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Determination of ferric iron chelators by high-performance liquid chromatography using luminol chemiluminescence detection

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ABSTRACT

Iron is an essential element for higher plants, and its acquisition and transportation is one of the greatest limiting factors for plant growth because of its low solubility in normal soil pHs. Higher plants biosynthesize ferric iron [Fe(III)] chelator (FIC), which solubilizes the iron and transports it to the rhizosphere. A high-performance liquid chromatography (HPLC) post-column method has been developed for the analysis of FICs using the luminol/H_2O_2 system for chemiluminescence (CL) detection. A size-exclusion column was the most suited in terms of column efficiency and CL detection efficiency. Mixing of the luminol with H_2O_2 in a post-column reaction was feasible, and a two-pump system was used to separately deliver the luminol and H_2O_2 solutions. The luminol and H_2O_2 concentrations were optimized using Fe(III)–EDTA and Fe(III)–citrate (Cit) solutions as analytes. A strong CL intensity was obtained for Fe(III)-Cit when EDTA was added to the luminol solution, probably because of an exchange of Cit with EDTA after separation on the HPLC column; CL efficiency was much higher for Fe(III)–EDTA than for Fe(III)–Cit with the luminol/H_2O_2 system. The present method can detect minute levels of Fe(III)–FICs; the detection limits of Fe(III)–EDTA, Fe(III)–Cit and Fe(III)–nicotianamine were 0.77, 2.3 and 1.1 pmol, respectively.

1. Introduction

Iron (Fe) is an essential element for all higher plants. While it is the fourth-most abundant element in the earth's crust [1], Fe deficiency, a severe nutrient problem for higher plants, may occur when Fe ions exist exclusively as Fe(III). This oxidation state is only sparingly soluble in normal soil pHs. For this reason, higher plants have developed Fe acquisition systems. Two such systems have been identified, which are known as Strategy I and Strategy II. Non-graminaceous plants adopt Strategy I, where plant roots secrete phenolic compounds such as protocatechuic acid and chlorogenic acid to chelate and solubilize Fe(III) [3–5]. Graminaceous plants, on the other hand, adopt Strategy II, where the plants secret a series of Fe(III)-chelating compounds known as mugineic acids (MAs) from their roots to cope with the problem [2]. Thus, ferric ion chelators (FICs) play a pivotal role in Fe acquisition in higher plants. Furthermore, after absorption from the roots, Fe is translocated to the shoots *via* the xylem and also to developing organs *via* the phloem. Xylem sap is only slightly acidic (pH 5.0–6.0) while phloem sap is alkaline (pH 8.0), in which the solubility of Fe(III) ion is very low. Thus, Fe(III) is believed to be transferred as FIC complexes [6–11]. Nicotianamine (NA;2(S), 3′(S), 3″(S)-*N*-[*N*-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]-azetidine-2-carboxylic acid; Fig. 1A) is an FIC and also a precursor for the biosynthesis of MAs in graminaceous plants [12]. In contrast to MAs, NA is merely involved in the internal translocation of Fe in higher plants, especially in phloem sap [13–15].

High-performance liquid chromatography (HPLC) has been frequently applied to the analysis of FICs. For example, low-molecular-weight organic acids were directly detected by monitoring the ultraviolet (UV) absorbance near 214 nm attributed to their carboxylic acid groups [16–18], and ethylenediamine-*N,N,N′,N′*-tetraacetic acid (EDTA; Fig. 1B) was detected at 258 nm [19]. The detection limit of citric acid (Cit) and EDTA reported in these papers was 210 and 1.71 pmol, respectively [18,19]. The organic acids were also labeled either in a pre-column or a post-column fashion, where the primary or secondary amine groups were target sites. Derivatization with phenacyl [20,21], naphthacyl [22] or *p*-nitrobenzyl [23,24] prior to the UV detection gave detection limits of 44, 67, 15 and 39 pmol for oxalic acid, oxamic acid, malonic acid and adipic acid, respectively [17,23]. MAs were mainly analyzed using post-column derivatization with *o*-phthaldialdehyde [25,26] or pre-column derivatization with 9-fluorenylmethyl chloroformate

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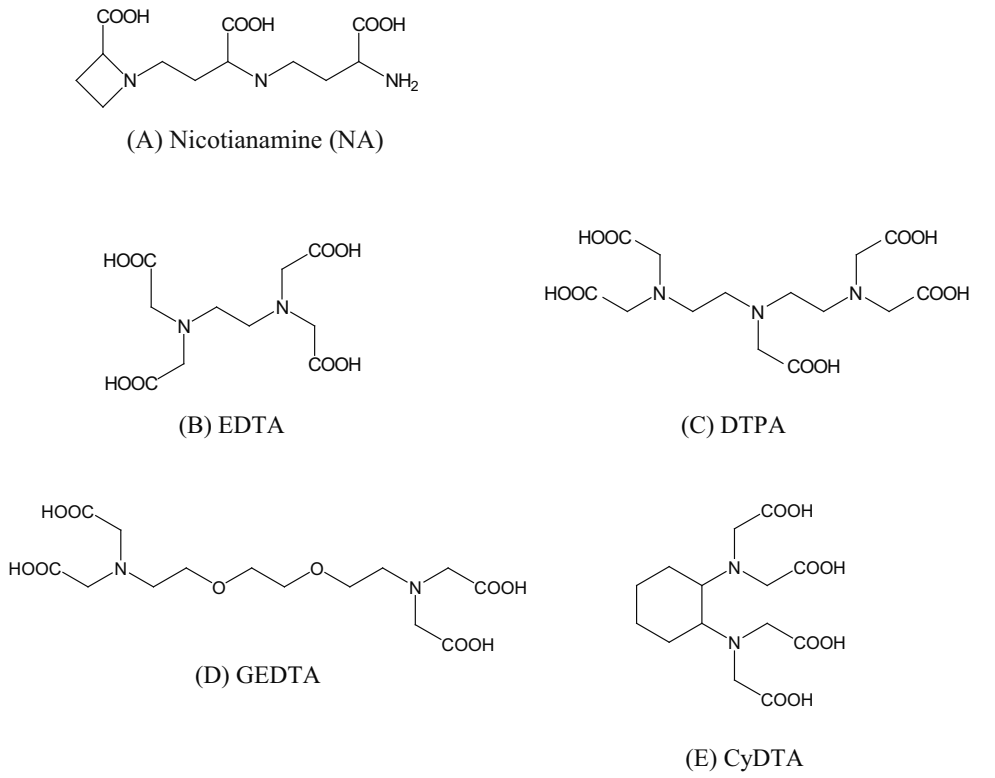

