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Short Communication

Anion-exchange high-performance liquid chromatography with post-column detection for the analysis of phytic acid and other inositol phosphates

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ABSTRACT

The use of gradient anion-exchange HPLC, with a simple post-column detection system, is described for the separation of *myo*-inositol phosphates, including "phytic acid" (*myo*-inositol hexaphosphate). Hexa-, penta-, tetra-, tri- and diphosphate members of this homologous series are clearly resolved within 30 min. This method should facilitate analysis and quantitation of "phytic acid" and other inositol phosphates in plant, food, and soil samples.

INTRODUCTION

Inositol phosphates, currently of considerable interest to biochemists [1], have long been studied by soil scientists and food chemists because of the metal-chelating properties of *myo*-inositol hexaphosphate, also known as "phytic acid" [2,3]. A recent review by Xu *et al.* [4] summarizes the methods which have been used for analysis of these molecules in foods. Nutrition-related studies have indicated that it may be important to know relative amounts of *myo*-inositol hexaphosphate (IP6) *vs.* the penta-, tetra-, tri-, di- and monophosphate esters (IP5, IP4, IP3, IP2 and IP1, respectively), since even limited dephosphorylation of phytic acid can reduce its inhibitory effect on mineral absorption [5,6]. These researchers used ion-pair reversed-phase high-performance liquid chromatography (HPLC) to identify and quantitate IP6, IP5, IP4 and IP3. Ion-pair reversed-phase methods [7,8] give adequate separation of the above inositol phosphoesters (the mono- and diphosphates are not resolved). However, sample extracts must first be passed through anion-exchange resin to remove inorganic phosphate and concentrate the inositol phosphates. Acidic column effluent is then evaporated to dryness (to remove HCl) and reconstituted in water prior to injection onto a reversed-phase HPLC column. Direct analysis of sample extracts by anion-exchange HPLC would eliminate the need for these sample preparation steps.

Ion-exchange chromatography has long been used to fractionate and purify *myo*-inositol phosphoesters produced by the hydrolysis of phytic acid or its phytate salts [7,9,10]. To date, published procedures involving anion-exchange HPLC fall into two categories. Isocratic, ion-

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chromatographic techniques [11–13] do not resolve less-phosphorylated species as well as the ion-pair reversed-phase methods cited earlier. In contrast, the elegant, isomer-specific gradient elution protocols (with post-column detection) developed by Mayr [14] and Phillippy and Bland [15] can provide more information than what was needed for our purpose, which is to screen a variety of plant materials for phytic acid and its partially-hydrolyzed homologues. The method described in this paper affords a compromise, enabling one to clearly resolve and, potentially, quantitate the hexa-, penta-, tetra-, tri- and diphosphate esters of *myo*-inositol with minimal sample preparation and equipment.

EXPERIMENTAL

Materials

Sodium phytate (from corn) and phytase (crude; from wheat) were obtained from Sigma (St. Louis, MO, USA). (Moisture content of the former was 14.5% and this was taken into account when preparing solutions.) Pure *myo*-inositol phosphates used as chromatographic standards were the pentaphosphate from Calbiochem (San Diego, CA, USA); 3,4,5,6-tetraphosphate, 1,4,5-triphosphate, 1,4-biphosphate and 2-monophosphate from Sigma. (Isomeric composition of the phosphoesters was not important for our purposes, but is given here for the sake of completeness.) Also purchased from Sigma were ferric chloride hexahydrate and sulfosalicylic acid. Aldrich (Milwaukee, WI, USA) supplied 1-methylpiperazine (99%), and sodium nitrate (reagent grade) was from J.T. Baker (Phillipsburg, NJ, USA). Distilled, deionized water was used for the preparation of solutions.

Due to the expense of commercially-purified *myo*-inositol phosphoesters IP5, IP4, IP3, and IP2, sodium phytate (10 mg/ml) was hydrolyzed with phytase (1 mg/ml; 10:1 phytate:phytase) following the preparative enzymatic method of Phillippy *et al.* [10]. This produced a mixture of *myo*-inositol phosphoesters which was used for preliminary examination of chromatographic conditions. The exact composition of this mixture varied depending upon initial incubation time and age of the hydrolysate. (For example, IP-5 produced during a 24-h incubation was completely converted to lower phosphoesters during a two-month storage period.) Once optimum separation parameters had been established, the commercially-purified, individual *myo*-inositol phosphates described above were used to verify identity of each hydrolysate component.

