Condensed and hydrolyzable tannins in two soybean (*Glycine max* L. cv. Madarin) cell lines that differ in their ploidy levels

KHALEQUZ ZAMAN

Institute of Molecular Biology, Jagiellonian University, Al. Mickiewicza 3, 31-120 Kraków, Poland

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Abstract

Comparison of SB₁ and SB₃ cell lines in both suspension and callus cultures showed that the latter, which produce much more dark brown pigments, had higher levels of condensed tannins. They also had higher total phenolics and flavonoid contents and L-phenylalanine ammonia lyase activity.

Key words: condensed and hydrolyzable tannins, ploidy levels, soybean, suspension and callus cultures

INTRODUCTION

Tannins are one of the polyphenolics, which are divided into hydrolyzable and condensed tannins, depending on their structure and responses to hydrolytic agents or enzymes (Bonner and Varner 1965, Haslam 1966).

Condensed tannins are important for brown pigmentation in plants (Goodwin 1965, Haslam 1966). Previously it was demonstrated that SB₁ and SB₃ cell lines differ from each other in several ways (Shargool and Jain 1984, 1986, Zaman 1988). SB₃ cells were higher in condensed tannins and lower in hydrolyzable tannins. The aim of this work was to examine the levels of tannins in SB₁ and SB₃ cells, which are respectively tetraploid and hexaploid (Shargool and Jain 1984, 1986, Zaman 1988).

MATERIAL AND METHODS

Two soybean cell lines referred in this report as SB₁ and SB₃ growing in suspension and callus cultures, were kindly provided by Dr. P. D. Shargool (Department of Biochemistry, Saskatchewan University, Saskatoon, Canada).

SB₁ cells attain their maximum growth in 4 days, while SB₃ cells take 6 days to reach that stage (Shargool and Jain 1984, 1986, Zaman 1988). After the SB₁ and SB₃ lines reached maximum growth in suspension and callus cultures, the cells were collected by filtration through miracloth and used for the extraction of tannins.

Extraction and identification of hydrolyzable and condensed tannins in SB₁ and SB₃ cells in suspension culture: Hydrolyzable tannins were extracted following the method of Barua and Houghton (1940). Five grams of soybean cells were boiled in 120 cm³ of water for one hour using a reflux condenser. The extract was filtered through cotton wool and the volume was made up to 150 cm³ after cooling and centrifuged at 12000 × g for 10 min. Condensed tannins were extracted following methods similar to those used by Strumeyer and Malin (1975). Five grams of soybean cells were boiled in 120 cm³ of 95% ethanol at 70°C for one hour. The extract was filtered through cotton wool and the volume was made up to 150 cm³ after cooling and centrifuged 12000 × g for 10 min. The same method was also used for the callus culture.

Preparation of non-tannins: The non-tannins preparation was determined by the Barua and Houghton (1940) procedure. One hundred cm³ of tannin extract from both cells were pipetted into a 250 cm³ volumetric flask. Fifty cm³ of gelatin were added and the volume was made up to 250 cm³ with acidic acid sodium chloride. The mixture was poured into an Erlenmeyer flask containing 20 g kaolin. After shaking for 15 minutes the mixture was filtered through filter paper. The filtrate was centrifuged at 12000 × g for 10 min. and the supernatant was saved for tannin titration.

Tannin titration: Quantitative estimation of tannins in extracts was carried out by an alkaline permanganate method of Barua and Houghton (1940). Ten cm³ of tannin extracts were pipetted into a 1000 cm³ Erlenmeyer flask, 25 cm³ of indigo-carmin was added next with a pipette and the mixture was diluted to 750 cm³, 0.04 N potassium permanganate was then run from a burette, one cm³ at a time, with brisk shaking.

As titration proceeded, the blue of the indigo-carmin passed through many shades to a final yellow with a faint pink at the rim. This was taken as the end point.

An aliquot of 25 cm³ of the non-tannin preparation was titrated in the same way as above. The amount of tannins was calculated by subtracting the non-tannins titre from tannin titre.

