myo-Inositol Phosphates in Corn Steep Water

Steven R. Hull† and Rex Montgomery*,‡

Departments of Pathology and Biochemistry, University of Iowa, Iowa City, Iowa 52242

Wet corn milling results in large volumes of steep water, the contents of which include *myo*-inositol in the form of phosphate esters. The principal *myo*-inositol derivative is the hexakisphosphate, IP6, which is accompanied by several isomers of IP3, IP4, and IP5. Analysis of the steep water at the early, middle, and later stages gave evidence that the lower phosphates are the result of enzymatic hydrolysis and autohydrolysis of the IP6, all species increasing as the steeping progresses. Calcium precipitation of the *myo*-inositol phosphates from the steep water results in the isolation of IP5 and IP6.

Keywords: *myo-Inositol phosphates; phytic acid; corn steep water*

INTRODUCTION

The steeping of corn is a necessary prerequisite to the isolation of corn starch and gluten in the wet-milling process. It involves the countercurrent flow of water, initially containing some sulfur dioxide, and corn in a number, around 10, of steeping tanks at 50–55 °C over a period of approximately 30 h. The details of the process vary among different industries. It follows that the fresh, dried corn enters the tank that contains the steep water from exposure to the partially steeped corn in the previous tanks.

The volume of steep water produced in the corn wet-milling industry is large. It is primarily handled by evaporation to a concentrated thick liquor that is a complex mixture of carbohydrates, amino acids, peptides, organic acids, minerals, and *myo*-inositol phosphates.

Corn steep water is a valuable coproduct of the corn wet-milling industry presently used in the manufacture of feed for cattle, providing for energy needs, weight gain from the amino acid content, and an essential nutrient in the form of inositol. Corn steeps are a source of *myo*-inositol hexakisphosphate (IP6), which is reported to comprise 8% on a dry weight basis of the total solids of corn steep liquor (Sands et al., 1986). To a lesser extent corn steep water serves as a supplement in antibiotic and other fermentations (Wright, 1987). IP6 has potential uses ranging from pharmaceutical to engineering applications (Graf, 1986). This study is concerned with the *myo*-inositol phosphate content of corn steep water, the distribution of the various *myo*-inositol phosphates at different times during the steeping process, and the recovery of IP5 and IP6 from the steep water.

MATERIALS AND METHODS

Materials. Standard *myo*-inositol phosphates were obtained from Sigma Chemical Co. (St. Louis, MO) or Calbiochem (San Diego, CA) and shown to be chromatographically pure (>95%) by IP-RP-HPLC or HPAEC-CD. Cation- and anion-exchange resins were obtained from Bio-Rad (Hercules, CA). Phytase (wheat bran), acid phosphatase (wheat germ), and alkaline phosphatase (type VII-N, bovine intestinal mucosa) were purchased from Sigma.

Collection of Steep Waters. Corn steep water was collected from four wet corn milling companies, identified as 1, 2, 4, and 15, in sterile glass containers at early (1–3 h), middle (14–17 h), and late (27–30 h) stages in the steeping prior to concentration to a heavy steep liquor (Table 1). The samples were stored at −20 °C until analyzed.

Methods. *Ion-Pair Reversed-Phase HPLC (IP-RP-HPLC).* IP3, IP4, IP5, and IP6 from steep water were initially identified and quantitated (Table 1) using IP-RP-HPLC as described by Sandberg and Ahderinne (1986). Samples were chromatographed on a Waters C_{18} Nova-Pak column (0.46 × 10 cm) using isocratic elution with buffer at a flow rate of 2 mL/min with refractive index detection. The buffer was prepared by the adjustment of a 50 mM formic acid/MeOH (49:51) solution to pH 4.3 with the ion-pairing reagent tetrabutylammonium hydroxide (TBA-OH, 1.5 mL/100 mL). The HPLC column was calibrated before each analysis with 2,4,5-IP3, 1,3,4,6-IP4, 1,3,4,5,6-IP5, and IP6.

High-pH Anion-Exchange Chromatography with Conductimetric Detection (HPAEC-CD). *myo*-Inositol phosphate isomers were separated by high-pH anion-exchange chromatography and detected by conductimetric means (HPAEC-CD) according to procedures outlined by the manufacturer (Dionex Application Note AN65). Chromatographic separation of IP6 and various isomers of IP3, IP4, and IP5 was effected on an Omni-Pak PAX-100 column (0.46 × 25 cm), using a gradient elution generated by mixing 200 mM NaOH, 50% 2-propanol, and water as follows: T_0 = 90 mM NaOH, 1% 2-propanol, T_{10} = 90 mM NaOH, 1% 2-propanol, T_{25} = 110 mM NaOH, 4% 2-propanol, T_{30} = 110 mM NaOH, 4% 2-propanol. Frequent calibration of the column was performed with IP3 standards, (1,3,4-, 1,4,5-, 1,5,6-, and 2,4,5-IP3), IP4 standards (1,2,5,6-, 1,3,4,5-, 3,4,5,6- and 1,3,4,6-IP4), 1,3,4,5,6-IP5, and IP6. The column was regenerated after every fifth or sixth analysis by elution at 1 mL/min with 140 mM NaOH/4% 2-propanol for 30–45 min, followed by re-equilibration in initial eluant for at least 1 h.

Determination of Phosphate:myo-Inositol Ratios. Phosphate and *myo*-inositol contents of the *myo*-inositol phosphates were determined after hydrolysis with 4 N HCl at 125–130 °C for 48 h. Phosphate was determined colorimetrically by either of two methods dependent upon the level of sensitivity dictated by the availability of analyte. The Fiske and Subbarow (1925) method was utilized when phosphate was expected at a concentration >2 μg. The method of Ames (1966) was used when phosphate was expected at a concentration <2 μg. *myo*-Inositol was determined by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) utilizing an MA1 column (0.46 × 25 cm) with 400 mM NaOH as eluant at a flow rate of 0.4 mL/min according to detailed procedures outlined by the manufacturer (Dionex Document

* Author to whom correspondence should be addressed [fax (319) 335-9570; e-mail rex.montgomery@uiowa.edu].

† Department of Pathology.

‡ Department of Biochemistry.

