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Separation of phytic acid and other related inositol phosphates by high-performance ion chromatography and its applications

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Abstract

A high-performance anion-exchange chromatographic method was developed for the separation of phytic acid and other inositol phosphates (*myo*-inositol bis-, tris-, tetrakis-, and pentakisphosphates) with gradient elution and ultraviolet absorbance detection after post-column derivatization. With the acidic eluents, the combination of anion-exchange and ion suppression retention mechanisms led to the separation of 35 inositol phosphates (excluding enantiomers) into 27 peaks for the first time, and the retention behaviors of all *myo*-inositol bis- to hexakisphosphate isomers were studied. The whole separation procedure was completed within 65 min. Based on the investigations of nonenzymatic hydrolysis of phytic acid under different conditions by using this method, an in-house reference standard solution was produced, which can be used for method development. In addition, by applying this method to in vitro kinetic studies, at least one new enzymatic hydrolysis pathway of phytic acid was found, and one rule of enzymatic dephosphorylation of inositol phosphates (position effect) was proposed and another one (neighboring effect) was confirmed. The principle of the proposed identification approach for several inositol phosphate isomers based on hydrolysis products study will be applicable to other natural products analysis, for which standards are very expensive or not available.
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Keywords: Retention mechanism; Phytic acid; Inositol phosphates; Phytase; Enzymes; Phosphates

1. Introduction

Phytic acid, *myo*-inositol hexakisphosphate ($InsP_6$, Fig. 1) is a naturally occurring constituent and the major storage form of phosphorus in cereals, legumes, nuts, and other crops. Historically, it was considered solely as an antinutrient because it can bind essential dietary minerals such as calcium, iron, and zinc, and thus decrease their bioavailability in humans. It also can react directly with charged groups of proteins or indirectly with the negatively charged groups of proteins mediated by a mineral cation, and thus adversely influence protein digestion and bioavailability [1–3]. In the past few years, numerous in vitro and in vivo studies have shown that $InsP_6$, either endogenous or exogenous, has also beneficial effects, such as

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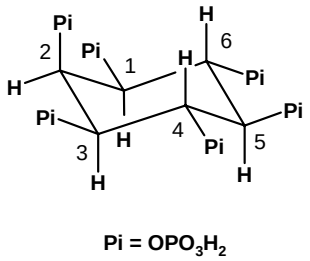

Fig. 1. Structure of phytic acid ($InsP_6$).

protection against cancers, heart diseases, diabetes, and renal calculi [1–3]. During food processing and digestion, $InsP_6$ can be degraded to *myo*-inositol pentakis-, tetrakis-, tris-, bis-, monophosphates ($InsP_5$–$InsP_1$) and in some cases free *myo*-inositol by enzymatic or nonenzymatic means. Enzymatic hydrolysis usually occurs in the gastrointestinal tract of humans, or during food processing, such as baking, malting, and fermenting by the action of intrinsic plant phytases, extrinsic microbial phytases, or both. Nonenzymatic hydrolysis generally takes place when foods are heated (e.g. autoclaving, canning) or treated with a strong acid [4]. With the removal of phosphate groups from the inositol ring, the mineral binding capacity decreases, resulting in an increased bioavailability of minerals. D-*myo*-inositol 1,4,5-trisphosphate (D-Ins(1,4,5)P_3), as a second messenger, plays an essential role in cell activation by mobilizing calcium from intracellular compartments [5]. Several isomers of inositol phosphates have shown other important physiological functions, such as anti-inflammatory effects [6] and prevention of diabetes complications [7]. The position of phosphate groups on the inositol ring determines the physiological functions; for example, even enantiomers, D-*myo*-inositol 1,4,5,6-tetrakisphosphate (D-Ins(1,4,5,6)P_4) and D-*myo*-inositol 3,4,5,6-tetrakisphosphate (D-Ins (3,4,5,6)P_4 or L-Ins(1,4,5,6)P_4), play distinct but critical roles in cellular signaling [8]. Therefore, in order to fully understand the physiological effects of individual inositol phosphates, it is crucial to establish an accurate and reliable method that can be used for the analysis of different isomeric forms of inositol phosphates including $InsP_6$.

In recent years, although a great variety of analytical techniques [9–11] have been applied to the analysis of $InsP_6$ or other inositol phosphates, only a few approaches, mainly ion-pair chromatography [12–17] and high-performance anion-exchange chromatography [18–30], are capable for the simultaneous determination of $InsP_6$ and other inositol phosphates. Most ion-pair chromatographic procedures were based on isocratic reversed-phase chromatographic separation with methanol or acetonitrile in weak acidic buffer containing cationic ion-pair reagents as mobile phase, and refractive index detection. These methods have been used for the separation of $InsP_3$–$InsP_6$ based only on the number of phosphate groups without differentiating isomeric forms of inositol phosphates [12–17]. Nowadays, high-performance anion-exchange chromatography has proven to be an isomer-specific analytical technique, which has the capability not only to separate inositol phosphates with different numbers of phosphate groups, but also to separate the different isomeric forms (excluding enantiomers) of inositol phosphates with the same number of phosphates. However, due to the slight differences in charges and structures between isomers, usually the separation is not very satisfactory. Because many isomer standards are not commercially available, some chromatographic peaks that had been separated were not identified, or were recognized mainly according to the elution sequence of these isomers in other studies [22,26], which might lead to misidentification. The aims of the present study are: (1) to develop a reliable analytical method that can separate a large number of inositol phosphate isomers, (2) to definitively identify each peak that has been separated, and (3) to apply the proposed method to study the hydrolysis of $InsP_6$ under different conditions, and prepare an in-house reference standard solution for further method development. Also, a method for quantitative determination of $InsP_6$ and some selected inositol phosphates in foods is being investigated in our laboratory.

