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MALDI-TOF ANALYSIS OF MIXTURES OF 3-DEOXYANTHOCYANIDINS AND ANTHOCYANINS

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Key Word Index—*Sorghum bicolor*; Poaceae; sorghum; mass spectrometry; MALDI; flavonoid; anthocyanins; anthocyanidins; 3-deoxyanthocyanidins.

Abstract—The mass spectrometric analysis of 3-deoxyanthocyanidins and anthocyanins present in crude extracts from sorghum plant tissue was carried out by matrix-assisted laser desorption ionization mass spectrometry (MALDI). Sensitivities to as low as 5 pmol/μl were easily attained for pure samples of the anthocyanidin, pelargonidin, and the anthocyanin, malvin. Sensitivities to the level of 15 pmol/μl were attained for 3-deoxyanthocyanidins present in crude extracts from sorghum plant tissue. Through its capability for analyzing very small quantities of these compounds in unpurified samples, MALDI provided a sensitive means for the detection of these flavonoid pigments in plant tissues. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Most plants use products of secondary metabolism to protect themselves from disease. When such compounds are synthesized in response to infection they are considered components of an active defense mechanism. Such secondary metabolites accumulate within the area of the infection site and, because of their toxicity they aid in restriction of the pathogen. Often, these compounds are also toxic to the plant that synthesizes them. Therefore, they must be compartmentalized, a process considered to require the specific channeling of biosynthetic intermediates to effectively separate them from the primary metabolic machinery of the cell [1–3].

A group of these compounds, known as phytoalexins, accumulate in response to the stress of infection, particularly attempted fungal infection [4, 5]. We have demonstrated the rapid accumulation of four 3-deoxyanthocyanidin phytoalexins in sorghum after inoculation with various fungal pathogens and nonpathogens [6–8]. It also was shown that phytoalexin synthesis occurs in a highly restricted manner within inclusions in the specific host cells that are under fungal attack [9]. Complete toxicity to fungal pathogens occurs at concentrations less than 10 μM for each compound as reported previously [7, 8].

Plasma desorption mass spectrometry (PDMS) has proven to be relatively successful in analyzing phenolic compounds [10, 11]. In particular, PDMS was used to identify and chemically characterize both anthocyanins and deoxyanthocyanidin phytoalexins [10–13]. However, our attempts to elucidate the potential intermediates in the biosynthesis of these compounds by PDMS have been frustrated by constraints of instrument sensitivity and sample purification procedures.

Matrix-assisted laser desorption ionization (MALDI) coupled with time-of-flight (TOF) mass analysis has proven to be particularly useful in the analysis of biomolecules [14, 15]. MALDI is considered a soft desorption ionization technique that enables M_r information to be obtained on both fragile and non-volatile high-mass molecules. This occurs because samples are included in crystals of a matrix material, usually an organic acid, which absorbs a substantial amount of the laser energy and allows for a soft desorption ionization of the sample. Sensitivity in the pmol μl^{-1} range for proteins can routinely be obtained with this method [14, 15]. The nominal M_r range accessible to MALDI analysis is up to 100 kDa and has been reported in certain studies to be well in excess of 200 kDa [16]. A wide range of biomolecular compounds, besides peptides and proteins, have been analyzed using MALDI, including oligonucleotides [17], oligosaccharides [18], and synthetic polymers [19] to name a few. In the present work, MALDI was used as a more sensitive tool to identify the deoxyanthocyanidin phytoalexins as well as anthocyanins