Instrumentation

Chromatography was performed with a Varian 5000 liquid chromatograph (Varian, Palo Alto, CA, USA) equipped with a Model 7125 Rheodyne injector (Rheodyne, Cotati, CA, USA). Post-column reagent was pumped with an Eldex Model A-60-S metering pump (Eldex, Menlo Park, CA, USA), and mixed with eluate from the analytical column via a Valco tee and a coil consisting of a 290 cm length of 0.76 mm I.D. polyether ether ketone (PEEK) tubing (1.3 ml volume). Detection was at 500 nm (0.1 AUFS) using an Isco V^4 absorbance detector (Isco, Lincoln, NE, USA). Check valves (SSI soft seat; Alltech, Deerfield, IL, USA) were installed prior to both inlet ports of the tee to prevent backflow of HPLC eluents into the low-pressure system or contamination of the analytical column with detection reagent. An externally adjustable pressure relief valve (Nupro "R3A" series; Indianapolis Valve and Fittings, Indianapolis, IN, USA) was installed in front of the reagent pump as a precaution against exceeding the pressure limit of this pump. An empty 5×0.41 cm column used to plumb this valve into the system also served as a pulse dampener. A diagram of the complete system is shown in Fig. 1.

Chromatography

Separation of the *myo*-inositol phosphates was achieved using a 5×0.46 cm I.D. polystyrene-based strong anion-exchange column (1000 Å PL-SAX; Polymer Labs., Amherst, MA, USA). (A PL guard cartridge system containing a 5×3 mm I.D. cartridge of PL-SAX packing was subsequently added to protect the analytical column during chromatography of plant extracts.) Optimum elution conditions were a

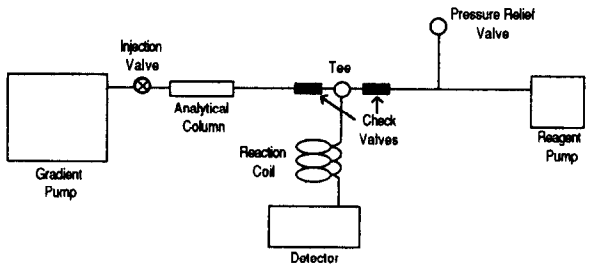

Fig. 1. Schematic diagram of HPLC/post-column detection system.

30-min linear gradient from 0.01 *M* 1-methylpiperazine (pH 4.0) to 0.5 *M* $NaNO_3$ in 0.01 *M* 1-methylpiperazine (pH 4.0) at a flow-rate of 1 ml/min. Effluent from the analytical column combined with a post-column reagent (also pumped at 1 ml/min) which consisted of 0.015% $FeCl_3 \cdot 6H_2O$ (w/v) plus 0.15% (w/v) sulfosalicylic acid [12]. The decrease in absorbance at 500 nm, as eluting phosphates complex with iron in the post-column reagent, was recorded as "positive" peaks by reversing polarity of the detector-chart recorder connections. Both HPLC mobile phase and post-column reagent solutions were filtered through 0.45-μm nylon 66 filters and degassed thoroughly prior to use. The post-column reagent, stored in an amber bottle at room temperature, is stable for several weeks.

Extraction procedure

Extraction of phytic acid and lower inositol phosphates from plant materials was based on the procedures of Cilliers and Van Niekerk [12] and others [4]. To 1.0 g of finely ground, dry sample in a 50-ml, screw-cap centrifuge tube (Nalgene 3119-0050; Nalge, Rochester, NY, USA) was added 10.0 ml of 0.5 *M* HCl. The tubes were shaked mechanically (*ca.* 150 rpm) for 2 h at room temperature. At the end of this time, the tubes were centrifuged for 20 min at 20 000 rpm (48 400 *g*). Supernatant was passed through a 0.2-μm porosity membrane filter (and stored at 4°C, if necessary) prior to injection.

RESULTS AND DISCUSSION

The HPLC column and mobile-phase conditions described in the preceeding section gave

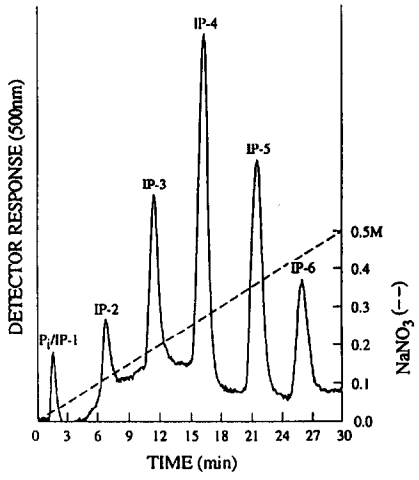

Fig. 2. Resolution of hexa-, penta-, tetra-, tri-, di-, and monophosphate esters of *myo*-inositol (IP6, IP5, IP4, IP3, IP2, and IP1, respectively.) (Inorganic phosphate, P_i, co-elutes with IP1.) A sample of hydrolyzed sodium phytate, spiked with pure IP5 and unhydrolyzed phytic acid, IP6, was chromatographed on a strong anion-exchange column (1000 Å PL-SAX; 5 × 0.46 cm). Elution conditions were a 30-min linear gradient from 0 to 0.50 *M* sodium nitrate in 0.01 *M* 1-methylpiperazine buffer (pH 4.0) at a flow-rate of 1 ml/min. "Detector response" denotes the change in absorbance at 500 nm as explained in Experimental.