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Table 1. *myo*-Inositol Phosphates in Industrial Corn Steep Water[a]

		myo-inositol phosphates (g/L)				
stream[b]	dry wt (g/L)	IP3	IP4	IP5	IP6	% dry wt
1-E	27.5	0	0	0	1.3	4.7
1-M	68.9	0	0	0.5	4.8	7.7
1-L	112.2	0	0.5	0.9	9.2	9.5
1-H	486.6	3.5	10.8	9.3	12.2	7.4
2-M	81.6	0	0	0.3	4.3	5.6
2-L	126.1	0	1.0	2.8	14.9	14.8
2-H	544.8	1.0	5.0	11.4	29.4	8.6
4-E	25.9	0	0	0	0.7	2.7
4-M	40.2	0	0	0	2.1	5.2
4-L	79.0	0	0	0.8	7.1	10.0
4-H	498.9	0.4	4.5	9.3	25.2	2.9
15-E	24.2	0	0	0	0	0
15-M	63.4	0	0	0.2	2.1	3.6
15-L	149.1	0	0.7	1.1	12.2	9.4

[a] *myo*-Inositol phosphates recovered from each corn steep sample using the acidic isolation method (see Methods) were analyzed by IP-RP-HPLC with refractive index detection. From response factors generated using standard IP3–IP6, the quantitation of each identifiable *myo*-inositol phosphate was performed and expressed in grams per liter in the original stream. [b] Stream codes: E, early steep (1–3 h steeps); M, mid steep (14–17 h steeps); L, late steep (27–30 h steeps); H, heavy steep. The numbers 1, 2, 4, and 15 are codes for four corn-milling companies.

034752). Quantitation was effected by comparison to a standard curve generated at each time of analysis using standard solutions of authentic *myo*-inositol. IP6 was used as a standard to validate the P/I ratio determination. The IP6 standard was shown to be free from IP5, IP4, and IP3 when analyzed by IP-RP-HPLC and HPAEC-CD.

Isolation of myo-Inositol Phosphates. (a) Isolation as Free Acids. Equal volumes of corn steep water (0.5 mL) and 1 N HCl (0.5 mL) were mixed with gentle shaking for 2 h at 4 °C. Any precipitate formed was removed by centrifugation or filtration, the supernatant was frozen overnight and allowed to thaw, and the resulting suspension was centrifuged (8000*g*, 30 min). The supernatant was diluted 20-fold and passed through an Ag1×8(Cl^-), 200–400 mesh (Bio-Rad) anion-exchange column (0.6 × 1 cm for analysis or 0.6 × 5 cm for preparations). Bound phosphate was eluted with 15–20 column volumes of 0.025 N HCl. The *myo*-inositol phosphates were then eluted from the Ag1 column with 10 mL of 2 N HCl. The acid was removed by evaporation at room temperature under reduced pressure to leave a dry residue containing a mixture of *myo*-inositol phosphates. A control experiment showed that IP6 did not undergo any partial hydrolysis during such isolation conditions.

(b) Isolation as Calcium Salts. The clear supernatant obtained after centrifugation of corn steep water (25 mL) was brought to pH 6–7 with 0.1% $Ca(OH)_2$ (425 mL). After 1 h at room temperature, a flocculent off-white precipitate formed, which was recovered by centrifugation (8000*g* for 30 min), washed several times with water, and resuspended in water (150 mL). In preparation for anion-exchange chromatography the calcium salts of the *myo*-inositol phosphates were converted to their free acids using Ag50×8(H^+), 200–400 mesh. A portion (50 mL) of the suspension was mixed with an equal volume of a 50% aqueous suspension of Ag50 (H^+) cation-exchange resin with occasional mixing at 4 °C for 1 h. The suspension was vacuum-filtered through a glass-sintered funnel and the filtrate neutralized with 10 N NaOH and passed through an Ag1×8(Cl^-), 200–400 mesh column (0.6 × 5 cm). The resin was washed with 100 mL of 0.025 N HCl, and the *myo*-inositol phosphates were eluted with 50 mL of 1 N HCl. The 1 N HCl eluant was evaporated in vacuo to a syrup, dissolved in water (3 mL), and subjected to IP-RP-HPLC and/or HPAEC-CD as described below.

Separation of myo-Inositol Phosphates by Anion-Exchange Chromatography. Preparative fractionation of *myo*-inositol phosphates was accomplished by anion-exchange chromatography (Figure 1) using an Ag1×8(Cl^-), 200–400 mesh, column (0.6 × 5 cm) and elution with a gradient of HCl (0–1 N), essentially as described by Bartlett (1982). Fractions were monitored for their *myo*-inositol phosphate content by IP-RP-HPLC, and fractions containing only one species were pooled for future analysis. Intermediate fractions were rechromatographed as before. In this manner *myo*-inositol phosphate fractions defined by IP-RP-HPLC as containing three to six phosphate groups per *myo*-inositol residue were isolated.

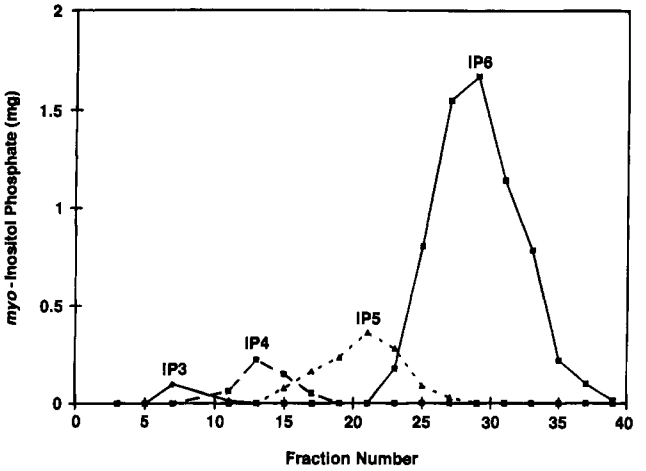

Figure 1. Anion-exchange chromatography of *myo*-inositol phosphates of corn steep water. *myo*-Inositol phosphates recovered from 0.1 mL of a late corn steep fraction (1.7, Table 1) were chromatographed on a column (0.6 × 5 cm) of Ag1 anion-exchange resin, eluted with an acid gradient (0–1 N HCl). Fractions (3.0 mL) were analyzed for *myo*-inositol phosphate content by IP-RP-HPLC. Purified IP3, IP4, IP5, and IP6 were pooled as outlined under Methods.

Hydrolysis of IP6. (a) Enzymatic Hydrolysis of IP6. IP6 was subjected to enzymatic digestion with phytase or acid phosphatase to generate 1,2,3,4-IP4 and 1,2,3,4,5-IP5 (Phillippy et al., 1987). Details of the slightly modified procedure are as follows:

IP6 (10 mg) in 2.5 mL of 10 mM sodium acetate/2 mM $MgSO_4$, pH 3.7 (pH of corn steep water) was incubated with 5 mg of wheat bran phytase or wheat germ acid phosphatase at 52 °C. At timed intervals corresponding approximately to early (1–3 h), middle (14–17 h), and late (27–30 h) times of corn steeping, a 0.25 mL aliquot was mixed with 3 volumes of ice-cold ethanol to terminate the enzymatic reaction. The ethanol concentration was adjusted to 50% and the sample filtered through a 0.2 μm nylon-based filter (Gelman Sciences) to remove the protein precipitate. The filtered sample after appropriate dilution was directly analyzed by HPAEC-CD.

