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Inhibitors of neomycin phosphotransferase II enzyme-linked immunosorbent assay in grapevine (*Vitis vinifera* L.) leaves

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Summary

Grapevine tissue extracts are rich in compounds that may inhibit detection and/or extraction of protein, DNA, and RNA. One such example can be found in the use of enzyme-linked immunosorbent assays (ELISA) to detect neomycin phosphotransferase II (NPTII) in leaf tissue. The objective of this study was to identify grape leaf components that interfere with protein detection via ELISA. A series of compounds were identified, and tartaric and ellagic acids were most inhibitory to NPTII detection. Polyphenolics as well as the low pH of grape leaf extracts also reduced the effectiveness of ELISA detection.

K e y w o r d s : ELISA, ellagic acid, NPTII detection, polyphenols, tartaric acid, transgenic grapevine, *Vitis vinifera.*

Introduction

ELISA has been widely used to quantify bioactive components, proteins, hormones and virus titers because of its high efficiency and throughput (Li *et al*. 2001). However, detection of a target molecule may be severely restricted in the presence of other tissue components that interfere with ELISA reactions. It has been established that grapevine tissues contain relatively high concentrations of polyphenols and polysaccharides. Their presence strongly inhibits detection of other molecules including DNA (Demeke and Adams 1992) and certain enzymes (Loomis and Battaile 1996). However, the precise tissue components that interfere with detection have not been identified.

The *npt-II* gene is routinely used in transgenic research as a selectable marker. NPTII is not readily detectable in the leaves of transgenic grapevines because certain grapevine leaf components apparently interfere with ELISA procedures. This may be due to interactions between the molecule of interest and phenolic compounds (Bell *et al*. 1960). We report here on specific compounds in grapevine leaves that inhibitor NPTII detection via ELISA. These same compounds may also interfere with other ELISA detection procedures, or with the extraction of other compounds from grapevine tissues.

Material and Methods

P l a n t m a t e r i a l s : *Vitis vinifera* 'Chardonnay' (clone 95) and transgenic lines of this clone expressing NPTII were grown in a greenhouse, with 250 µmol·m^{-2}·s^{-1} PPF (Photosynthetic photon flux) light supplement during the daytime (14 h) at 22-28 °C.

N P T I I d e t e c t i o n i n l e a f e x t r a c t s a n d s u b - f r a c t i o n s : Fully expanded fresh leaf tissues (200 mg) were collected. Homogenized samples were suspended in 1 ml of a protein extraction buffer (PEB1). After centrifugation at 13,000 g for 10 min at 4 °C, the supernatant was used for ELISA, per manufacturer's recommendations (Agdia Inc., Elkhart, Indiana, USA).

S e p a r a t i o n o f l e a f c o m p o n e n t s : Twelve grams of dried leaf powder were extracted in quadruplicate using cold MeOH (80 %, v/v) at 5 °C for 24 h in the dark. Samples were centrifuged at 13,000 g for 5 min and the supernatant collected. Fractionations using organic solvents and further separations using silica column chromatography were conducted with modifications per Eom *et al.* (2006). Total phenolic content of each fraction was measured (Singleton and Rossi 1965).

L C - E S I / M S (l i q u i d c h r o m a t o g r a p h y - e l e c t r o s p r a y i o n i z a t i o n m a s s s p e c t r o m e t r y) a n a l y s i s : Bioactive fractions (25 µl) were injected into a High Performance Liquid Chromatography (HPLC) system (Agilent 1100 Microbore), using a Vydac C18 column (5 µm/300A/0.1 x 15 cm) with a Brownlee C18 guard column. Gradient elution HPLC was performed with solvent A, 0.2 % formic acid in water, and solvent B, 0.2 % formic acid in acetonitrile. LC-ESI/MS analysis was carried out on a mass spectrometer (Bruker Esquire LC), equipped with a 2795 separation module, 2996 photodiode array detector, and Masslynx software (Waters, Milford, Massachusetts). The flow rate of sample elution was 0.1 ml·min^{-1}. Column effluent was monitored at 280 nm and the mass spectrometer data were acquired in positive ion mode $(M+H)^+$ at a range of 100 to 1000 m/z. Mass data obtained were compared with chemical libraries in which grape metabolites had been identified previously. Selected compounds were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA) and tested for their effects on NPTII ELISA.