2. Experimental

2.1. Apparatus

A Dionex DX-500 ion chromatograph (Sunnyvale, CA, USA) equipped with a GP40 gradient

pump was employed along with a Dionex PeakNet chromatography workstation (Version 5.2) for instrument control as well as data acquisition and processing. The separation was performed by a linear gradient elution program on a Dionex CarboPac PA-100 guard column (50 mm × 4 mm) and a CarboPac PA-100 analytical column (250 mm × 4 mm, 10 μm; retention mechanism: anion exchange), and the detection by a Dionex AD25 absorbance detector at 295 nm after post-column reaction with a solution of 1 g/l $Fe(NO_3)_3$ in 0.33 M $HClO_4$. The gradient elution was effected with a mixture of two eluents: (A) 500 mM HCl and (B) H_2O; 0–16 min, 8–20% A, 92–80% B; 16–33 min, 20–37% A, 80–63% B; 33–49 min, 37–100% A, 63–0% B; 49–50 min, 100% A, 0% B; and 50–50.1 min, 100–8% A, 0–92% B. The flow rates of eluent and post-column reaction solution were 1.0 and 0.4 ml/min, respectively. A Dionex knitted coil (750 μl) for post-column reaction was used. The column temperature was maintained at 30 °C, and the injection volume of standard and sample solutions at 100 μl with a Dionex AS3500 autosampler. Equilibration time between each chromatographic run was 15 min. Prior to the chromatographic analysis, aliquots of hydrolysis solutions were filtered through 0.22 μm Millipore Millex-GV membrane filters (Bedford, MA, USA).

2.2. Reagents

The water for the preparation of all solutions was purified by a Millipore Milli-Q academic system. All chemicals were of analytical grade or higher purity unless otherwise stated.

Phytic acid (dodecasodium salt hydrate) and phytic acid (40%, w/w, solution in water) were purchased from Aldrich (Milwaukee, WI, USA). Phytase (EC 3.1.3.26, from wheat), phytase (EC 3.1.3.8, from *Aspergillus ficuum*), Ins(2)P_1, D-Ins(1,4)P_2, D-Ins(2,4)P_2, D-Ins(4,5)P_2, D-Ins(1,3,4)P_3, D-Ins(1,5,6)P_3, D-Ins(2,4,5)P_3, D-Ins(1,2,5,6)P_4, D-Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4, D-Ins(3,4,5,6)P_4, and Ins(1,3,4,5,6)P_5 were obtained from Sigma (St. Louis, MO, USA). Ins(1,2,3)P_3, Ins(1,3,5)P_3, D-Ins(1,4,6)P_3, D-Ins(2,3,5)P_3, D-Ins(3,4,5)P_3, D-Ins(1,2,3,4)P_4, Ins(1,2,3,5)P_4, D- Ins(1,2,4,5)P_4, and D-Ins(1,4,5,6)P_4 were obtained from Alexis Biochemicals (San Diego, CA, USA). Ins(1,3)P_2 and D-Ins(1,5)P_2, were obtained from A. G. Scientific (San Diego, CA, USA), and D-Ins(3,4)P_2, from Calbiochem–Novabiochem (La Jolla, CA, USA). D-Ins(1,2,4)P_3, Ins(2,4,6)P_3, Ins(4,5,6)P_3, D-Ins(1,2,4,6)P_4, Ins(2,4,5,6)P_4, D/L-Ins(1,2,3,4,5)P_5, and D/L-Ins(1,2,4,5,6)P_5, were kindly provided by Professor Sung-Kee Chung and Dr. Yong-Uk Kwon at Pohang University of Science & Technology (Pohang, South Korea), D-Ins(1,4,5)P_3 (product of LC Labs., Woburn, MA, USA), and Ins(1,2,3,4,6)P_5, by Dr. Brian Q. Phillippy at Southern Regional Research Center, Agricultural Research Service, US Department of Agriculture (New Orleans, LA, USA), as well as D-Ins(1,2,6)P_3 (product of Perstorp AB, Perstorp, Sweden), by Mr. Richard Helms at Grain Processing Corp. (Muscatine, IA, USA).

2.3. Preparation of in-house reference standard solution

Transfer 2 ml of $InsP_6$ (dodecasodium salt hydrate) solution (5 mg/ml) into a glass tube with PTFE lined screw cap, and add 0.40 ml HCl (to a final concentration of 2 M at pH ca. 0.5–0.6). Cap the tube tightly, and heat the solution in an oven at 140 °C for 1 h. Cool, put the tube in a 40 °C water bath, and dry the content under a stream of nitrogen. Add water to redissolve residue and make up to a final volume of 10 ml. This solution can be stored in a 4 °C refrigerator for at least 1 year.

2.4. Identification of inositol phosphate isomers

All the $InsP_2$–$InsP_6$ isomers, except DL-Ins(1,2)P_2, Ins(4,6)P_2, and Ins(2,5)P_2, were identified by comparison of the retention times with available standards. For the isomers with unsatisfactory resolution peaks, the identification was further confirmed by addition of particular standards to the reference standard solution before analysis.

In case of DL-Ins(1,2)P_2, Ins(4,6)P_2, and Ins(2,5)P_2, for which the standards were not available, the identification was performed by comparing the retention times of available or identified $InsP_2$ isomers with the hydrolysis products of D-Ins(1,2,4)P_3, D-Ins(1,4,6)P_3, and D-Ins(2,3,5)P_3 solutions after heating in a 100 °C oven for 1–2 h, respectively.

2.5. Nonenzymatic hydrolysis of $InsP_6$

Transfer 4 ml of 2.5 mg/ml $InsP_6$ (dodecasodium salt hydrate) solution into a PTFE vial with a cap, adjust pH with diluted HCl. Cap the vial tightly, and autoclave the solution at 121 °C or heat in an oven at 100 °C for 1 h. Cool, transfer the entire solution to a 10 ml volumetric flask, and dilute to volume with water.

2.6. Enzymatic hydrolysis of $InsP_6$

2.6.1. Hydrolysis of $InsP_6$ by wheat phytase

Dissolve 100 mg $InsP_6$ (dodecasodium salt hydrate) in 4 ml water, and adjust pH to 5.15 with diluted HCl. Add 50 mg wheat phytase, dissolve, and immediately dilute to 5 ml with water, and then incubate the solution at 55 °C in a water bath. At 0, 15, 30, 60, 120, 180, 240 min and finally 22 h, withdraw 0.5 ml hydrolysis solution, add 2 ml of ice-cold ethanol, shake vigorously, put into a 100 °C oven for 2 min to terminate the enzymatic reaction, and dilute to 10 ml with water. Centrifuge the solution at 12,100 × *g* for 10 min, and then withdraw the supernatant, filter, and analyze.

2.6.2. Hydrolysis of $InsP_6$ by Aspergillus ficuum phytase

Dissolve 100 mg $InsP_6$ (dodecasodium salt hydrate) in 4 ml water, and adjust pH to 2.50 with diluted HCl. Add 5 mg *A. ficuum* phytase, dissolve, and immediately dilute to 5 ml with water, and then incubate the solution at 37 °C in a water bath. At 0, 15, 30, 60, and 120 min, withdraw 0.5 ml hydrolysis solution, and the subsequent procedures are the same as mentioned above.