(b) Autohydrolysis of IP6. Autohydrolysis was used to generate 1,2,4,5,6-IP5 (Phillippy and Bland, 1988). IP6 (5 g) in 50 mL of 10 mM sodium acetate/2 mM $MgSO_4$ was adjusted to pH 3.7 with HCl. This solution (60 mL after pH adjustment) was autoclaved in a 250 mL Erlenmeyer flask covered with aluminum foil for 60 min at 132 °C (AMSCO general purpose autoclave). The cooled solution was brought to a final volume of 200 mL and, after appropriate dilution, was directly analyzed by HPAEC-CD.

Analytical Enzymatic Hydrolysis of myo-Inositol Phosphates from Steep Water. Phytase and/or alkaline phosphatase hydrolysis of suspected IP species from corn provided further evidence that most, if not all, of the HPAEC-CD-separable components in each IP-RP-HPLC-defined IP species are *myo*-inositol phosphates. Hydrolysis of aqueous solutions of *myo*-inositol phosphates with alkaline phosphatase and/or phytase were carried out essentially according to manufacturer's specifications for up to 90 h. At 24 h intervals the susceptibility of the IP species to enzymatic hydrolysis was monitored by HPAEC-CD. Details of the enzymatic hydrolysis conditions follow:

(a) Phytase. An aqueous solution of sample suspected to contain *myo*-inositol phosphate (100 μL) was mixed with 4 volumes of buffer (15 mM sodium acetate/1 mM $MgCl_2$, pH 4.5). Phytase (0.1 unit, 1 unit will hydrolyze 1.0 μmol of inorganic phosphorus from 1.5×10^{-3} M phytate per minute at pH 5.15 at 55 °C) was added every 24 h, and incubation was at 55 °C. A control sample with no enzyme was treated similarly, and in each case the IP content was determined by HPAEC-CD.

(b) Alkaline Phosphatase. An aqueous solution of sample suspected to contain *myo*-inositol phosphate (100 μL) was mixed with 4 volumes of buffer (20 mM triethanolamine/1 mM $MgCl_2$/1 mM $ZnCl_2$, pH 8.0). Enzyme (10 units, 1 unit will hydrolyze 1.0 μmol of *p*-nitrophenyl phosphate per minute at 37 °C) was added every 24 h, and incubation was at 37 °C. A control sample with no enzyme was treated in parallel and the IP content determined in each case by HPAEC-CD.

RESULTS AND DISCUSSION

The analysis and identification of the phosphate esters of *myo*-inositol in corn steep water presented problems in the analytical separation of the many compounds, the availability of reference materials, and the determination of the P/I ratio to establish the class of the *myo*-inositol phosphate.

Determination of P/I Ratios. P/I ratio determinations from hydrolysates of *myo*-inositol phosphates have been reported with a variance ranging from 4 to 18% (Grado and Ballou, 1961; Cosgrove, 1963; Sandberg and Ahderinne, 1986). This variation could lead to difficulties in differentiating between IP3, IP4, IP5, and IP6 species. The analysis of the *myo*-inositol phosphate mixtures from the corn steep water necessitated the development of a reliable analytical procedure to establish the phosphate:*myo*-inositol (P/I) ratios in the fractions. Approaches using initial phosphatase or phytase hydrolysis were not universally applicable because of the variety of *myo*-inositol phosphate structures, some of which were only slowly hydrolyzed (see discussion below). Acid hydrolysis with sulfuric acid (Bartlett, 1982) or with 2 N TFA for 16 h (data not shown) resulted in low recovery of *myo*-inositol and/or phosphate, causing significant error in the P/I ratios. Optimal conditions ascertained by performing a timed acid hydrolysis of standard IP6 with 4 N HCl were determined to be 125–130 °C for 48 h, conditions similar to those used by others (Grado and Ballou, 1961; Cosgrove, 1963). Reliable data were routinely achieved only after an authentic *myo*-inositol was concomitantly heated to correct for any loss of *myo*-inositol by thermal and/or acid-catalyzed degradation or production of unidentifiable side products. Routinely, 85–90% of the *myo*-inositol was identifiable as such by HPAEC-PAD analysis after hydrolysis with 4 N HCl at 125–130 °C for 48 h. Validation of this hydrolysis procedure was demonstrated by determination of the P/I ratio of standard IP6. Four separate determinations resulted in a P/I for IP6 of 5.9 ± 0.1. Despite this validation, in view of the documented variance in P/I ratio determinations by various authors as mentioned above, the P/I ratio data must be viewed with caution and cannot serve as the sole criterion for IP isomer determination. The P/I ratios for the IP3, IP4, and IP5 classes and their subfractions are summarized in Table 2.

Chromatographic Analysis of *myo*-Inositol Phosphates. Using the IP-RP-HPLC methodology of Sandberg and Ahderinne (1986), the classes of IP3, IP4, IP5, and IP6 of corn steep water could be separated and quantitated (Table 1). More definitive separation of the isomers within the IP3, IP4, and IP5 classes was achieved with HPAEC-CD.

Table 2. Phosphate:*myo*-Inositol Ratios of *myo*-Inositol Phosphates of Corn Steep Water[a]

myo-inositol phosphate	phosphate (μmol/mL)	*myo*-inositol (μmol/mL)	P/I
IP3, total	4.90	1.60	3.1
peak I[b]	2.00	0.72	2.8
peak II[b]	0.67	0.24	2.8
peak III[b]	0.48	0.26	1.9
IP4, total	15.30	0.36	4.3
peak I[c]	1.37	0.37	3.7
peak II[c]	–[d]	0.01	–
peak III[c]	–	0.02	–
IP5, total	34.90	7.20	4.8
peak I[c]	2.38	0.48	5.0
peak II[c]	1.52	0.33	4.6
peak III[c]	0.61	0.11	5.5

[a] Phosphate, determined colorimetrically, and *myo*-inositol, determined chromatographically, were compared to calculate the phosphate:*myo*-inositol ratio for each *myo*-inositol phosphate species purified from corn steeps using conventional anion-exchange chromatography as in Figure 1. In addition, the P/I ratios for isomeric species of IP3 (Figure 6), IP4 (Figure 7), and IP5 (Figure 8) isolated by HPAEC-CD were determined. Phosphate and *myo*-inositol determined as described under Methods. [b] Phosphate analyzed according to Ames (1966). [c] Phosphate analyzed according to Fiske and Subbarow (1925). [d] Below detection limits.

