglucose. Two of the major peaks clearly co-chromatographed with β-1,2,3,6-tetragalloylglucose and β-1,2,3,4,6-pentagalloylglucose; in one case, a significant peak was ascribed to 1,6-digalloylglucose. It should be noted that we could detect only traces of β-1,2,4,6-tetragalloylglucose [*cf.* compound (c) in Fig. 2] in all cultures. This substance has been proposed as a biosynthetic intermediate, together with the 1,2,3,6-isomer [5].

To our knowledge, only one earlier report exists on the occurrence of gallotannins in cell cultures. Haslam and coworkers [3, 5] reported that oak calli contained small

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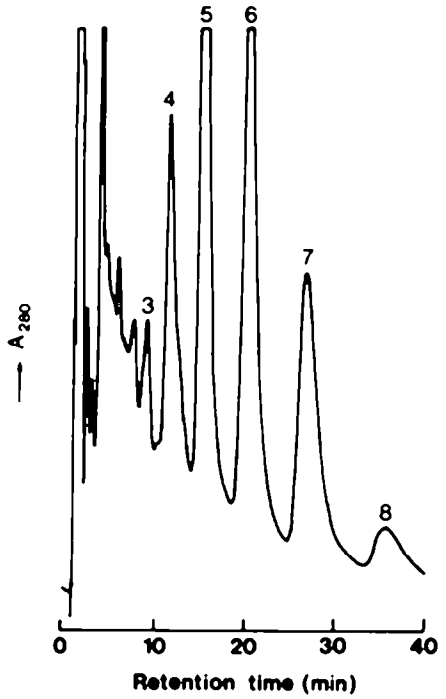

Fig. 1. Normal-phase HPLC on silica gel of an extract from oak callus tissue grown on LS medium. For details see Experimental. (3)–(8): Tri-, tetra-, penta-, hexa-, hepta- and octagalloylglucose.

quantities of pentagalloylglucose, together with gallic acid and pyrogallol. In extension of this observation, our results show that such cultures have the potential to synthesize and accumulate a much wider spectrum of gallotannins.

In this context, it is of interest that assays with cell-free extracts from these callus cultures revealed the presence of two enzymes which had originally been isolated from intact oak leaves, and which have been proposed to be related to gallotannin biosynthesis. These were a β-glucogallin forming, UDP-glucose-dependent glucosyltransferase [1, 6], and an acyltransferase catalysing a rapid exchange reaction between β-glucogallin and free glucose [7]. It was particularly surprising that these enzymes were active *in vitro* in spite of the presence of enormous amounts of phenolics which occasionally resulted in almost black-coloured cell-free extracts. It is conceivable that this fact reflects a high degree of adaptation of these enzymes to their phenolic substrates, and perhaps also to their cellular compartment.

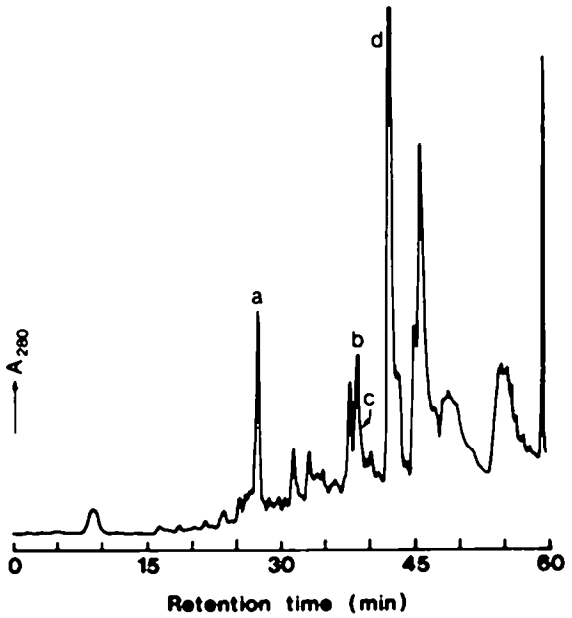

Fig. 2. Reversed-phase HPLC on RP-18 of an extract from oak callus. Gradient: 0–5 min (0% acetonitrile), 5–50 min (0–30% acetonitrile), 50–60 min (30–100% acetonitrile) [2, 5]. For further details see Experimental. (a) 1,6-Digalloylglucose, (b) 1,2,3,6-tetragalloylglucose, (c) 1,2,4,6-tetragalloylglucose (shoulder), (d) 1,2,3,4,6-pentagalloylglucose.

EXPERIMENTAL

Callus cultures were obtained from shoot segments of 1- to 3-month-old *Q. robur* seedlings grown in the greenhouse. The plant stems were surface-sterilized (5% NaOCl, 5–10 min), rinsed with sterile water, cut into segments of *ca* 0.5 cm length, applied to agar plates with LS medium (Linsmeier and Skoog [8]) or 4x-medium [9], and kept at 25° in the dark. Developing calli were transferred to fresh media for further culture.

Extraction of tannins was done according to ref. [10]. Briefly, fresh callus (5–10 g) was homogenized in MeOH (3 ml/g fr. wt) under ice-cooling. After filtration, the liquid was partitioned three times with petrol (60–80°). The MeOH-phase was concentrated *in vacuo* at 30°, diluted with H_2O, and extracted eight times with EtOAc. After evaporation of the organic phase at 30°, the solid residue was taken up in EtOH and analysed.

HPLC analyses were carried out with a Merck-Hitachi apparatus equipped with a low-pressure-mixing gradient system. Normal-phase HPLC was performed with silica gel columns (Merck LiChrosorb Si 60 CGC glass cartidges, particle size 5 μm, 180 × 3 mm i.d.) using the solvent *n*-hexane–MeOH–THF–formic acid (63:27:9:1; by vol.) plus 320 mg oxalic acid per 1000 ml (from ref. [11], as modified by Gross [7]). Reversed-phase HPLC was carried out on LiChrosorb RP-18 columns (Merck CGC glass cartridges, particle size 5 μm, 180 × 3 mm i.d.) with the acetonitrile–H_3PO_4 gradient given by Haslam and coworkers [2, 5]. Flow rates were 1 ml/min throughout.

Reference compounds. Authentic samples of the various galloylglucose esters mentioned above were generous gifts of Dr. G.-I. Nonaka (Fukuoka) and Prof. E. Haslam (Sheffield), respectively.

Enzyme studies. Callus tissues (15–20 g fr. wt) were homogenized and partially purified by ammonium sulphate fractionation as described previously [6]. With these cell-free extracts, the enzymatic formation of β-glucogallin from UDP-glucose and gallate was determined as given in refs [1, 6]; the galloyl exchange reaction between β-glucogallin and glucose was measured according to ref. [7].

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