3. Results and discussion

3.1. Analytical system

3.1.1. Selection of separation and detection systems

In theory, there are 63 inositol phosphates. Because *myo*-inositol contains five equatorial and one axial hydroxyl groups (see Fig. 1), the molecule is symmetrical on either side of an axis formed by positions 2 and 5, and thus positions 1 and 3 are equivalent, as are positions 4 and 6. Excluding the enantiomers that cannot be separated on ion-exchange stationary phase (it was also confirmed in this study: the retention times of two pairs of enantiomers, D-Ins(1,4,5,6)P_4 and D-Ins(3,4,5,6)P_4, D-Ins(1,5,6)P_3 and D-Ins(3,4,5)P_3, are identical), there are 39 inositol phosphates, including 4 $InsP_1$, 9 $InsP_2$, 12 $InsP_3$, 9 $InsP_4$, and 4 $InsP_5$ isomers as well as $InsP_6$. Due to their strong acidity, all of them exist as anions with different valences in solution, making it possible for separation by anion-exchange chromatography. Both basic [23–27,29] and acidic [18–22,26,28,30] elution programs have been used, coupled with chemically suppressed conductivity detection and post-column derivatization absorbance detection, respectively.

In this study, the basic elution system was initially tested by using a Dionex OmniPac PAX-100 guard column (50 mm × 4 mm) and an OmniPac PAX-100 analytical column (250 mm × 4 mm, 8.5 μm; retention mechanism: anion exchange) with conductivity detection after chemical suppression. When the optimal separation program was used, in which eluent was A (200 mM NaOH)–B (water)–C (50% isopropanol, v/v) = 55:41:4 (v/v) and injection volume was 25 μl, some selected inositol phosphates eluted isocratically in the order of DL-Ins(1,5,6)P_3, DL-Ins(1,2,6)P_3, $InsP_6$, DL-Ins(1,3,4)P_3, DL-Ins(1,4,5)P_3, DL-Ins(1,2,5,6)P_4, DL-Ins(2,4,5)P_3, Ins(1,3,4,5,6)P_5, and Ins(1,3,4,6)P_4, which was very similar to the results obtained by a gradient elution program [25]. However, the retentions of inositol phosphates on the stationary phase did not increase with increasing number of phosphate groups on the inositol ring as expected. Also, the retention times of all analytes were not reproducible, which made the peak identification very difficult. This may be accounted for by the fact, in basic solution, inositol phosphates are completely deprotonated, and thus highly charged; however, due to the steric hindrance, not all the deprotonated phosphate groups can effectively interact with the anion-exchange sites on the stationary phase. Therefore, the retentions are probably determined by the steric structures of both the stationary phase and analytes in addition to the charges on analytes. Besides, due to the high valences of inositol phosphates, the column capacity can be easily approached or exceeded, making the retention times irreproducible. Hence, although the basic elution system is useful in separating the less phosphorylated inositol phosphates such as $InsP_1$ and

$InsP_2$ [27], it is not suitable for the separation of a large number of inositol phosphates, and thus not adopted for this study.

Two kinds of analytical columns (tandem with their guard columns), OmniPac PAX-100 and CarboPac PA-100 columns, were compared in the acidic elution system, both of which were compatible with pH 0–14 eluents. HCl was chosen as an eluent because the relatively weak elution power of Cl^- was beneficial for separating isomers with slightly different charges compared to other acids such as HNO_3 and $HClO_4$, which also could be used. The optimal separation conditions for CarboPac PA-100 column were described in Section 2.1, and the optimal conditions for OmniPac PAX-100 column were at 30 °C when the gradient elution was carried out with a mixture of three eluents: (A) 500 mM HCl, (B) water, and (C) 50% isopropanol (v/v), in which the proportion of eluent C was maintained at 2%, and a series of linear gradients changed the proportions of eluents A and B: 0–28 min, 8–29% A, 90–69% B; 28–45 min, 29–80% A, 69–18% B; and 45–45.1 min, 80–8% A, 18–90% B. The comparison results are summarized in Table 1. On both columns, inositol phosphate isomers eluted in the order of increasing numbers of phosphate groups on the inositol ring (it must be pointed out that in acidic elution system, only the specific elution programs can realize this; for example, when using a different elution program, Ins(1,2,3,4,6)P_5 elutes earlier than DL-Ins(1,4,5,6)P_4), and $InsP_1$ and inorganic phosphate, which were poorly retained on the stationary phase, eluted just after the void time (t_0). Compared with OmniPac PAX-100 column, CarboPac PA-100 column gave better separation for $InsP_2$–$InsP_6$ isomers because its higher ion-exchange capacity was advantageous for increasing the selectivity and thus improving the separation. Although OmniPac PAX-100 column gave one more $InsP_4$ isomer peak, the resolutions for most $InsP_4$ peaks were not satisfactory. Neither the addition of organic solvent (methanol or isopropanol) to the eluent for CarboPac PA-100 column, nor the concentration change of organic solvent in the eluent for OmniPac PAX-100 column, could improve the separation. Also, for OmniPac PAX-100 column, it was necessary to maintain at least 1% (v/v) organic solvent in the eluent to wet the hydrophobic substrate. Taking all the above factors into consideration, CarboPac PA-100 column was finally chosen and no organic solvent was added in the eluent.

Table 1
Characteristics and separation performances of two columns

	OmniPac PAX-100	CarboPac PA-100
Column ion-exchange capacity (μeq.)	40	90
Separated peaks[a] for $InsP_2$–$InsP_6$	21	27
$InsP_2$	3	6
$InsP_3$	5	9
$InsP_4$	8	7
$InsP_5$	4	4
$InsP_6$	1	1

[a] In the in-house reference standard solution.

Furthermore, the effects of column temperature on the retentions of different inositol phosphates on CarboPac PA-100 column were different. By increasing the column temperature in the range of 20–45 °C, for some isomers, the retention times increased; while for others, the retention times initially increased and then decreased, resulting in poor resolutions between the $InsP_2$ isomers and the $InsP_3$ isomers, along with improved resolutions between the $InsP_4$ isomers. No significant change for separation of the $InsP_5$ isomers and $InsP_6$ was found with changing the column temperature. Comparing the results obtained at 30 °C, the retentions increased with increasing column temperature for DL-Ins(1,2,4,6)P_4 and even more so for Ins(1,2,3,5)P_4. Sequentially, when the column temperature reached 45 °C, they can be separated albeit not baseline separation. In order to obtain more isomer peaks, finally, 30 °C was chosen as column temperature.