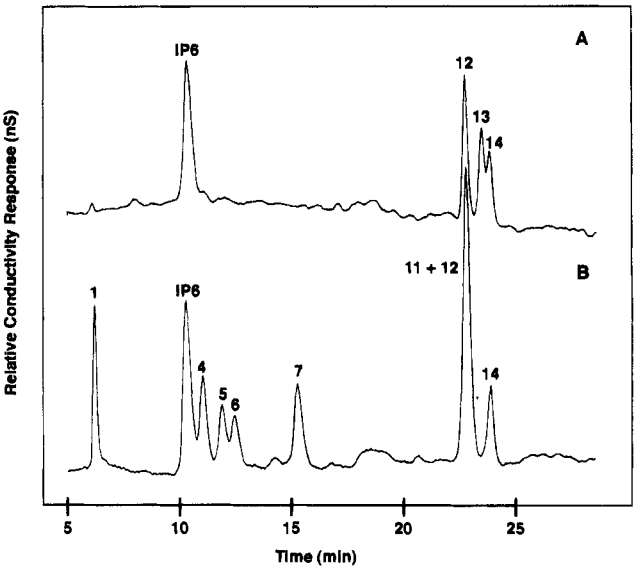

Figure 2. HPAEC-CD analysis of reference *myo*-inositol phosphates. *myo*-Inositol phosphates of known isomeric composition were analyzed by HPAEC-CD using the chromatographic conditions described under Methods. (A) A standard mixture was analyzed whose composition was (1) 1,5,6-IP3, (4) 1,3,4-IP3, (5) 1,4,5-IP3, (6) 2,4,5-IP3, (7) 1,2,5,6-IP4, (11) 3,4,5,6-IP4, (12) 1,3,4,5,6-IP5, and (14) 1,3,4,5-IP4. (B) A standard mixture was analyzed whose composition was (12) 1,3,4,5,6-IP5, (13) 1,3,4,6-IP4, and (14) 1,3,4,5-IP4. IP6 was added as an internal standard in each analysis. Each chromatogram has been baseline-corrected by subtraction of a water blank analyzed concurrently.

That HPAEC-CD provided a suitable means of chromatographic separation of isomers of *myo*-inositol phosphates was established using reference standards as well as the major IPs generated by enzymatic hydrolysis and autohydrolysis of IP6 species, which have been well characterized by others (Phillippy et al., 1987; Phillippy and Bland, 1988). Adequate resolution of all the available IPs, except the pair 1,3,4,5,6-IP5 and 3,4,5,6-IP4, was achieved within 30 min by HPAEC-CD (Figure 2 and Table 3).

Table 3. Relative Retention Times of *myo*-Inositol Phosphates[a]

peak no.	*myo*-inositol phosphate	relative retention time[b]
1	1,5,6-IP3	0.58 ± 0.01 (15)[c]
2	1,2,6-IP3[d,e]	0.63 ± 0.02 (8)
3	1,2,3-IP3[d,e]	0.85 ± 0.03 (8)
4	1,3,4-IP3	1.07 ± 0.02 (15)
5	1,4,5-IP3	1.16 ± 0.01 (14)
6	2,4,5-IP3	1.21 ± 0.02 (15)
7	1,2,5,6-IP4	1.50 ± 0.02 (15)
8	1,2,4,5,6-IP5[d]	1.64 ± 0.03 (3)
9	1,2,3,4,5-IP5[e]	1.87 ± 0.02 (6)
10	1,2,3,4-IP4[e]	2.12 ± 0.04 (6)
11	3,4,5,6-IP4	2.28 ± 0.03 (11)
12	1,3,4,5,6-IP5	2.28 ± 0.03 (18)
13	1,3,4,6-IP4	2.35 ± 0.03 (5)
14	1,3,4,5-IP4	2.39 ± 0.03 (18)

[a] *myo*-Inositol phosphates of known identity were analyzed by HPAEC-CD analysis as described under Methods. IP6 was added as an internal standard. The amount of sample injected was such that the total "equivalents" subjected to chromatography did not exceed the loading capacity of the column (40 μequiv). Reproducible analytical chromatography was achieved when the amount of individual IP species did not exceed 5 μg/mL in a 25 μL injection. Unless otherwise noted, all injections hence were in a total volume of 25 μL. [b] Retention times are relative to IP6 added as internal standard. [c] Number in parentheses represents the number of experiments. [d] Obtained from an autohydrolysis of IP6 at pH 3.7 according to the procedure of Phillippy and Bland (1988). [e] Obtained from an enzymatic hydrolysis (phytase or acid phosphatase) as described by Phillippy et al. (1987).

Table 4. Relative Retention Times of the *myo*-Inositol Trisphosphates of Corn Steep Water[a]

peak no.	relative retention time[b]	identity
1	0.58 (2)[c]	1,5,6-IP3
2	0.61 (2)	1,2,6-IP3
3	0.84 (2)	1,2,3-IP3
4	1.07 (2)	1,3,4-IP3
5	1.16 (2)	1,4,5-IP3
6	1.21 (2)	2,4,5-IP3

[a] IP3 isolated from corn steep water was mixed with IP6 and analyzed by HPAEC-CD to determine relative retention times. The results from the indicated number of experiments are summarized. Identity of each component is based upon relative retention time and confirmed by cochromatography with authentic IP species and in the case of 1,5,6-, 1,2,6- and 1,2,3-IP3 by P/I ratio determinations. [b] Relative to IP6 added as an internal standard. [c] Number in parentheses represents the number of experiments.

Several points need to be made concerning the reproducibility of the chromatography. The elution times of the IPs are dependent to a great extent upon the loading capacity of the column, making it somewhat difficult to overlay chromatograms from different experiments. The column capacity problem is reduced if care is taken to frequently regenerate the column (see Methods). The use of IP6 as an internal standard allowed for the attainment of reproducible relative retention times for the purpose of identification (Tables 3–6). When column capacity was compromised, the column was subjected to washing procedures as outlined by the column manufacturer.