good resolution of the hexa-, penta-, tetra-, tri- and diphosphates of *myo*-inositol (Fig. 2). Inorganic phosphate and *myo*-inositol monophosphate co-elute as non-retained species. Resolution of this homologous series of phosphate esters by gradient anion-exchange chromatography exceeds that achieved by isocratic ion chromatography [12] or ion-pair reversed-phase HPLC [7,8], with IP6 through IP2 all separated by at least 3 min. Maintaining the mobile phase at pH 4.0 insures that each phosphate moiety possesses only one negative charge, since six of the 12 replaceable protons in the phytic acid molecule are strongly dissociated ($pK_a \leq 2$) with the remainder being more weakly acidic ($pK_a \geq 5.7$) [2]. The use of a polystyrene-based strong anion-exchange column [16,17] prevents the potential problem of packing material deterioration which could be encountered during prolonged use of a silica-based column at this mobile-phase pH.

Minear *et al.* [18] also employed gradient elution (at pH 10) with a polymeric strong anion-

exchange column (25 × 0.4 cm I.D.) to achieve good resolution of *myo*-inositol phosphates, IP6–IP1. However, longer elution times (58 min for IP6) and off-line detection (fractions were collected and analyzed for phosphorous) are definite disadvantages of that procedure. The gradient ion-chromatographic method of Phillippy and Bland [15], which is potentially applicable to food samples, can resolve isomers of the various *myo*-inositol phosphoesters (*e.g.* all four pentaphosphates). However, the chromatographic information provided may be more appropriate to qualitative or kinetic studies than to quantitation of relative amounts of phytic acid *vs.* partially-hydrolyzed species regarding mineral bioavailability [5,6].

A standard curve for the quantitation of *myo*-inositol hexaphosphate, IP-6, was obtained by injecting various quantities of sodium phytate. The plot of peak area *vs.* amount injected (not shown) was linear from 2.5–100 μg ($r > 0.999$). Precision for replicate injection (10 μl; 100 μg load) of sodium phytate was *ca.* 1% for retention time and 2% for peak area (calculated as $h \times w_{1/2}$). A recovery study was performed with wheat flour that contained 0.1% IP-6 as determined by HPLC. An aliquot of concentrated sodium phytate solution was added to each dry sample so as to provide an addition of 1.0% phytic acid. Samples were then extracted and chromatographed as previously described. Recovery was 97 ± 1% ($n = 3$).

To date, the method described in this communication has been applied to extracts of cowpea seeds (Fig. 3) and leaves, rice grain, and taproots from four species of perennial forage legumes. (Other components of these samples did not interfere with detection, although the high sugar content of taproot extracts necessitated periodic cleaning of the column with 1 *M* sodium chloride.) Results of this work will be the subject of a future publication.

The detection method employed involves a ligand-exchange reaction between the iron(III)-sulfosalicylic acid complex and eluting inositol phosphates. It was first described by Wade and Morgan [19] for the detection of phosphate esters on paper chromatograms, and has subsequently been applied to both colorimetric [20]

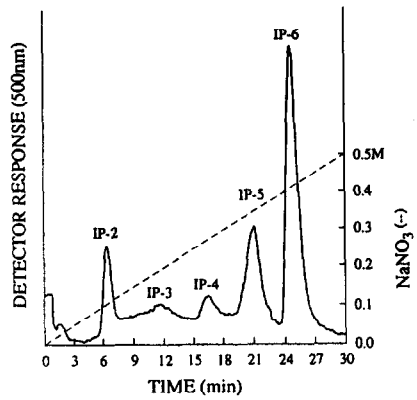

Fig. 3. Chromatogram of *myo*-inositol phosphates extracted from cowpea seed. An aliquot of 20 μl of acidic extract (see Experimental) was chromatographed using the column and elution conditions described in Fig. 2. (Peak identification abbreviations are the same as in Fig. 2.)

and chromatographic [12] methods for the determination of "phytate" or phytic acid, *i.e.* *myo*-inositol hexaphosphate. Although Cilliers and Van Niekerk [12] detected the lower phosphate esters via post-column reaction with this reagent, chromatographic resolution of these homologues was relatively poor, owing to isocratic elution.

In summary, the use of gradient anion-exchange HPLC in conjunction with post-column detection via "Wade reagent" offers several advantages over previously published procedures for the analysis of *myo*-inositol phosphates in non-physiological samples, *i.e.* where discrimination between phosphate isomers is not needed. Minimal preparation of samples is required —acidic sample extracts need only be centrifuged and filtered prior to injection. Resolution of the lower phosphoesters and peak shape of strongly-retained components is considerably better than that afforded by isocratic elution from an ion chromatography column. This HPLC protocol can be implemented in laboratories with a gradient pumping system and a variable-wavelength absorbance detector; all hardware needed to construct the post-column detection system is commercially available. The addition of an electronic integrator would facilitate routine analysis of large numbers of samples, but is not absolutely necessary. Given appropriate sample extraction procedures and

calibration curves, this chromatographic method should be applicable to the analysis and quantitation of homologous inositol phosphates in various plant, food and soil samples.

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