Inositol phosphates have no characteristic absorption spectra in ultraviolet or visible region, therefore, three post-column derivatization approaches have been previously employed for the acidic elution system: $Fe^{3+}/HClO_4$ system [19,26,28,30,31], Fe^{3+}/sulfosalicylic acid (SA) system [18], and YCl_3/4-(2-pyridylazo)resorcinol (PAR) system [20–22]. In this study, all three detection systems were compared, and the results are summarized in Table 2 (according to the reaction conditions, when YCl_3/PAR detection system was used, YCl_3/HCl solution must be added into eluent and, accordingly, the elution gradient program was modified and a little

Table 2
Comparison of three post-column detection systems used in the acidic elution system

	$Fe^{3+}/HClO_4$	Fe^{3+}/SA	YCl_3/PAR
Post-column solution	1 g/l $Fe(NO_3)_3$ in 0.33 M $HClO_4$	0.24 g/l $Fe(NO_3)_3$ in 7 mM SA	0.3 mM PAR in 1.6 M triethanolamine (pH 9.1)
Detection wavelength (nm)	295	500	520
Detection peak mode	Positive peak	Negative peak	Negative peak
Detection sensitivity	Medium	Lowest	Highest
Effect on separation	No	No	Yes
Baseline stability	Good	Good	Poor

different from that shown in Section 2.1, which was used when the other two detection systems were tested). Although the YCl_3/PAR system gave the highest detection sensitivity, the separation was very unsatisfactory, and the baseline very unstable. Several "ghost peaks" eluted after $InsP_6$ peak, and a longer equilibration time (more than 30 min) was also needed. It was probably because Y^{3+} and Cl^- in the eluent formed some anionic complexes, and eluted in high concentration of HCl during the latter part of the gradient program. The high viscosity of this post-column reaction solution and possible adsorption of reaction products on the wall of reaction coil also degraded the peak resolutions, and the adoption of shorter coils could not solve this problem. Finally, $Fe^{3+}/HClO_4$ detection system was chosen for this study because it gave satisfactory detection sensitivity for all inositol phosphates. As this post-column reaction was not very fast, a longer reaction coil was beneficial for improving the detection sensitivity. However, the larger volume of coil also increased the void volume of the analytical system and produced peak tailing. So, a 750 μl coil was finally chosen as a compromise.

Under the optimized experimental conditions (as described in Section 2.1), a total of 27 peaks were detected in the in-house reference standard solution for all 35 possible $InsP_2$–$InsP_6$ isomers (excluding enantiomers, Fig. 2), and the reproducibility of retention times (%, $n = 8$) were not more than 0.11, 0.05, and 0.03 for $InsP_4$, $InsP_5$, and $InsP_6$ peaks, respectively. Although the retention times of several isomers differed slightly when the pure standards were individually chromatographed, e.g. DL-Ins(1,2,4,6)P_4 eluted earlier than Ins(1,2,3,5)P_4, and DL-Ins(1,2,3,4)P_4 earlier than Ins(1,3,4,6)P_4, they co-eluted when the mixed solutions were analyzed.

3.1.2. Separation mechanism

To fully understand the separation mechanism of inositol phosphates in acidic elution system, experiments were conducted with isocratic elution programs using $InsP_5$ and $InsP_6$ as examples. A solution containing only $InsP_5$ and $InsP_6$ was prepared by hydrolyzing $InsP_6$ solution (pH 2.0) at 100 °C for 1 h (see Section 3.4.1), and t_0 was determined by using the water dip for each chromatographic run. Firstly, according to theory [32,33], the relationship between an ion's retention factor k, and the eluent concentration C, is given by:

$$\log k = -\left(\frac{Z}{E}\right) \log C + \log I \quad (1)$$

where Z is the effective charge of analyte, E the charge of eluent (here, $E = 1$), and I a constant that depends on the column and eluent. At two different pH values, the Cl^- concentration was changed by adding KCl in the eluent. When HCl was kept at 0.25 M (pH ca. 0.60), the KCl concentration was increased from 0 to 0.15 M; when HCl was 0.35 M (pH ca. 0.46), the KCl concentration from 0 to 0.10 M. As shown in Fig. 3, keeping the acidity constant in eluent, the retention times of all $InsP_5$ and $InsP_6$ decrease with increasing Cl^- concentration, and there is a very good linear relationship between the log[Cl^-] and log k for each analyte. The effective charges of $InsP_5$ and $InsP_6$ were calculated according to the slopes (Table 3). By increasing the acidity of eluent, the effective charges of all $InsP_5$ and $InsP_6$ decrease, which proves the existence of ion suppression mechanism in eluent. Also, the higher the effective charge of the analyte, the longer the retention time, indicating that at least for $InsP_5$ isomers, the effective charge is the determining factor for the retention times in acidic elution system.