Enzymatic Hydrolysis and Autohydrolysis of IP6. Several IPs, which are not available commercially, were prepared from enzymatic hydrolysis and autohydrolysis of IP6 according to the method of Phillippy et al. (1987, 1988). Two IP4s (1,2,5,6-IP4 and 1,2,3,4-IP4) and one IP5 (1,2,3,4,5-IP5) are major components of the enzymatic digestion of IP6 at early times of incubation (Phillippy et al., 1987), and 1,2,4,5,6-IP5 is a major constituent among the many IP species generated by autoclaving IP6 at pH 4 (Phillippy et al., 1988). The

Table 5. Relative Retention Times of the *myo*-Inositol Tetrakisphosphates of Corn Steep Water[a]

peak no.	relative retention time[b]	identity
7	1.48 ± 0.02 (4)[c]	1,2,5,6-IP4
10	2.10 ± 0.04 (4)	1,2,3,4-IP4
14	2.36 ± 0.05 (4)	1,3,4,5-IP4

[a] IP4 isolated from corn steep water was mixed with IP6 and analyzed by HPAEC-CD to determine relative retention times. The results from the indicated number of experiments are summarized. Identity of each component is based upon relative retention time and confirmed by cochromatography with authentic IP species and in the case of 1,2,5,6-IP4 by P/I ratio determination. [b] Relative to IP6 added as an internal standard. [c] Number in parentheses represents the number of experiments.

Table 6. Relative Retention Times of the *myo*-Inositol Pentakisphosphates of Corn Steep Water[a]

peak no.	relative retention time[b]	identity
8	1.67 ± 0.03 (4)[c]	1,2,4,5,6-IP5
9	1.84 ± 0.04 (4)	1,2,3,4,5-IP5
12	2.27 ± 0.04 (4)	1,3,4,5,6-IP5

[a] IP5 isolated from corn steep water was mixed with IP6 and analyzed by HPAEC-CD to determine relative retention times. The results from the indicated number of experiments are summarized. Identity of each component is based upon relative retention time and confirmed by cochromatography with authentic IP species and P/I ratio determinations. [b] Relative to IP6 added as an internal standard [c] Number in parentheses represents the number of experiments.

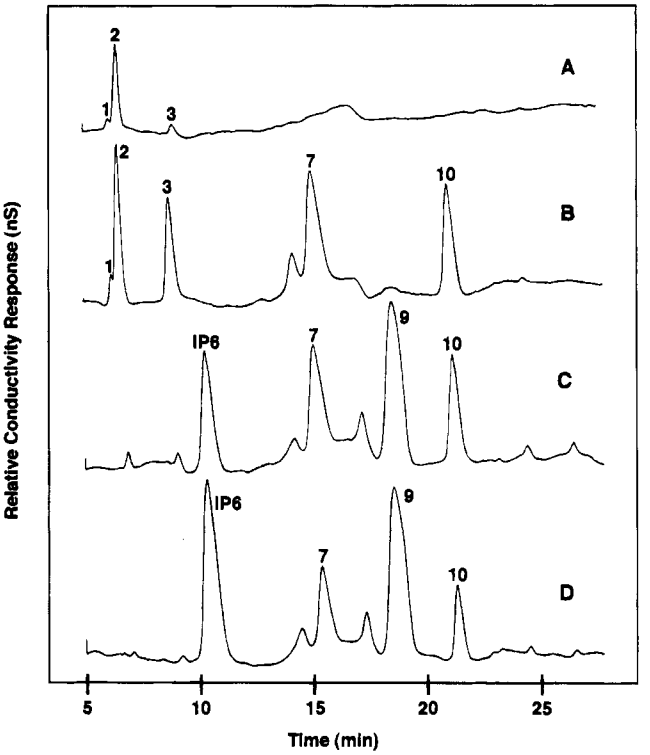

Figure 3. HPAEC-CD analysis of phytase-digested IP6. IP6 was subjected to enzymatic digestion with phytase according to the procedure described under Methods. A $^1/_{10}$ aliquot after digestion for (A) 30, (B) 14, (C) 5, and (D) 3 h was processed and each diluted (40-fold) similarly before analysis by HPAEC-CD. (1) 1,5,6-IP3; (2) 1,2,6-IP3; (3) 1,2,3-IP3; (7) 1,2,5,6-IP4; (9) 1,2,3,4,5-IP5; (10) 1,2,3,4-IP4.

three predominant IP species enzymatically generated from IP6 during the early times of hydrolysis with either phytase (Figure 3C, D) or acid phosphatase (Figure 4C, D) were readily identifiable by HPAEC-CD on the basis of the following arguments: Since the retention behavior of 1,2,5,6-IP4 (peaks 7 in Figures 3 and

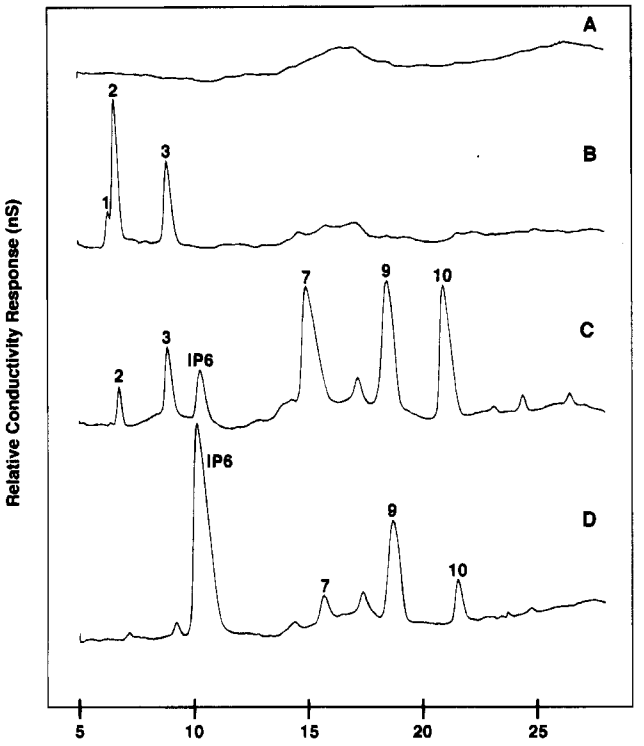

Figure 4. HPAEC-CD analysis of acid phosphatase-digested IP6. IP6 was subjected to enzymatic digestion with acid phosphatase according to the procedure outlined under Methods. A $^1/_{10}$ aliquot after digestion for (A) 30, (B) 14, (C) 5, and (D) 1 h was processed and each diluted (40-fold) similarly before analysis by HPAEC-CD. (1) 1,5,6-IP3; (2) 1,2,6-IP3; (3) 1,2,3-IP3; (7) 1,2,5,6-IP4; (9) 1,2,3,4,5-IP5; (10) 1,2,3,4-IP4.