Fig. 1. Structures of (A) nicotianamine and (B)–(E) synthetic ferric iron chelators (FICs) added to the post-column solution A (PCS-A).

[27] prior to fluorescence detection, and using pre-column derivatization with phenylisothiocyanate [28] prior to UV detection. The detection limits of MAs reported in these papers were 370 (mugineic acid), 70 (2′-deoxymugineic acid), 100 (3-*epi*-hydroxymugineic acid) and 500 pmol (nicotianamine) [27,26]. A novel detection system for FIC was presented, with which the dequenching of Fe(III)–Calcein Blue (CB; 4-methylumbelliferone-8-methyleneiminodiacetic acid) was exploited. This system has the feature that the signal intensity correlates with the binding strength of the FICs to Fe(III) [29]. Using this methodology, the detection limit of Cit was 72 pmol.

Chemiluminescence (CL) has been widely exploited as an HPLC detector and in flow-injection analysis because of its high sensitivity, wide linear range and relatively simple instrumentation. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) is a reagent frequently employed in CL spectrometry because it provides an intense CL emission near 425 nm when it is oxidized in the presence of oxidants such as dioxygen, hydrogen peroxide, hexacyanoferrate(III) and permanganate [30]. In the reaction, a co-oxidant is required for the oxidation to occur. As a Fe(III)–FIC complex may function as a co-oxidant, a novel detection system can be devised in which Fe(III)–FIC complexes alone are specifically detected. This paper reports analytical instrumentation for the detection of FICs based on an HPLC post-column derivatization with CL detection using a luminol/H_2O_2 system.

2. Material and methods

2.1. Reagents

Luminol, hydrogen peroxide, 1 M ammonium acetate solution (HPLC grade), acetic acid (HPLC grade), 25% ammonia solution, potassium chloride and Cit monohydrate were purchased from WAKO Pure Chemical Industries (Tokyo, Japan). Ammonium bicarbonate (LC–MS grade) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethylenediamine-*N,N,N′,N′*-tetraacetic acid iron(III) [Fe(III)–EDTA] sodium salt trihydrate, ethylenediamine-*N,N,N′,N′*-tetraacetic acid (EDTA) disodium salt dihydrate, diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA; Fig. 1C), *o,o′*-bis(2-aminoethyl)ethyleneglycol-*N,N,N′,N′*-tetraacetic acid (GEDTA; Fig. 1D) and *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid (CyDTA; Fig. 1E) monohydrate were obtained from Dojindo Laboratories (Kumamoto, Japan). Ferric chloride hexahydrate and boric acid were purchased from Kokusan Chemical (Tokyo, Japan). Synthetic NA was kindly provided by Professor Emeritus Satoshi Mori at The University of Tokyo. All other reagents were of analytical grade. Milli-Q Ultrafree water (Merck Millipore, Darmstadt, Germany) was used to dissolve and dilute the reagents.

2.2. HPLC analytical system

Fig. 2 shows the HPLC analysis system used in this study. The system consisted of pumps 1 (PU-980, Jasco, Tokyo, Japan), 2 and 3 (PU-2080, Jasco), a dynamic mixer (MX-2080-32, Jasco), a three-line degasser (DG-2080-53, Jasco), an automatic sampler (AS-950-10, Jasco), two column ovens (column oven 1 (CO1), CO-965(Jasco); and column oven 2 (CO2), SSC-2120(Senshu Scientific Co., Ltd., Tokyo, Japan)) and a CL detector (CL-2027, Jasco). An eluent with a pH of 5.5 was prepared by adding dilute acetic acid to a 10 mM ammonium acetate solution. An eluent with a pH of 8.0 was made by adding a diluted ammonia solution to a 10 mM ammonium bicarbonate solution. The pH values corresponded to the pHs of xylem sap (pH 5.5) and phloem sap (pH 8.0).

The Fe(III)–FIC complex was separated by isocratic elution of a degassed eluent that was prepared daily and delivered by pump 1 at a flow rate of 0.5 mL/min. Twenty microliters of a sample were loaded on a size-exclusion column (8.0 mm × 300 mm, Superdex Peptide 10/300 GL, GE Healthcare UK Ltd., Buckinghamshire, UK) at a temperature of 39 °C in oven CO1. After separation on the column, the Fe(III)–FIC complex was reacted in the reaction coil in oven CO2