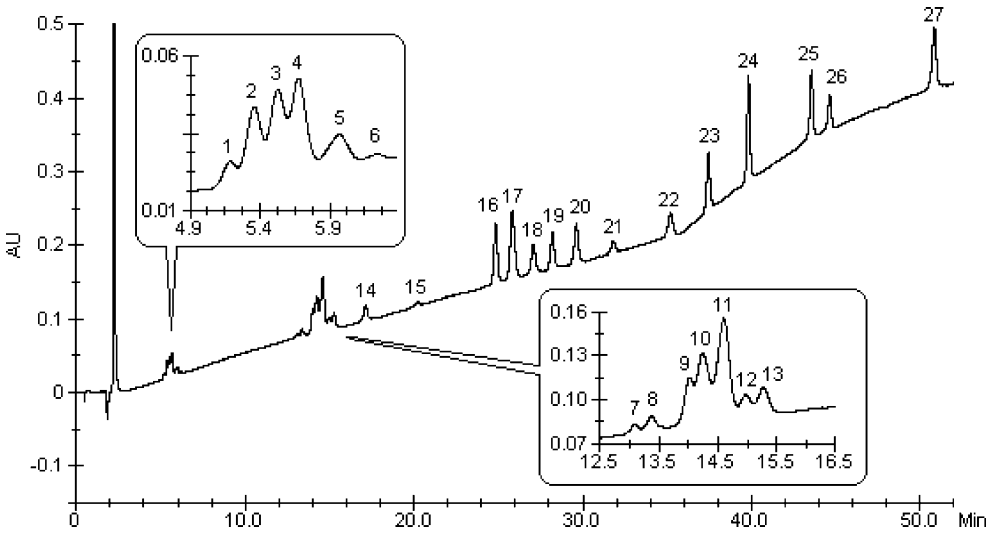

Fig. 2. Chromatogram of the in-house reference standard solution. Peaks: (1) Ins(1,3)P_2; (2) DL-Ins(3,4)P_2, DL-Ins(1,5)P_2; (3) DL-Ins(1,2)P_2; (4) DL-Ins(2,4)P_2, DL-Ins(1,4)P_2; (5) DL-Ins(4,5)P_2, Ins(2,5)P_2; (6) Ins(4,6)P_2; (7) Ins(1,3,5)P_3; (8) Ins(2,4,6)P_3; (9) DL-Ins(1,3,4)P_3; (10) DL-Ins(1,2,4)P_3, DL-Ins(2,3,5)P_3; (11) Ins(1,2,3)P_3, DL-Ins(1,2,6)P_3, DL-Ins(1,4,6)P_3; (12) DL-Ins(1,4,5)P_3; (13) DL-Ins(2,4,5)P_3; (14) DL-Ins(1,5,6)P_3; (15) Ins(4,5,6)P_3; (16) DL-Ins(1,2,4,6)P_4, Ins(1,2,3,5)P_4; (17) DL-Ins(1,2,3,4)P_4, Ins(1,3,4,6)P_4; (18) DL-Ins(1,2,4,5)P_4; (19) DL-Ins(1,3,4,5)P_4; (20) DL-Ins(1,2,5,6)P_4; (21) Ins(2,4,5,6)P_4; (22) DL-Ins(1,4,5,6)P_4; (23) Ins(1,2,3,4,6)P_5; (24) DL-Ins(1,2,3,4,5)P_5; (25) DL-Ins(1,2,4,5,6)P_5; (26) Ins(1,3,4,5,6)P_5; and (27) InsP_6. With regard to enantiomers not separated, both possible isomers are denoted according to this rule (DL-Ins). Detailed experimental conditions as in the text.

Secondly, the Cl^- concentration was kept at 0.4 M (thus, the elution power was constant), and the proportions of HCl and KCl in eluent varied. As shown in Fig. 4, by decreasing the acidity, in the beginning, the retention times of all InsP_5 and InsP_6 increase because ion suppression is reduced, i.e. ionization is enhanced, and the effective charges of all isomers increase. When the K^+ concentration increases to a certain range, the retention times of analytes decrease, which maybe due to K^+ forming ion-pair complexes with these isomers. Among the InsP_5 isomers, DL-Ins(1,2,4,5,6)P_5 and Ins(1,3,4,5,6)P_5, especially the latter, can more easily form ion-pair complexes with K^+, which is probably related to their structures. Thus, in this experiment, ionization and ion-pair interactions exist simultaneously. At the beginning of acidity reduction, ionization is dominant; and later, ion-pair interaction becomes dominant. So, in physiological conditions, high concentration of K^+ and other monovalent cations can possibly form ion-pair complexes of different strengths with inositol phosphate isomers, at least with InsP_6 and InsP_5.

In summary, the high concentration of HCl in eluent not only provides the eluting anion Cl^-, but also suppresses the ionization of highly charged inositol phosphates, both of which are enhanced with the steady increase of HCl concentration in the gradient program. All of these make it possible to separate these multivalent anions with similar structures by anion-exchange chromatography.

Table 3
The effective charges of InsP_5 and InsP_6 in eluents at different pH

pH	Ins(1,2,3,4,6)P_5	DL-Ins(1,2,3,4,5)P_5	DL-Ins(1,2,4,5,6)P_5	Ins(1,3,4,5,6)P_5	InsP_6
0.60	2.19	2.33	2.43	2.54	3.06
0.46	1.85	1.97	2.10	2.21	2.68

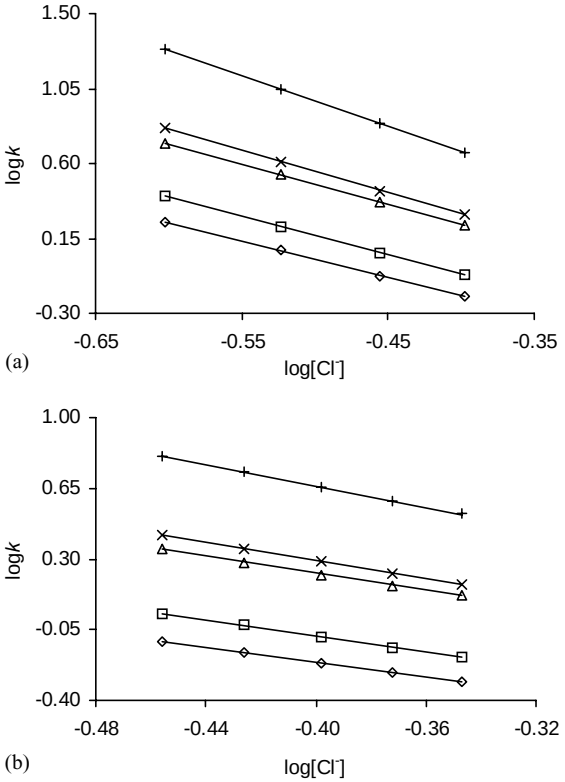

Fig. 3. The relationship between retention of analytes and the total concentration of Cl^- in eluent when HCl concentrations were: 0.25 M (a) and 0.35 M (b). (◇) Ins(1,2,3,4,6)P_5, (□) DL-Ins(1,2,3,4,5)P_5, (△) DL-Ins(1,2,4,5,6)P_5, (×) Ins(1,3,4,5,6)P_5, and (+) InsP_6.