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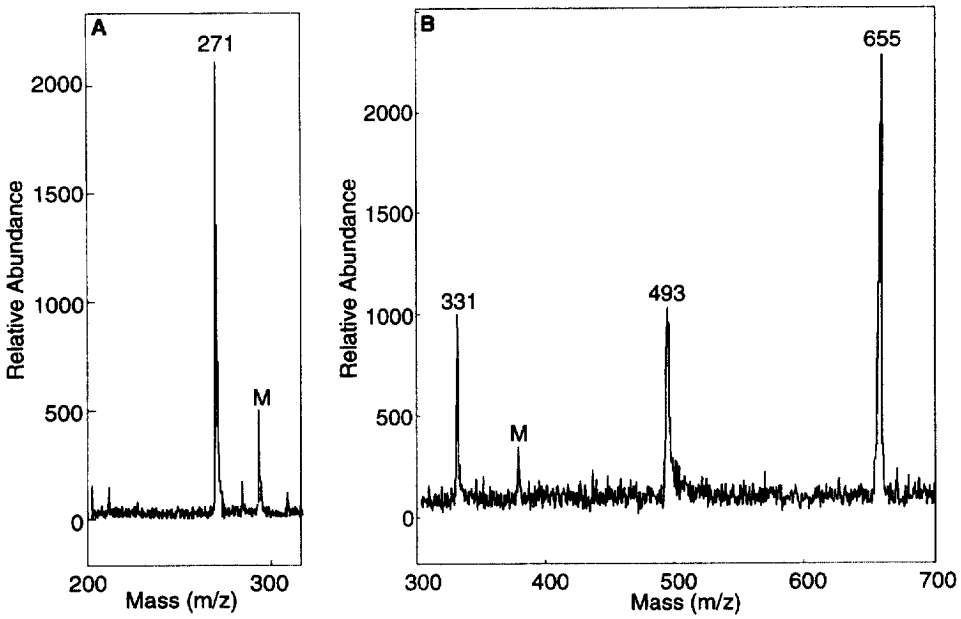

Fig. 1. MALDI mass spectra of the anthocyanidin pelargonidin chloride and the anthocyanin malvin chloride. Concentration for each was 50 pmol μl^{-1}. (A) The peak at m/z 271 corresponds to the pelargonidin cation. M indicates a matrix peak. (B) The peak at m/z 655 corresponds to the malvin cation. The peaks at m/z 493 and 331 represent deglycosylated fragments of the molecular ion. M indicates a matrix peak.

at pmol concentrations. To our knowledge, MALDI has not been used for the analysis of complex flavonoids such as anthocyanins and deoxyanthocyanidins.

RESULTS AND DISCUSSION

The ability of the MALDI-TOF method to analyze anthocyanins and anthocyanidins was first evaluated with the pure compounds pelargonidin chloride and malvin chloride. Pelargonidin chloride is an anthocyanidin with a M_r of 306. The mass spectrum obtained showed a predominant peak at m/z 271, which corresponds to the pelargonidin cation after the loss of the chloride anion (Fig. 1A). Other than minor matrix peaks no other peaks were evident indicating that pelargonidin itself was not degraded. This profile is consistent with the one previously observed by PDMS analysis [10, 11]. Upon MALDI analysis of the anthocyanin malvin chloride, a spectrum with a peak at m/z 655, which corresponds to the malvin cation was obtained (Fig. 1B). Two other peaks in the mass spectrum, m/z 493 and 331, represent fragments resulting from the subsequent losses of two glucose units. Although the same pattern of peaks was observed when PDMS was used to analyze malvin [10, 11], the relative intensity of the peaks was significantly different from that observed by MALDI. Moreover, in the MALDI spectrum, the predominant peak is the malvin cation m/z 655, whereas in the PDMS spectrum the major peak was the aglycone of malvin, the malvidin cation (m/z 331). The MALDI conditions used in this study cause less fragmentation of the anthocyanins than PDMS. Although mass spectra shown in Fig. 1 represent analyte concentrations of 50 pmol μl^{-1}, similar spectra were obtained with as little as 5 pmol μl^{-1} for malvin as well as for pelargonidin.

After observing that MALDI was able to detect the molecular cations of the pure anthocyanin malvin and the pure anthocyanidin pelargonidin, the method was used to analyze similar compounds in extracts isolated from sorghum. Exposure of etiolated sorghum seedlings to light results in the synthesis of the anthocyanin pigment cyanidin 3-dimalonyl glucoside. However, if the seedlings are both exposed to light and inoculated with conidia of the fungus *Cochliobolus heterostrophus*, they synthesize 3-deoxyanthocyanidin phytoalexin pigments in addition to the anthocyanin cyanidin 3-dimalonyl glucoside [20].