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Results

NPTII in transgenic grapevines: NPTII could not be detected via ELISA in leaf extracts of transgenic grapevines, although NPTII could be detected in root tissue and xylem sap (data not shown). ELISA detection of NPTII was strongly inhibited even when purified NPTII was added to extracts of non transgenic leaves.

Fractionation of leaf extract of 'Chardonnay': The methanol-soluble components comprised 9.4 % of the dried leaf tissues. We found that certain components of the ethyl acetate (M2) and aqueous (M3) fractions (M2-4, M2-7 and M3-2) were most strongly inhibitory of NPTII detection (Table). M2 had abundant phenolics, whereas M3 contained 5-fold less phenolics than M2. In M2 sub-fractions, NPTII detection was inhibited the most by fractions with the greatest concentration of phenolics, specifically M2-4, M2-5, M2-6 and M2-7. Among M3 sub-fractions, M3-2 had the strongest inhibitory effect upon NPTII detection, even though this fraction contained relatively low phenolic levels. We hypothesize that phenolic compounds were at least partially associated with inhibition of NPTII detection in M2 fractions, whereas other components must have been responsible for the inhibition observed in M3 fractions.

Chemical identification of active fractions using LC-ESI/MS: Numerous flavonoid compounds were identified in M2-4, including flavonols (kaempferol, quercetin and myrucetin), a flavononol (dihydrokaempferol), anthocyanidin derivatives (delphinidin-3-(6-p-coumaroyl)-glc-pyruvic acid and delphinidin-3-glc-acetaldehyde), and a flavan-3-ol (galloatechin). Three acidic compounds, ellagic acid, ethylcaffeoyltartrate and diethylcaffeoltartrate, were also detected in M2-4. Masses in M2-7 indicated that most chemical components were anthocyanidins combined with glucose. Ellagic acid was detected in both M2-4 at 28.8 min retention time and M2-7 at 30 min retention time on LC-ESI/MS. In the M3-2 fraction, major chromatogram peaks were observed within 10 min at 280 nm wavelength with HPLC. Mass data for five peaks in fraction M3-2 (MW = 120, 129, 202, 364 and 382) detected through 3 min retention time could not be matched with any previously reported grape phytochemicals.

Inhibitory effect of selected chemical components upon NPTII detection: Of the chemical components detected via LC-ESI/MS, thirteen were tested for inhibitory effects upon NPTII detection (Figure). Tartaric acid caused 73 % inhibition compared to a control, while ellagic acid inhibited NPTII detection by 39 %. Kaempferol and caffeic acid inhibited NPTII detection by 30 %. Other chemicals tested caused 20-30 % inhibition, except quercetin, genistein and xylose, which caused little inhibition (13, 7, and 0 % inhibition, respectively).

Discussion

Limitations in the detection of protein in grapevines may be due to several factors, including secondary metabolites, extract pH, as well as the conditions of the ELISA procedure. It has been reported that certain proteins interact with polyphenols and may precipitate, forming protein-

Table

NPTII detection and total phenolic contents in sub-fractions of Chardonnay leaf extracts