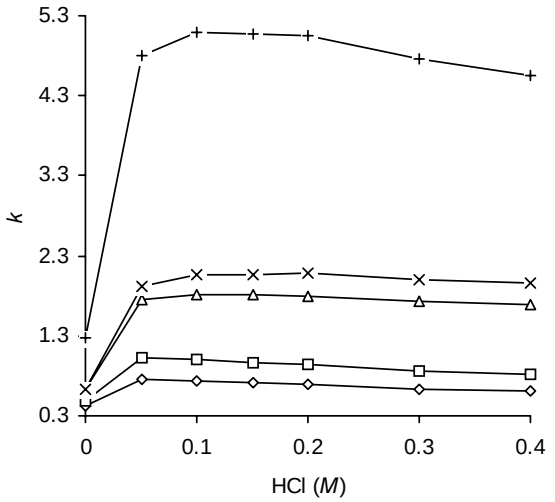

Fig. 4. Effect of HCl concentration in eluent on the retention of analytes. (◇) Ins(1,2,3,4,6)P_5, (□) DL-Ins(1,2,3,4,5)P_5, (△) DL-Ins(1,2,4,5,6)P_5, (×) Ins(1,3,4,5,6)P_5, and (+) InsP_6.

3.2. Identification of inositol phosphates

Up to now, not all the inositol phosphate isomers were commercially available. Therefore, it was always a challenge to identify all the chromatographic peaks that were separated in any particular study. Several InsP_5 and InsP_4 isomers were indirectly identified in accordance with InsP_6 enzymatic hydrolysis products under the specific experimental conditions [25,26,28], which were considered reliable because the hydrolysis was highly selective, and more importantly, the hydrolysis products had been well recognized by different means [34,35]. In some studies [22,26], several isomers were recognized mainly based on their retention behaviors on other different columns using different elution programs, which might be problematic. In this study, InsP_6 and all InsP_2–InsP_5 isomers, with the exception of DL-Ins(1,2)P_2, Ins(4,6)P_2, and Ins(2,5)P_2, were unambiguously identified by using available standards.

When a solution of D-Ins(1,2,4)P_3 was heated at 100 °C, in theory, there probably would be three InsP_2 hydrolysis products, namely, D-Ins(1,2)P_2, D-Ins(1,4)P_2, and D-Ins(2,4)P_2. In the hydrolysis solution, two InsP_2 peaks were found, and the smaller one was identified as co-eluting D-Ins(1,4)P_2 and D-Ins(2,4)P_2 by comparison with available standards. Therefore, the remaining larger unknown peak should be D-Ins(1,2)P_2, indicating that the isolated phosphate group (position 4) of D-Ins(1,2,4)P_3 is the easiest to be removed. Another solution containing Ins(1,2,3)P_3 was heated at 100 °C for 1–4 h, or at 120 °C for 1 h. In the resulting hydrolysis solutions, only one InsP_2 peak for DL-Ins(1,2)P_2 was found. That may be because the phosphate group of position 2 of Ins(1,2,3)P_3 is in the middle of two phosphates (positions 1 and 3), and thus the most difficult to be removed. Using similar approaches, Ins(4,6)P_2 and Ins(2,5)P_2 were identified by examining the hydrolysis products of D-Ins(1,4,6)P_3 and D-Ins(2,3,5)P_3, respectively. In D-Ins(1,4,6)P_3 hydrolysis solution, the peak of D-Ins(1,6)P_2 was much larger than the other two peaks—Ins(4,6)P_2 and D-Ins(1,4)P_2,

indicating that the isolated phosphate group (position 4) of D-Ins(1,4,6)P_3 is the easiest to be removed. While in D-Ins(2,3,5)P_3 hydrolysis solution, the peak of D-Ins(2,3)P_2 was much larger than the other two peaks (Ins(2,5)P_2 and D-Ins(3,5)P_2), and the peak of D-Ins(3,5)P_2 was the smallest, showing that for D-Ins(2,3,5)P_3, the isolated phosphate group (position 5) is the easiest, while the phosphate of position 2 the most difficult to be removed.

3.3. Interference study

Using this method, the common anions, such as nitrate and carbonate, which have no detectable peak, as well as formate, acetate, citrate, and glucose-1-phosphate, which elute around t_0, do not interfere with the detection. The retention time of sulfate is around 10.0 min, between the $InsP_2$ and $InsP_3$ groups, and thus, does not interfere with the detection. Oxalate elutes just before $InsP_2$ group, when present in high concentration, it will most likely interfere with the detection of $InsP_2$.

3.4. Hydrolysis of $InsP_6$

Due to the cost and limited availability, many inositol phosphate isomers described in other studies were frequently prepared by hydrolyzing $InsP_6$ solution [12,15,19,36,37]. The $InsP_6$ hydrolysis solution can also be obtained commercially, such as Aldrich phytic acid solution (40%, w/w), which contains many inositol phosphate isomers as well as iron and some unknown substances [14]. An ideal reference standard solution, which can be used for analytical method development, should contain all possible inositol phosphate isomers. In addition, $InsP_6$ is the predominant inositol phosphate in raw dry cereals and legumes [13,14,17,38]. During food processing, to some extent, $InsP_6$ is often nonenzymatically or enzymatically dephosphorylated to yield a large number of $InsP_5$–$InsP_1$ isomers. Consequently, processed foods may contain varying amounts of lower dephosphorylated inositol phosphates. On the other hand, because monogastric animals including humans lack sufficient phytase in their guts to adequately break down $InsP_6$ in whole grains or legumes, the dietary phytases of plant or microbial origin actually play a vital role in $InsP_6$ degradation in the gastrointestinal tract of humans. Also, because the position of phosphate groups on the inositol ring is of great significance for the biological properties, it is of great interest in the study of human nutrition to investigate $InsP_6$ hydrolysis pathway and products under different conditions, and the results will be useful for preparing some specific inositol phosphate isomers as well.

3.4.1. Nonenzymatic hydrolysis of $InsP_6$ and preparation of in-house reference standard solution

Generally, enzymatic hydrolysis is more specific than nonenzymatic hydrolysis for isomer formation, whereas nonenzymatic hydrolysis is more efficient for producing more even distribution of isomers. Phillippy et al. studied the hydrolysis of $InsP_6$ solution at pH 4–10.7 by autoclaving [36]. In the present study, upon hydrolysis of $InsP_6$ solution by autoclaving at 121 °C for 1 h, the percentages of $InsP_6$ decomposed at pH 1.0, 2.0, 4.0, 6.0, 8.0, and 10.8 were 67.7, 76.8, 89.6, 81.9, 65.8, and 45.1%, respectively. The results show that by decreasing pH from 10.8 to 4.0, the decomposition percentages of $InsP_6$ increase, which is consistent with the result of the earlier study [36], while with further decrease in pH from 4.0 to 1.0, the decomposition percentages decrease. Also, in the pH range of 1.0–10.8, the lower the pH, the more even distribution of inositol phosphate isomers. Even for the hydrolysis solution at pH 2.0, which gave the highest number of isomer peaks (22 peaks for $InsP_2$–$InsP_6$), the isomers profile was not very good. Hence, autoclave hydrolysis is not appropriate for preparing reference standard solution.