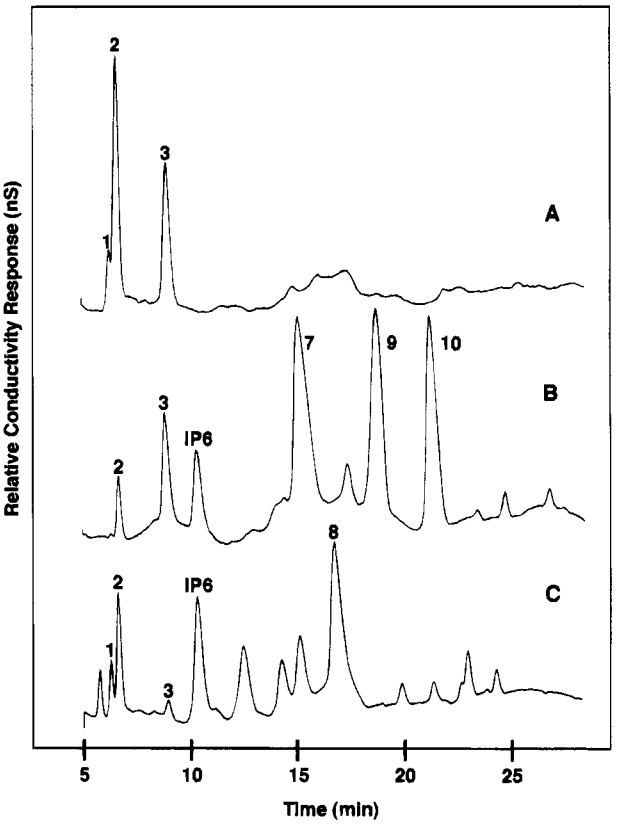

Figure 5. HPAEC-CD analysis of autohydrolyzed IP6. IP6 was autoclaved according to the procedure described under Methods. HPAEC-CD chromatographic comparison of (A) a 14 h acid phosphatase digest and (B) a 5 h phytase digest with (C) the autohydrolysate was done to aid in the identification of the 1,2,4,5,6-IP5. Before chromatography, IP6 was added as an internal standard to the autohydrolysate of IP6. (1) 1,5,6-IP3; (2) 1,2,6-IP3; (3) 1,2,3-IP3; (7) 1,2,5,6-IP4; (8) 1,2,4,5,6-IP5; (9) 1,2,3,4,5-IP5; (10) 1,2,3,4-IP4.

4) has already been established from analysis of authentic standard (peak 7 in Figure 2), only two major species in the early enzyme digests remained to be assigned. The kinetics of appearance and disappearance of these two species (Figure 3B–D) show that one component (peak 9) is more prominent early and does not persist beyond 5 h of digestion, whereas the other component (peak 10) is not prominent until 5 h of digestion and persisted up to 14 h, kinetics that parallel that of authentic 1,2,5,6-IP4 (peak 7). These kinetics are consistent with the assignment of peak 9 as an IP5 and peak 10 as an IP4. Thus, the HPAEC-separable components 7, 9, and 10 from enzymatic digestion of IP6 are identified as 1,2,5,6-IP4, 1,2,3,4,5-IP5, and 1,2,3,4-IP4, respectively. It is evident that at later times of enzymatic hydrolysis (Figures 3A and 4B) several major species are prominent, which are most likely IP3 species (see below). Furthermore, it is noteworthy that the phytase and acid phosphatase generate the same isomers, but some isomers (1,2,5,6-IP4 and 1,2,3,4-IP4) are more resistant to phytase than toward acid phosphatase (compare Figure 3B with Figure 4B).

Autohydrolysis of IP6 has been demonstrated to generate a more diverse mixture of IPs, dominated by 1,2,4,5,6-IP5, IP3, and IP2 species (Phillippy et al., 1987, 1988), when compared to the enzymatic digest of IP6. An HPAEC-CD chromatographic comparison of an autohydrolysate of IP6 with reference standards (data not shown) revealed the presence of several common species, allowing for the identification of 1,5,6-IP3, 2,4,5-IP3, and 1,2,5,6-IP4 among the autohydrolysis products. An HPAEC-CD chromatographic comparison of the enzymatic digests of IP6 (Figure 5A,B) with the autohydrolysate of IP6 (Figure 5C) shows that the most prominent component (peak 8) of the autohydrolyzed IP6 does not elute with any of the reference or enzymatically generated IPs and by exclusion is 1,2,4,5,6-IP5. In addition, Phillippy noted that two IP3s, 1,2,6-IP3, and 1,2,3-IP3 were common to the two different hydrolysates of IP6. Two prominent components (peaks 2 and 3) are present in both the enzymatic hydrolysates and autohydrolysates of IP6 and, on the basis of trends in the order of elution of other IP3 isomers, are 1,2,6-IP3 and 1,2,3-IP3, respectively.

On the basis of the separation of well-characterized IP species, either reference compounds or those generated by enzymatic hydrolysis or autohydrolysis of IP6, it is clear that HPAEC-CD is a rapid and convenient chromatographic method by which isomers of IP3, IP4, and IP5 can be separated and identified. With this chromatographic tool in hand, the IPs of corn steep water were characterized with respect to their isomeric content using a threefold strategy: (1) demonstration of the susceptibility of HPAEC-separable species to phytase and/or phosphatase digestion, (2) characterization of their retention behavior on HPAEC-CD analysis and confirmation of their identity by cochromatography with authentic standards, and (3) determination of the P/I ratios of HPAEC-separable species.

***myo*-Inositol Phosphates of Corn Steep Water.** *IP3.* The total IP3 gave three fractions (peaks I–III, Figure 6C) upon preparative HPAEC, but upon analytical scale chromatography revealed a complex chromatogram that corresponded, by cochromatography, with

