Phytosiderophores of the mugineic acid family, and the respective iron species, have been separated by anion-exchange chromatography with NaOH gradient elution. Two different detection methods were used in parallel, pulsed amperometry (PAD) for phytosiderophores and atomic absorption spectrometry (AAS) for iron. This combination enables identification of separated iron species. Up to five different iron species were separated and detected within 30 min – two different phytosiderophore species, two amino acid species, and one species which has not yet been identified but which is most probably a decomposition product of phytosiderophores. The detection limit was in the low µmol L⁻¹ concentration range, which is sufficiently low for determination in real plant samples, even after dilution. The method has been applied to root washings of iron-deficient wheat and barley plants and to a xylem exudate of non-deficient maize.

Keywords Phytosiderophores · Speciation · HPLC · Pulsed amperometry · AAS

Introduction

Phytosiderophores of the mugineic acid family are highly effective iron chelators released from roots of graminaceous plant species in response to iron deficiency [1]. The respective mechanisms of iron mobilization and iron uptake have been investigated in some detail [2, 3, 4, 5], and the chelation of other metals by phytosiderophores (e.g. zinc [6]) has also been considered. Methods for accurate determination of phytosiderophores are a prerequisite for such investigations. Highly sensitive and simultaneous determinations of the different mugineic acids (i.e. mugineic acid (MA), 2’-deoxymugineic acid (DMA), 3-hydroxymugineic acid (HMA), and 3-epi-hydroxymugineic acid (epi-HMA)) are based on ion-exchange or ion-pair chromatography with fluorescence detection using orthophthalaldialdehyde (OPA) as post-column derivatization agent [7, 8, 9, 10]. We have recently shown that pulsed amperometric detection (PAD) can be used as an alternative detection system after anion-exchange HPLC of mugineic acids [11].

Uptake studies are usually performed by measuring the correlation of phytosiderophores and iron (or other metals) in plant compartments, often with the help of labeled phytosiderophores [1, 6, 8]. The direct determination of the intact Fe-phytosiderophore-chelate, however, has not yet been achieved. Model experiments, at higher concentrations, have been performed with the intention of elucidation of the structure of the Fe-mugineic acid complex [4, 9, 12, 13, 14]. In the work discussed in this paper at least two different iron–phytosiderophore complexes were identified in the µmol L⁻¹ concentration range after anion-exchange chromatography by using PAD and AAS in parallel as selective detectors for phytosiderophores and iron, respectively.

Experimental

Plant culture and collection of samples

Hydroponic cultures of wheat (Triticum aestivum L.), barley (Hordeum vulgare L., cv. Europa and cv. Minori), and maize (Zea mays L.) were used for the experiments. Plant culture, composition of the nutrient solution, and collection of the samples for phytosiderophore analysis were performed as described previously [10]. Briefly, the germination was for four to six days on filter paper or quartz sand moistened with saturated calcium sulfate solution. Seedlings were then transferred to 2.5-L pots (40 seedlings per pot) with continuously aerated nutrient solution, which was changed at three-day intervals. Plant culture was performed in a growth chamber at 60% to 70% RH, with a light intensity of 220 µmol photons m⁻² s⁻¹, and a 16/8 h (25/22 °C) day-night regime.

For collection of root exudates from whole root systems plants were removed from the nutrient solution 2 h after onset of the light
period and the roots were washed twice for 1 min in deionized water. The root systems were then submerged in 500 mL double-distilled water for 3 h with continuous aeration. The root washings were filtered (Blue Ribbon No 5893, Schleicher and Schüll, Dassel, Germany) and subsequently vacuum-concentrated at 50 °C to a volume of 50 mL, which was used for HPLC analysis.

Phytosiderophore reference substances

Phytosiderophores, which are not commercially available, were isolated from iron-deficient plants at the “Institut für Pflanzenernährung”, Stuttgart, by use of preparative cation-exchange chromatography, according to the method of von Wieren et al. [6], and were further purified by thin-layer chromatography [10]. In the purified samples phytosiderophores were quantified by HPLC [10]. The purity of DMA, MA, and epi-HMA standards isolated by use of this procedure was at least 95%.

HPLC

HPLC was performed with a Knauer K1001 gradient pump with K1500 solvent organizer, solvent degasser, titanium mixing chamber, and Rheodyne 9010 injection valve with 50-µL sample loop. The separation column was a Dionex AS12 (250 mm × 4 mm i.d.) with Dionex AG12 guard column (50 mm × 4 mm i.d.). All capillaries and connections were made of PEEK.