In order to maximize the number of inositol phosphate isomers in an even distribution, it is critical to obtain all possible isomers and the even distribution in each dephosphorylation step during the gradual hydrolysis of $InsP_6$. Therefore, the oven hydrolysis was studied because of its ease of control. When $InsP_6$ solutions were heated at 100 °C for 1 h, in pH 1.0–10.8, only $InsP_6$ and two to four $InsP_5$ peaks were found except at pH 4.0, in which a small peak for DL-Ins(1,2,5,6)P_4 also appeared. The decomposition percentages of $InsP_6$ at pH 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 10.8 were 4.5, 6.6, 10.1, 12.4, 9.5, 6.9, 5.6, 4.1, and 3.4%, respectively, showing the effect of pH on $InsP_6$ decomposition by oven heating was similar to that by autoclaving. Also, the stronger the acidity in pH 1.0–10.8, the more even distribution

of $InsP_5$ isomers, and at pH 1.0, the most even distribution of $InsP_5$ was obtained. Hence, to prepare for a reference standard solution with satisfactory isomer distribution, the pH of $InsP_6$ solution should be lowered further, and the relatively slow decomposition can be improved by prolonging the time or more effectively, by increasing the temperature. Eventually, after heating at 140 °C for 1 h, $InsP_6$ solution containing 2 M HCl (pH ca. 0.5–0.6) gave very satisfactory results. Because the very high concentration of HCl in the final hydrolysis solution is too strong for the initial eluent and thus, not good for separating the early eluting isomers ($InsP_2$ and $InsP_3$), the excess HCl need be removed by evaporation before analysis.

In this hydrolysis solution, a total of 27 peaks representing $InsP_2$–$InsP_6$ isomers are detected, and the decomposition percentage of $InsP_6$ is 95.3%. Comparing the Aldrich hydrolysis solution, this in-house reference standard solution gives higher proportions of Ins(4,6)P_2, Ins(1,3,5)P_3, Ins(2,4,6)P_3, Ins(4,5,6)P_3, and Ins(2,4,5,6)P_4, some of which only can be detected in very high concentration of Aldrich hydrolysis solution. Considering the convenience, the commercial hydrolysis solution can be used as an alternative.

The hydrolysis of $InsP_6$ solution by microwave was also carried out with a CEM MDS-2100 microwave digestion system (full power: 950 W, Matthews, NC, USA). Operating at 30% power (maximum pressure: 200 psi, highest temperature: 100 °C; and 1 psi = 6894.76 Pa), $InsP_6$ solution at pH 4.0 turned brown after heating for 20 min, indicating the decomposition of inositol molecule. Even using 10% power (maximum pressure: 15 psi, highest temperature: 100 °C), after $InsP_6$ solution (pH 4.0) was heated for 20 min, four $InsP_5$ peaks and all $InsP_4$ peaks except DL-Ins(1,3,4,5)P_4 appeared in the hydrolysis solution. These preliminary tests show that microwave heating is very effective for the dephosphorylation of $InsP_6$ and to reduce its antinutrient effect, which may be very useful in food industry.

3.4.2. Enzymatic hydrolysis of $InsP_6$

Phytases, which catalyze the sequential and specific hydrolysis of $InsP_6$, occur in a variety of organisms including plants, microorganisms, and animal tissues. With respect to the position of initial hydrolysis on the inositol ring, different types of phytases have been recognized: 3-phytases (D-3 position), 5-phytases, and 6-phytases (L-6 or D-4 position). Two commercially available phytases, wheat phytase (EC 3.1.3.26, 6-phytase) and *A. ficuum* phytase (EC 3.1.3.8, 3-phytase), have been intensively studied [27,34,35,39,40]. With wheat phytase, initial hydrolysis occurs at D-4 position of $InsP_6$ to predominately yield D-Ins(1,2,3,5,6)P_5, and the further hydrolysis products are D-Ins(1,2,5,6)P_4, D-Ins(1,2,3,6)P_4, Ins(1,2,3)P_3, and D- and L-Ins(1,2)P_2 [34]. With the same phytase, hydrolysis of D-Ins(1,2,5,6)P_4 produces a mixture of D-Ins(1,2,6)P_3 and D-Ins(1,5,6)P_3 (the former is the major product), while hydrolysis of D-Ins(1,2,3,6)P_4 gives only Ins(1,2,3)P_3 [35]. In contrast to wheat phytase breakdown, the initial dephosphorylation of $InsP_6$ by *A. ficuum* phytase gives D-Ins(1,2,4,5,6)P_5 [39], and the further hydrolysis product is D-Ins(1,2,5,6)P_4 [40]. Based on a study [41] for a different phytase (*Pseudomonas* sp. phytase), Skoglund et al. suggested that the hydrolysis of D-Ins(1,2,5,6)P_4 by *A. ficuum* phytase produced D-Ins(1,2,5)P_3 and D-Ins(1,2,6)P_3, and then DL-Ins(1,2)P_2 [27], which had not been definitively confirmed. Comparing the analytical methods used in the above studies, the present method can separate more $InsP_2$–$InsP_6$ isomers, therefore, by using a combination of this analytical method and kinetic studies, the enzymatic hydrolysis pathways from $InsP_6$ to $InsP_2$ could be more easily established. In this study, the hydrolysis pathways of $InsP_6$ by these two phytases were further studied at the optimal pH values and temperatures recommended by the manufacturer of phytases (Sigma). The addition of either phytase to the pH-adjusted $InsP_6$ solutions did not affect the pH of the final solutions.