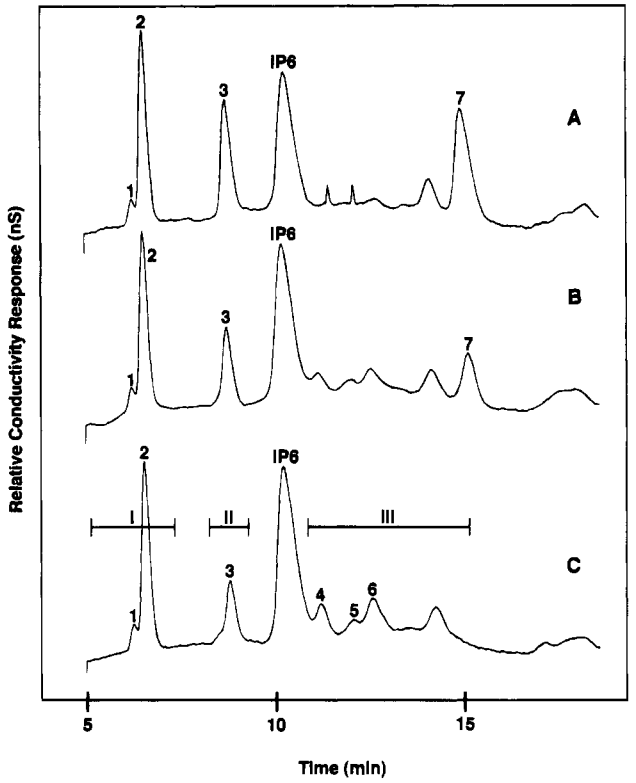

Figure 6. HPAEC-CD analysis of the *myo*-inositol trisphosphates of corn steep water. IP3 (Figure 1) was analyzed with (B) and without (C) a 14 h phytase digest of IP6 and compared with a 14 h phytase digest alone (A). In each analysis IP6 was added as an internal standard. Each chromatogram has been baseline-corrected by subtraction of a water blank analyzed concurrently. I–III represent the IP3 species recovered for P/I determinations (Table 2) by preparative HPAEC. (1) 1,5,6-IP3; (2) 1,2,6-IP3; (3) 1,2,3-IP3; (4) 1,3,4-IP3; (5) 1,4,5-IP3; (6) 2,4,5-IP3.

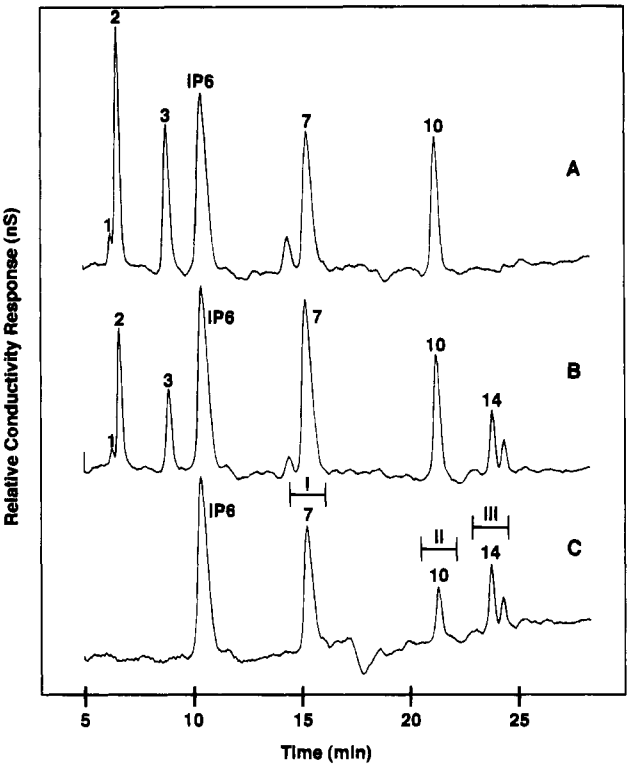

Figure 7. HPAEC-CD analysis of the *myo*-inositol tetrakisphosphates of corn steep water. IP4 (Figure 1) was analyzed with (B) and without (C) a 14 h phytase digest of IP6 and compared with a 14 h phytase digest alone (A). In each analysis IP6 was added as an internal standard. Each chromatogram has been baseline-corrected by subtraction of a water blank analyzed concurrently. I–III represent IP4 species recovered for P/I determinations (Table 2) by preparative HPAEC. (7) 1,2,5,6-IP4; (10) 1,2,3,4-IP4; (14) 1,3,4,5-IP4.

1,5,6-, 1,2,6-, 1,2,3-, 1,3,4-, 1,4,5-, and 2,4,5-IP3 and an unidentified peak in order of their elution times (Table 4; Figure 6B). After extensive digestion with alkaline phosphatase (up to 90 h), the unidentified peak persisted at 14% of the original peak area while all other IP3s were completely dephosphorylated to give *myo*-inositol as the only neutral species (data not shown). P/I ratios (Table 2) for 1,5,6- and 1,2,6-IP3 (peak I, Figure 6C) and 1,2,3-IP3 (peak II, Figure 6C) of 2.8 provided confirmation of their identity. A P/I ratio for the other IP3s and the unidentified peak (peak III, Figure 6C) was lower than expected (1.9, Table 2) but could be low because of the non-IP nature of the unidentified component present in this heterogeneous fraction.

IP4. IP4 was separable into four peaks by analytical scale HPAEC but only three peaks (peaks I–III, Figure 7C) upon preparative HPAEC. Only one IP4 fraction (peak I) was isolated in enough quantity to obtain a P/I ratio of 3.7 (Table 2). All of the components were susceptible to enzymatic hydrolysis, and after 72 h *myo*-inositol was the only product, with perhaps a trace (6%) of peak I still resistant. The chromatographic behavior (Table 4) of three of the four peaks was consistent with 1,2,5,6-, 1,2,3,4-, and 1,3,4,5-IP4 and confirmed by cochromatography with the partial enzymatic hydrolysate of IP6 (Figure 7). The minor peak (RRT = 2.41 ± 0.06, four experiments) was not identified.

IP5. HPAEC-CD analysis of IP5 showed 1,2,4,5,6-, 1,2,3,4,5-, and 1,3,4,5,6-IP5 as predominant species and a minor species that must be 1,2,3,5,6-IP5 since this is the only other chromatographically separable IP5 species. These assignments, based upon cochromatography with reference IP compounds (data not shown) or with an enzymatic hydrolysate (Figure 8) or autohydrolysate of IP6 (Figure 9), are consistent with their P/I ratios (Table 2). Furthermore, upon phytase digestion for 24 h each HPAEC-separable species showed complete dephosphorylation with concomitant detection of *myo*-inositol as the only product (data not shown).

In summary, the composition of IP isomers present in corn steep water strongly suggests that enzymatic hydrolysis and/or autohydrolysis of the IP6 extracted from corn during the steeping process is a likely mechanism for the accumulation of lower *myo*-inositol phosphates. The corn steeping process provides favorable conditions for both reactions to occur. Corn steeping provides an environment of acidic pH (Oberleas and Harland, 1986; Xu et al., 1992), elevated temperature (52 °C), prolonged incubation times (up to 30 h), and the presence of phosphatase enzymes (unpublished data).