Etiolated sorghum seedlings (cv. DK46) were inoculated and the plants were incubated in the light for 48 h. The pigments were then isolated from the mesocotyls by extraction into methanol. Controls were plants treated in the same manner but not inoculated. MALDI analysis of the extract isolated from the control plants exhibited only a peak at m/z 621. However, the mass spectrum obtained for the extract from seedlings that had been inoculated also exhibited peaks at m/z 255, 269, 271, and 285 in addition to the ion at m/z 621 (Fig. 2). These ions are consistent with the deoxyanthocyanidins apigeninidin, 7-methoxy apigeninidin, luteolinidin, 5-methoxy luteolinidin, and the anthocyanin cyanidin 3-dimalonyl glucoside,

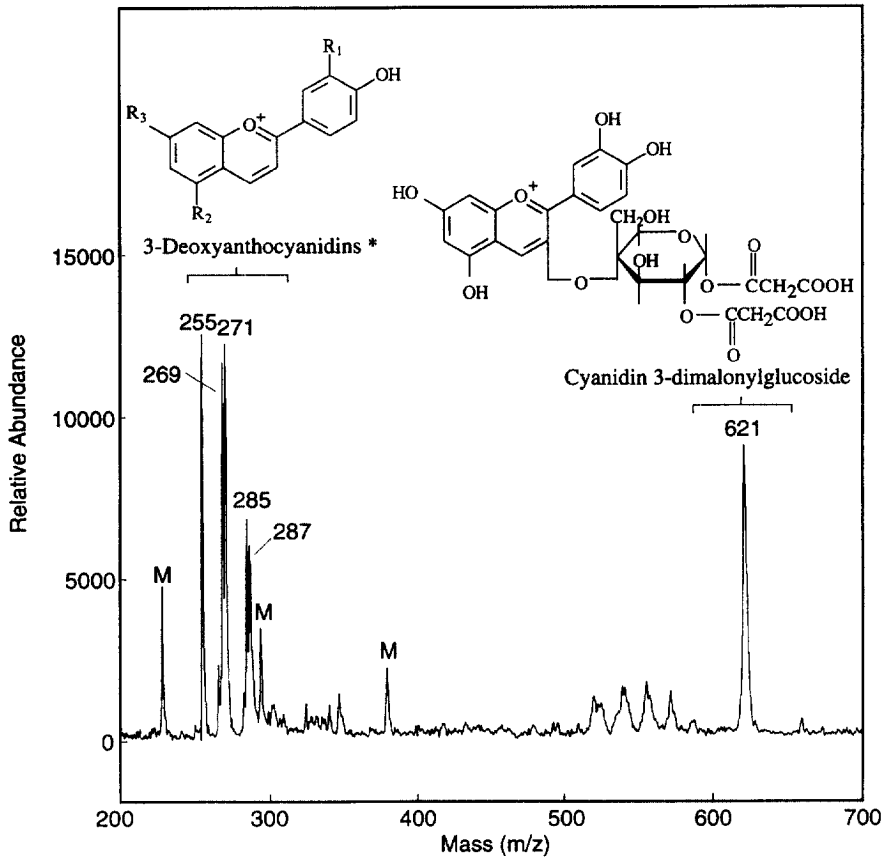

Fig. 2. MALDI mass spectrum of the pigment extract from mesocotyls of the sorghum cv. DK46 that had been exposed to light and inoculated with conidia of the fungus *Cochliobolus heterostrophus*. The spectrum shows the presence of the cation for cyanidin 3-dimalonyl glucoside (*m/z* 621) and of the aglycone fragment cyanidin (*m/z* 287). In addition, the 3-deoxyanthocyanidins apigeninidin (*m/z* 255), 7-methoxyapigeninidin (*m/z* 269), luteolinidin (*m/z* 271), 5-methoxyluteolinidin (*m/z* 285) were detected. M indicates matrix peaks. * Apigeninidin (*mw* = 255), R_1 = H, R_2 and R_3 = OH; 7-methoxy-apigeninidin (*mw* = 269), R_1 = H, R_2 = OH, R_3 = OCH_3; luteolinidin (*mw* = 271), R_1, R_2, and R_3 = OH; 5-methoxy-luteolinidin (*mw* = 285), R_1, R_3 = OH, R_2 = OCH_3.

respectively [20, 21]. An ion at *m/z* 287 was also present. This ion is indicative of cyanidin, which comes from the fragmentation of cyanidin 3-dimalonyl glucoside.