3.4.2.1. Wheat phytase. When wheat phytase was incubated with $InsP_6$ solution, at 0–60 min, besides $InsP_6$, three $InsP_5$ peaks, DL-Ins(1,2,3,5,6)P_5, Ins(1,2,3,4,6)P_5, and DL-Ins(1,2,4,5,6)P_5 peaks, were found, of which the DL-Ins(1,2,3,5,6)P_5 peak predominated. Of the two detected $InsP_4$ peaks, DL-Ins(1,2,3,6)P_4 and DL-Ins(1,2,5,6)P_4, the latter is bigger. According to an earlier study [34], the possibility of presence of Ins(1,3,4,6)P_4, which could co-elute with DL-Ins(1,2,3,6)P_4, was excluded. A small peak for DL-Ins(1,2,6)P_3 and Ins(1,2,3)P_3 also appeared (from the above hydrolysis products, it is not likely that DL-Ins(1,4,6)P_3 exists, which can co-elute with

DL-Ins(1,2,6)P_3 and Ins(1,2,3)P_3), and no InsP_2 peak was detected. At 120–180 min, DL-Ins(1,2)P_2 peak appeared. At 240 min, DL-Ins(1,5,6)P_3 peak was found. At 22 h, two other small peaks for DL-Ins(1,6)P_2 (from the above hydrolysis products, although the co-eluting DL-Ins(1,5)P_2 may exist, the possibility seemed to be slight according to the preceding hydrolysis pathways, which should be confirmed further) and DL-Ins(5,6)P_2 (from the above hydrolysis products, it is not likely that Ins(2,5)P_2 exists, which can co-elute with DL-Ins(5,6)P_2) appeared, and no InsP_6 or InsP_5 peak was observed. From these results and in view of the earlier studies [34,35], especially the optical identifications of the produced isomers, a more comprehensive diagram for hydrolysis pathways from InsP_6 to InsP_2 by wheat phytase is depicted in Fig. 5. Although D-Ins(1,2,6)P_3 was not detected in D-Ins(1,2,3,6)P_4 hydrolysis solution [35], the possibility that D-Ins(1,2,3,6)P_4 was hydrolyzed to yield D-Ins(1,2,6)P_3 by wheat phytase could not be definitely excluded because, according to the results of the same study [35], some contaminants did appear in the produced Ins(1,2,3)P_3 isolate, and Ins(1,2,3)P_3 would co-elute with D-Ins(1,2,6)P_3, if present. All the possible degradation pathways to D-Ins(1,6)P_2 and D-Ins(1,2)P_2 are shown in Fig. 5, which can be easily confirmed if sufficient quantity of relevant InsP_3 isomer standards are available.

3.4.2.2. Aspergillus ficuum phytase. When *A. ficuum* phytase was incubated with InsP_6 solution, at 0–15 min, the peaks for InsP_6, DL-Ins(1,2,4,5,6)P_5, DL-Ins(1,2,5,6)P_4, DL-Ins(1,2,6)P_3 (from the hydrolysis products, it is impossible to be DL-Ins(1,4,6)P_3 and Ins(1,2,3)P_3, which can co-elute with DL-Ins(1,2,6)P_3), DL-Ins(1,5,6)P_3, DL-Ins(1,2)P_2, and DL-Ins(5,6)P_2 (from the above hydrolysis products, it is impossible to be Ins(2,5)P_2, which can co-elute with DL-Ins(5,6)P_2) were found. At 30 min, InsP_6 and all InsP_5 peaks completely disappeared. At 60 min, DL-Ins(1,6)P_2 peak appeared, and no peak for InsP_4 was found. At 120 min, no InsP_3 or InsP_2 peak was found. From these results and in consideration of the earlier studies [39,40], especially the optical identifications of the isomers, a diagram for hydrolysis pathways from InsP_6 to InsP_2 by *A. ficuum* phytase is illustrated in Fig. 6. Because by using the present method, DL-Ins(1,2,5)P_3 and DL-Ins(1,2,6)P_3 can be separated, and DL-Ins(1,5,6)P_3 peak isolated with any other InsP_3 peak, the InsP_3 products will be unambiguously characterized as D-Ins(1,2,6)P_3 and D-Ins(1,5,6)P_3. That is to say, a new pathway from D-Ins(1,2,5,6)P_4 via D-Ins(1,5,6)P_3 to D-Ins(5,6)P_2 was found. Since both D-Ins(1,2,6)P_3 and D-Ins(1,5,6)P_3 can probably be hydrolyzed to yield D-Ins(1,6)P_2, these two possible pathways are also shown in Fig. 6.

From these results, it appears that, under the specific experimental conditions, there are two rules for in vitro enzymatic hydrolysis of inositol phosphates with these two phytases: (1) *neighboring effect*: once the phytase initially hydrolyzes InsP_6 at the specific positions (for wheat phytase, just the first step, D-4 position; for

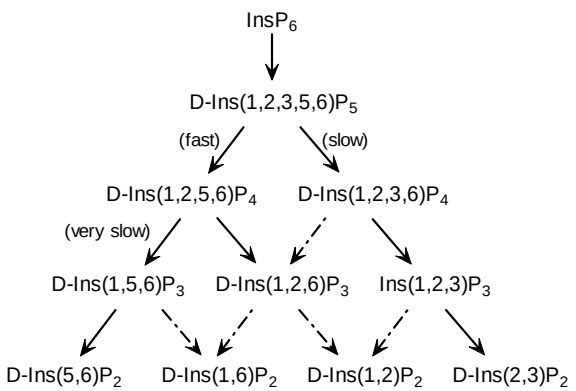

Fig. 5. Hydrolysis pathways of InsP_6 to InsP_2 by wheat phytase (pH 5.15, 55 °C): (solid arrows) confirmed, and (dashed arrows) need be further confirmed.

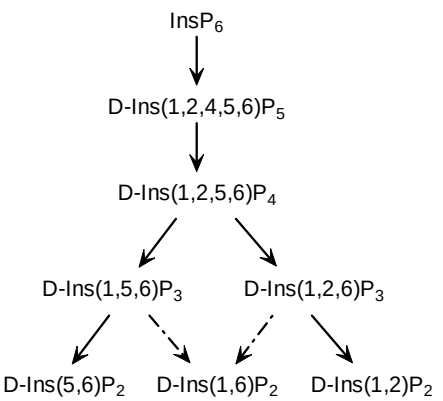

Fig. 6. Hydrolysis pathways of InsP_6 to InsP_2 by *A. ficuum* phytase (pH 2.50, 37 °C): (solid arrows) confirmed, and (dashed arrows) need be further confirmed.

A. ficuum phytase, the first two steps, D-3 and then D-4 positions), the following dephosphorylation always occurs at the consecutive position and adjacent to the free hydroxyl group, which was also pointed out in an early study [35]; and (2) *position effect*: for dephosphorylation, phosphate groups of positions 2 and 5, especially that of position 2, are more difficult to be removed. Attack adjacent to a free hydroxyl group could be due to greater nucleophilicity of the hydroxyl oxygen compared to the ester bond oxygen [42]. Under the experimental conditions in this study, neighboring effect seems to be more important than position effect.

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