Extraction and identification of condensed tannins in SB₁ and SB₃ cell lines in suspension and callus cultures by absorption of Sephadex LH-20: Condensed tannins in SB₁ and SB₃ cell lines in suspension culture were extracted and identified according to the methods of Strumeyer and Malin (1975). Five grams of cells were mixed with 95% ethanol and heated for 60 min. The extract was filtered and centrifuged 10 min. at 12000 × g. A sample of the extract was

applied to a column of Sephadex LH-20 (2.2×27 cm) equilibrated with 95% ethanol. The column was eluted with ethanol (400 cm^3) at a flow rate of 24 cm^3 per hour and 5 cm^3 fractions were collected. The absorbance was determined at 280 nm using a Bausch and Lomb spectrophotometer. The column was then eluted with 50% acetone-water (200 cm^3) at a flow rate of 24 cm^3 per hour. Fractions of 5 cm^3 were collected and the absorbance was measured at 400 nm. The fractions were assayed for tannin by gelatin precipitation test as before. Only the acetone eluate contained compounds with tannin-like properties (Strumeyer and Malin 1975).

Non-tannin component of soybean cells SB₁ and SB₃ in suspension culture:

The non-tannin components obtained by Sephadex LH-20 chromatography were examined for the presence of phenolic compounds by TLC analysis and exposure to a variety of spray reagents (Haslam 1966, Strumeyer and Malin 1975). Solvent systems used to develop the chromatograms were as follows — benzene:methanol:acetic acid (45:8:4), chloroform:ethyl acetate:formic acid (5:4:1) and acetone:water (9:1). The same procedure was also used for callus culture.

Protein determination: Protein was determined according to the method of Bradford (1976). Details have been described elsewhere (Zaman 1988).

Enzyme assay: PAL activity of the acetone powder was determined by the method of Harborne (1973), as described in a previous paper (Zaman 1988).

RESULTS

The hydrolyzable tannins were extracted with boiling water and condensed tannins were extracted with 95% boiling ethanol (Barua and Houghton 1940, Strumeyer and Malin 1975). Tannins were removed from the extract by absorbing them on gelatin and the extract was titrated before and after the removal of tannins with potassium permanganate solution (Barua and Houghton 1940). Differences in both titres were used to calculate the amount of tannins in each extract. Tables 1 and 2 represent the amounts of hydrolyzable and condensed tannins in the two cell lines in both cultures. A comparison shows that the level of condensed tannins in SB₃ cells was 1.3 times higher in suspension cultures and 1.5 times higher in callus cultures than in SB₁ cell lines. It is important to note that the SB₃ line is richer in condensed tannins and SB₁ line exist mainly in hydrolyzable form in both cultures.

However, by utilizing Sephadex LH-20 it was also possible to both separate the tannins from non-tannin compounds (Strumeyer and Malin 1975) and recover approximately 90% of the original material applied to the column. As illustrated by the chromatograms in Figs. 1-4, when a 95% ethanol extract was applied to a (2.2×27 cm) column of Sephadex LH-20, the non-tannins were removed by exhaustively washing with 95% ethanol. These non-tannin

Fig.1. Separation of tannins from non-tannins by absorption on Sephadex LH-20 from SB₁ cell line in suspension culture. (○—○) Elution with 95% ethanol, (●—●) elution with 50% acetone

Fig. 2. Separation of tannins from non-tannins by absorption on Sephadex LH-20 from SB₃ cell line in suspension culture. (○—○) Elution with 95% ethanol, (●—●) elution with 50% acetone

Fig. 3. Separation of tannins from non-tannins by absorption on Sephadex LH-20 from SB₁ cell line in callus culture. (○—○) Elution with 95% ethanol, (●—●) elution with 50% acetone

Fig. 4. Separation of tannins from non-tannins by absorption on Sephadex LH-20 from SB₃ cell line in callus culture. (○—○) Elution with 95% ethanol, (●—●) elution with 50% acetone

components were resolved into a series of fractions visually distinguishable by color (Strumeyer and Malin 1975). Each fraction contained numerous components which were subsequently resolvable by TLC (Haslam 1966).

The tannins, on the other hand, remained tightly absorbed to the top of the column in 95% ethanol and could be observed as an immobile, orange-brown band.