Gradient elution

The sodium hydroxide gradient was mixed from ultrapure (Sera-dest) water (solvent A) and analytical grade 200 mmol L⁻¹ NaOH (solvent B). The composition of the gradient was: 0–7 min, 94%A+6%B; 7–17 min, linear gradient to 100%B, 17–30 min, 100%B. The column was then re-equilibrated to the starting gradient composition (94%A+6%B). The flow rate was 1 mL min⁻¹.

Precautions

To obtain reproducible retention times the carbonate content of mobile phases should be kept to a minimum by using only freshly prepared, helium-degassed solvents. Also, because iron contamination of the column affects the retention times and resolution of phytosiderophores, the column should be regenerated periodically with 100 mmol L⁻¹ oxalic acid at a flow rate of 1 mL min⁻¹ for 1–2 h.

Electrochemical detection

For pulsed amperometric detection an ESA Coulochem II detector was used with an ESA 5040 analytical cell equipped with a gold working electrode. An ISMATEC peristaltic pump was used for post-column addition of NaOH to adjust the pH to 13. A triple-step waveform was used for PAD – detection at +0.1 V (time 500 ms, 300 ms acquisition delay), followed by oxidative cleaning at +0.6 V (time 100 ms) and regeneration at –0.8 V (time 150 ms).

HPLC–AAS

For element-specific detection of iron, fractions were collected every 6 s directly at the outlet of the HPLC column (no post-column addition of NaOH). These fractions (volume 100 µL) were acidified with 50 µL nitric acid and analyzed at 248.3 nm (slit 0.2, current 10 mA) by use of a Hitachi Z8000 atomic absorption spectrometer with Zeeman background correction. Acidification of the alkaline mobile phase is necessary to obtain good sensitivity from AAS measurements. Quantitation was performed by use of a calibration curve, constructed by use of iron standards dissolved in the HPLC mobile phase and acidified in the same way as the samples. By use of this procedure no sensitivity differences were observed for different iron species or chromatographic fractions. This was checked by comparing the HPLC–AAS results with the respective iron concentration as measured after complete mineralization of the sample.

The detection limit of HPLC–AAS is 22 ng mL⁻¹. It was calculated from the blank level (mean±3σ) of chromatographic fractions collected without injecting a sample.

Results and discussion

Chromatography and electrochemical detection of phytosiderophore species

Phytosiderophores of the mugineic acid family are tricarboxylic amino acids which differ in their hydroxylation pattern (Fig. 1). They can be separated chromatographically by use of an anion-exchange column at alkaline pH. Some typical chromatograms obtained from root washings of wheat and barley are shown in Fig. 2. All samples were injected after dilution (1:100) without further sample pretreatment. Phytosiderophores are eluted in order of increasing negative charge. The respective retention times are 20.4 min for DMA (peak 1), 21.0 min for MA (peak 3), and 23.9 min for epi-HMA (peak 2). With the exception of some minor peaks in the retention range below 6 min the phytosiderophores are the only analytes detectable by pulsed amperometric detection (PAD). This high selectivity and sensitivity of PAD for compounds containing aliphatic hydroxy groups is well-known and has been used mainly for detection of carbohydrates [15, 16, 17, 18, 19, 20, 21], although amino acids [22, 23] and even metal species [24, 25] are also detectable by PAD. The above mentioned peaks in the retention range below 6 min are amino acids, which are electroactive under the experimental conditions used. Serine, threonine, and tyrosine (containing aliphatic or phenolic hydroxy groups) are readily detectable and cysteine (containing a thiol group) was also detected. This was tested by injecting the respective amino acid standards. Although the sensitivity of PAD for phytosiderophores is comparable with that of the commonly used fluorimetric detection after post-column derivatization, it depends on the number of hydroxy groups present in the respective molecule [11]. Thus the slopes of the calibration graphs differ for DMA, MA, and epi-HMA.

![Fig. 1](image_url) The chemical structures of mugineic acids. R1=H, R2=β-OH – mugineic acid (MA); R1=R2=H – 2′-deoxymugineic acid (DMA); R1=R2=β-OH – 3-hydroxymugineic acid (HMA); R1=α-OH, R2=β-OH – 3-epi-hydroxymugineic acid (epi-HMA)