Solvent fractions from leaf tissues	Sub-fractions of solvent fractions	Amount of extracts (mg/12 g dry leaf tissue)	NPTII detection rate (% ± SE)	Total phenolics ($\mu g \cdot ml^{-1}$ ± SE)
M		1,128	85 ± 2	293 ± 2
M1		248	101 ± 2	225 ± 7
M2		583	56 ± 1	588 ± 8
	M2-1	265	74 ± 5	201 ± 1
	M2-2	145	90 ± 1	184 ± 5
	M2-3	13	92 ± 1	217 ± 5
	M2-4	7	31 ± 1	256 ± 5
	M2-5	10	48 ± 2	447 ± 6
	M2-6	21	46 ± 0	391 ± 5
	M2-7	78	33 ± 1	524 ± 4
	M2-8	10	53 ± 3	194 ± 4
M3		212	71 ± 1	112 ± 3
	M3-1	149	56 ± 2	101 ± 8
	M3-2	8	31 ± 4	59 ± 6
	M3-3	5	58 ± 1	92 ± 10
	M3-4	14	43 ± 3	57 ± 7
	M3-5	29	61 ± 1	57 ± 4
	M3-6	4	94 ± 3	67 ± 1

M: Methanolic extract of Chardonnay leaf. M1: hexane fraction. M2: ethyl acetate fraction. M3: Aqueous fraction. M2-1 to 8: Ethyl acetate fractions after column chromatography. M3-1 to 6: Aqueous fractions after column chromatography.

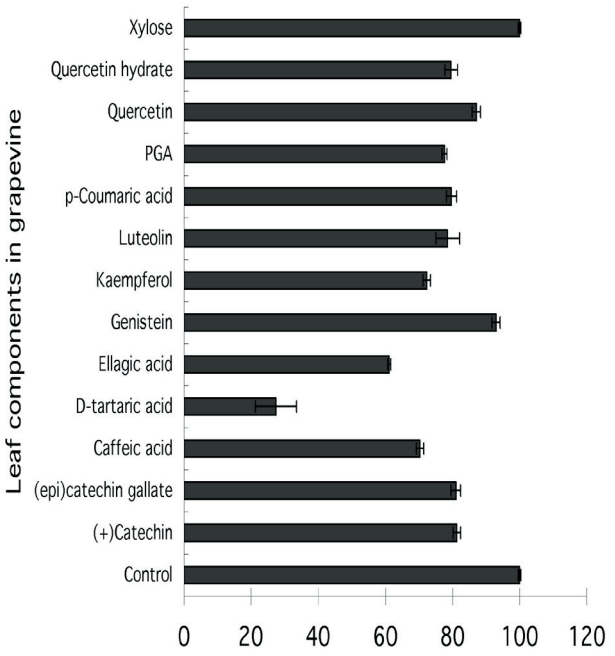

Figure: NPTII detection in the presence of selected chemical components. NPTII (150 pg) was mixed with each compound (1.0 mM) and dissolved in a protein extraction buffer. PGA: polygalacturonic acid.

polyphenol or protein-phenol-protein complex molecules (Haslam 1974). Protein precipitation caused by polyphenols (Quideau *et al*. 2003) may lead to reductions in protein present in a solution, and ELISA detection carried out with such a solution will be impaired. Our results showed that inhibitors of the ELISA reaction in grape leaf extracts were broadly distributed among the chemical groups separated by organic solvents (Table). The numerous widely distributed chemical groups may cause difficulty in separating inhibitors of ELISA detection from target proteins in leaf tissues of grapevine.

This project focused on the determination of which compounds in leaf tissue inhibited protein detection via ELISA. However, inhibition is not only caused by phenolic compounds, but also by pH. The extract of leaf debris after methanol extraction showed a very acidic pH, which led to inhibition of NPTII detection. Acidity may also be a factor in limiting protein detection. When NPTII standards were dissolved in a pH 3.5 version of PEB1 adjusted with hydrochloric acid, NPTII detection by ELISA was strongly inhibited (data not shown).

The complicated distribution of inhibitory chemical components makes it difficult to prepare samples free ELISA inhibitors. Experimental analyses with grapevine leaf tissue, including DNA, RNA and protein analysis (Demeke and Adams 1992, Loomis and Battaile 1996), may be more difficult than in other plant species due to the amount and distribution of phytochemicals with inhibitory effects. However, certain actions, such as controlling extract pH or eliminating polyphenols, may result in improved protein detection via ELISA, and possibly in other chemical detection and extraction procedures as well.

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