Elution of the column with 50% aqueous acetone subsequently released the tannins as a symmetrical peak. Approximately 60% of the dry matter from the ethanol extract was found in the non-tannin fraction while 30% was obtained as tannin. Together they represent a total recovery of 90% of the material applied to the column.

Table 1
Condensed and hydrolyzable tannins in SB₁ and SB₃ cells in suspension cultures

Cell lines	Tannins (mg g ⁻¹ of cells)		Difference condensed-hydrolyzable
	condensed	hydrolyzable	
SB ₁	0.072	0.08	-0.008
SB ₃	0.096	0.02	0.076

Table 2
Condensed and hydrolyzable tannins in SB₁ and SB₃ cells in callus cultures

Cell lines	Tannins (mg g ⁻¹ of cells)		Difference condensed-hydrolyzable
	condensed	hydrolyzable	
SB ₁	0.170	0.083	0.087
SB ₃	0.257	0.095	0.162

The non-tannin components were eluted with ethanol and the pattern obtained is shown in Figs. 1-4. Fractions were combined and subjected to TLC on silica gel G, using the benzene:methanol:acetic acid (45:8:4) solvent system (Randerath 1966). The non-tannin components were identified using standard substances, characteristic colours and R_f coefficients (Haslam 1966).

DISCUSSION

The biosynthetic pathway of hydrolyzable tannins and condensed tannins are quite different (Hills 1956, Haslam 1966). It is interesting to notice that the SB₃ cell line has higher PAL activity (Zaman 1988), but is lower in

hydrolyzable tannins, which are also phenolic compounds. This is because the biosynthetic pathway of hydrolyzable tannins does not involve the reaction catalyzed by PAL (Hills 1956, Haslam 1966). Therefore, the biosynthesis of hydrolyzable tannins is not regulated by PAL activity, which is known as a regulatory point for the synthesis of phenolic compounds (Hills 1956, Haslam 1966).

However, the biosynthetic pathway of condensed tannins does involve this reaction and it is closely related with flavonoid synthesis (Goodwin and Mercer 1983). In fact, condensed tannins are the polymers of flavan-3-ols and flavan-3,4-diols (Hills 1956, Harborne 1965), which are a class of flavonoids (Bonner and Varner 1965). There is no evidence for hydrolyzable tannins to be involved in brown pigmentation (Haslam 1966). The condensed tannins are themselves brown colored in plant tissues (Goldstein and Swain 1963).

It is noteworthy (Tables 1, 2) that the SB₃ line is higher in condensed tannins, which are directly involved in brown pigmentation in both cultures, but lower in hydrolyzable tannins, which have no known effect on brown coloration (Haslam 1966, Goodwin and Mercer 1983). This finding is very important in that the higher condensed tannins are significant contributors to the production of much more brown pigments in SB₃ cells.

The polyphenols isolated from SB₁ and SB₃ cell lines from both cultures were resolved into tannins and non-tannins identified by virtue of their affinity for proteins (Harborne 1965, Haslam 1966, Strumeyer and Malin 1975).

Various methods have been used by others for the separation of tannins from other substances but often these present serious problems in the recovery of the tannins, such difficulties have long been associated with the fractionation of tannins (Haslam 1966). This paper also presents a simple method for the separation of soybean tannins from non-tannin materials based on the adsorption of the tannins on Sephadex LH-20 in 95% ethanol. The phenolic pigments in the non-tannin fraction were resolved further by TLC. The tannin polyphenolics were removed from the column by elution with 50% aqueous acetone after exhaustively washing with 95% ethanol, which eliminated non-tannin components.

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Taniny skondensowane i hydrolizujące w dwóch liniach komórek soi (Glycine max L. cv. Mandarin) różniących się ploidalnością

Streszczenie

Komórki SB₃, które wytwarzają więcej ciemnobrązowych pigmentów zarówno w kulturach zawiesinowych, jak i kallusowych, mają większą zawartość tanin skondensowanych niż hydrolizujących. Mają one także większą zawartość związków fenolowych, flawonoidów i większą aktywność lizy fenyloalaninowej.