Based on HPLC analysis of these extracts, the concentrations of the deoxyanthocyanidins luteolinidin and apigeninidin were estimated to be approximately 15 pmol μl^{-1} in the tissue extract. The apparent concentration of the anthocyanin cyanidin 3-dimalonyl glucoside was about 80 pmol μl^{-1}. It is important to note that for MALDI analysis the extracts were analyzed without additional purification. In contrast, with PDMS analysis most samples had to be purified by HPLC or to be partially purified by partitioning with organic solvents prior to mass spectrometry [8, 13]. The ability of MALDI to analyze crude extracts is of significant importance as it avoids the loss of material that occurs during purification procedures.

This study demonstrates that MALDI can be used to detect pmol concentrations of flavonoid compounds in unpurified plant extracts. Although the 3-hydroxyanthocyanidins and the 3-deoxyanthocyanidins investigated here are structurally similar, current evidence indicates that they are synthesized via different pathways [20]. Standard biochemical analyses have not been successful for the evaluation of potential metabolic intermediates in deoxyanthocyanidin biosynthesis. Because of its sensitivity, MALDI, in combination with techniques such as diode array HPLC analysis, provides a means for the detection and identification of metabolic intermediates involved in deoxyanthocyanidin biosynthesis.

EXPERIMENTAL

Plant material and fungal inoculation

Seeds of the sorghum [*Sorghum bicolor* (L.) Moench] cultivar DK46 (Dekalb Pfizer Genetics) were imbibed in water at 28° overnight. The imbibed seeds were then planted in rolls of germination paper and

grown in the dark at the same temperature for 4 days. Half of the etiolated plants were inoculated with conidia of the fungus *Cochliobolus heterostrophus* (Drechs.) Drechs., an organism that causes resistance expression and the synthesis of phytoalexins by the plant. Seedlings were inoculated with a conidial suspension (1.0×10^5 conidia ml^{-1}) to which Tween 20 (1 μl ml^{-1}) was added as a wetting agent. The plants were incubated at room temperature and 100% relative humidity under constant light (60 mE m^{-2} s^{-1}) for 48 h. Another half was incubated under the same conditions with no inoculation.

Samples of mesocotyl tissue (approx. 200 mg) were collected 48 h after incubation/inoculation. The mesocotyls were excised from the seedlings, and 1 cm segments were removed from the base and the apex of each mesocotyl. The remaining tissue was cut into segments, weighed, and placed in 1 ml of HPLC grade MeOH. The anthocyanins and phytoalexins that had accumulated were allowed to leach from the tissue at 4 for 24 h after which the extracts were analyzed.

Matrix-assisted laser desorption ionization (MALDI) mass spectrometer

A PerSeptive Biosystems Voyager linear time-of-flight MALDI mass spectrometer was used in this study. The instrument used a nitrogen laser operating at 387 nm with a typical accelerating voltage of 28 kV. For sample preparation, the matrix, α-cyano-4-hydroxycinnamic acid (10 mg), was solubilized in 1 ml of a mixture of 1% TFA–H_2O–CH_3CN (1:4:5). The analytes were mixed with the matrix for 1 min after which 1 μl of the mixture was applied to the sample plate and the solvents were allowed to evaporate at room temp. Pigments selected as known analytes were commercial samples of pelargonidin and malvin. One μl of a pelargonidin stock solution of 100 pmol μl^{-1} and a stock solution of 10 pmol μl^{-1} was each mixed with 1 μl of matrix solution. The same procedure was carried out for the analyte malvin. For the extract from uninoculated sorghum mesocotyl tissue (cv. DK46), 1 μl of extract was mixed with 1 μl of matrix, and for the extract from inoculated mesocotyl tissue 1 μl of extract was mixed with 2 μl of matrix.

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