Determination of IP6 in Food. The IP6 content of foodstuffs when analyzed by the determination of covalently bound phosphate presumes that no other *myo*-inositol phosphates are present in appreciable amounts (Oberleas and Harland, 1986; Xu et al., 1992). The presence of significant levels of IP3, IP4, and IP5 species in heavy steeps would have a significant effect on the phytate determination of this byproduct by the official method for analysis (Harland and Oberleas, 1986), which calls for phosphorus analysis of a strong

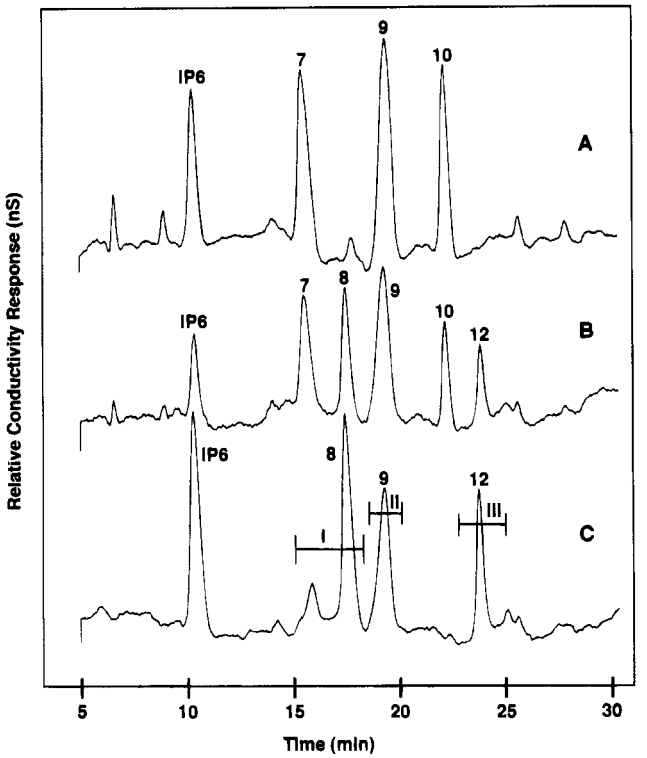

Figure 8. HPAEC-CD analysis of the *myo*-inositol pentakisphosphates of corn steep water. IP5 (Figure 1) was analyzed with (B) and without (C) a 5 h phytase digest of IP6 and compared with a 5 h phytase digest alone (A). IP6 was added as an internal standard to the IP5 corn sample (panel C) but not the 5 h phytase digest. Each chromatogram has been baseline-corrected by subtraction of a water blank analyzed concurrently. I–III represent IP5 species recovered for P/I determinations (Table 2) by preparative HPAEC. (8) 1,2,4,5,6-IP5; (9) 1,2,3,4,5-IP5; (12) 1,3,4,5,6-IP5.

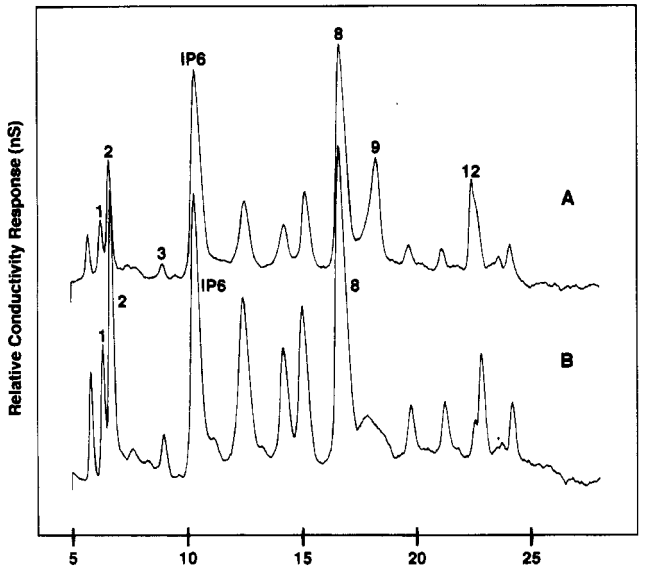

Figure 9. HPAEC-CD cochromatography of IP5 with autohydrolyzed IP6. IP5 (Figure 1) was cochromatographed with autohydrolyzed IP6 (A) and compared with autohydrolyzed IP6 alone (B). Each chromatogram has been baseline-corrected by subtraction of a water blank analyzed concurrently. This comparative analysis allowed for the identification of 1,2,4,5,6-IP5 in IP5 (8). (1) 1,5,6-IP3; (2) 1,2,6-IP3; (3) 1,2,3-IP3; (8) 1,2,4,5,6-IP5; (9) 1,2,3,4,5-IP5; (12) 1,3,4,5,6-IP5.

acid hydrolysate performed on an anionic fraction recovered from an acidified corn steep. Since the anionic exchange step does not resolve the lower IPs from IP6, their presence would lead to a significant overestimation of phytic acid.

Isolation of *myo*-Inositol Phosphates. Recent biomedical and engineering research suggests possible new applications for IP5 and IP6 (Sands et al., 1986); in particular, recent evidence suggests that these two IP species are potentially valuable anti-inflammatory agents (Cecconi et al., 1994). In addition, Hawkins and co-workers recently demonstrated the likelihood of the role of IP6 as a physiologically relevant antioxidant (Hawkins et al., 1993). Corn steep water could be a source for these *myo*-inositol polyphosphates. Given the potential value of *myo*-inositol phosphates, precipitation of IPs from corn steep as their calcium salts was investigated with the view to providing a more rapid and convenient recovery procedure as compared to ion-exchange methods. Calcium precipitation of stream 4H resulted in the recovery of IP5 and IP6 as monitored by IP-RP-HPLC (data not shown) in only 18% (1.7 mg/mL, Table 1) and 48% (11.9 mg/mL, Table 1) of theoretical yield, respectively. HPAEC-CD analysis of the calcium precipitate indicated that all isomeric forms of IP5 were present (data not shown). These results clearly indicate that the calcium precipitation of corn steep would not be as practical as the ion-exchange method for recovery of *myo*-inositol phosphates from large volumes of corn steep.

ABBREVIATIONS USED

IP, *myo*-inositol phosphate; IP3, *myo*-inositol trisphosphate; IP4, *myo*-inositol tetrakisphosphate; IP5, *myo*-inositol pentakisphosphate; IP6, *myo*-inositol hexakisphosphate; HPAEC-PAD or HPAEC-CD, high-performance anion-exchange chromatography with either pulsed amperometric detection or conductimetric detection; IP-RP-HPLC, ion-pair reversed-phase high-performance liquid chromatography.

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