Journal of Biological Methods | **2015** | Vol. 2(1) | e16
DOI: 10.14440/jbm.2015.58

ARTICLE

Assessing phytase activity–methods, definitions and pitfalls

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Competing interests: The authors have declared that no competing interests exist.

Received April 2, 2015; Revision received May 21, 2015; Accepted May 21, 2015; Published June 4, 2015

ABSTRACT Phytases are nutritionally important for increased bioavailability of dietary minerals and phosphate for monogastric animals including humans. Release of minerals and phosphate is accomplished by the enzymatic stepwise degradation of phytate (inositol hexaphosphate, IP_6). Activity determinations of phytase is often based on analysis of total released phosphate (P_i), but phytase activity in its purest form represents released product per time from IP_6 only. Microbial and plant preparations often also contain mixtures of phosphatases and organic phosphate compounds; hence some released phosphate in enzymatic assays may originate from non-phytase phosphatases degrading non-phytate molecules. Moreover, even purified enzyme extracts assessed via P_i release may result in errors, since commercial IP_6 commonly contains contamination of lower inositol phosphates, and further, the products of phytase IP_6 hydrolysis are also substrates for the phytase. These facts motivate a quantitative comparative study. We compared enzyme activity determination in phytase assay samples at four different time points, based on analyzing the substrate IP_6 versus the product P_i using different selected methods. The calculated activities varied substantially. For example, at 15 min into enzymatic assay, variations from 152 mU/ml (by IP_6 analysis on HPIC) to 275–586 mU/ml (by P_i analysis using several methods) was detected. Our work emphasizes the importance of defining the type of activity assessed, showing that phytase activity based on released P_i may yield false positive results and/or overestimations. We propose to differentiate between phytase activity, being the activity by which IP_6 is degraded, and total inositol phosphatase activity, corresponding to total released phosphate during the enzymatic reaction.

Keywords: inositol phosphatase activity, inositol phosphate, phosphate analysis, phytase activity, phytate

INTRODUCTION

Phytases are well known for their ability to improve animal and human nutrition by degradation of phytate (inositol hexaphosphate, IP_6), which releases minerals otherwise unavailable for intestinal uptake (for recent reviews see e.g. Dersjant-Li et al [1]). Assessing phytase activity in a comparable way is therefore important for screening, selecting and improving enzymes as well as product organisms.

Phytase activity determinations may seem straightforward, but can be deceptive and involve a number of uncertainties. Phytase activity determinations are often based on analyzing released phosphate by colorimetric analysis methods [2-6]. This is many times sufficient since the released amount of P_i per time is often the relevant parameter, for example in feed applications. The ISO 30024:2009 method can, for instance, be used for determination of released phosphate in complete feed materials [6].

Measuring released P_i per time is however a black box, and does not represent only the enzymatic activity on the specific substrate phytate (IP_6). Scientifically, P_i-based measurements may therefore be insufficient. The present study was undertaken to investigate differences in detected phytase activity using different methods, and to propose a more precise terminology, distinguishing between the actual phytase activity (defined as IP_6 hydrolysis) and total inositol phosphatase activity (defined as the sum of all IP_n hydrolysis).

Assessing phytase activity can be tricky in several ways: First, one of the products of the phytase reaction (IP_5) is also substrate for the same enzyme and so is the subsequent products (IP_4 and down). This means that if we define phytase activity as degradation of phytate (IP_6 only), there will be competing substrates present in such assays. The contribution of IP_6 hydrolysis - the phytase reaction - in relation to the total inositol phosphatase reactions cannot be accurately determined by assessing P_i. Second, phytases tend to be broad in substrate specificity which means that some extracts may contain other organic P-compounds degradable by phytase enzymes. Third, it is well known that non-phytase phosphatases may degrade IPs from IP_5 and downwards. These will, if present, contribute to the P_i production in an assay without degrading phytate through phytase reaction. Fourth, commercial IP_6 used in assays contains impurities in the form of lower IPs (up to 12% IP_5 has been found by Carlsson et al 2001 [7]). This may lead to false positive results from P_i-production catalyzed by non-phytase inositol phosphatases, and may in fact not even involve any degradation of IP_6.

Awareness of these pitfalls, and of the fact that a certain released P_i data in a phytase assay may come from very different proportions of phytase activity versus other phosphatase activity, is important. Specifying this would be highly valuable when screening or comparing phytases. The only way to open the black box is to analyze inositol phosphates

in addition to inorganic phosphate. This would also contribute to fair comparison of different phytases.

Yet, various phosphate analysis methods are widely used for determination of phytase activity [5,8-11], and are recognized as fast and simple methods. Other studies [7,12,13] instead determine the phytase activity by analyzing IP_6 concentration using high-performance liquid ion chromatography (HPIC). To the best of our knowledge, there are no studies on how substrate (IP_6) versus product (P_i) based assays match, and hence a need for comparing commonly used methods for phytase activity determinations.

Several of the colorimetric phosphate analysis methods used today (e.g. [10,11]) are modified versions of a method presented by Fiske and Subbarow in 1925 [14]). In the original method, acid ammonium molybdate solution was added to a phosphate containing sample to form the yellow phosphomolybdic acid, subsequently reduced by 1-amino-2-naphtol-4-sulfonic acid (ANSA) to molybdenum blue, which can be determined spectrophotometrically. Since ANSA is difficult to prepare in solution [14], and may be toxic, modified methods have been developed to exclude ANSA. One alternative is spectrophotometric detection of the formed yellow phosphomolybdic acid [5,15,16] without reduction to molybdenum blue. Another alternative is to exchange ANSA for a different reducing agent, such as ferrous sulfate [17], stannous chloride [18,19] or ascorbic acid [20] to produce molybdenum blue. Finally, a different way of analyzing phosphate, without prior processing of the samples, is by HPIC [21].

The first aim of this study was to compare principally different phytase activity determinations based on either product degradation or substrate formation. The second aim was to compare selected colorimetric phosphate analysis methods with analysis of phosphate by HPIC, both to investigate possible differences in detected phosphate levels as well as to compare method stability and usability, which are all factors that may affect phytase data.

MATERIALS AND METHODS

Substrates for analysis

It is well known that expression of yeast phytase is affected by the surrounding phosphate concentration, yet, phosphate analysis of yeast media components such as yeast extract and peptone is not always included in the manufacture standard analysis. For this reason, three yeast extracts and one peptone sample were included in the study, along with four phytase assay samples (**Table 1**).

The yeast extract and peptone solutions were prepared in NaOH/succinic acid buffer pH 5.5 (0.1 M) and filter sterilized using 0.2 µm filters (Merck Millipore), the exact concentrations are listed in **Table 1**. There was no further processing of the samples before undergoing the different analysis methods, except dilutions in deionized water to achieve theoretical concentrations between 1-20 mg/L phosphate.

The phytase assay samples were achieved by addition of 1 ml cell-free and phytase-containing supernatant from yeast cultivations, into 4 ml freshly prepared NaAc/HAc buffer (pH 5) with 1 g/L phytate (Phytic acid sodium salt, Sigma). The assay was carried out at 40°C and samples were withdrawn at times 0 min, 15 min, 30 min and 60 min, and the enzymatic reaction was immediately stopped by addition of HCl to a final concentration of 0.5 M. Triplicates of all samples were kept at -20°C until analysis.

Rapid IP_6 analysis by IP_6-HPIC

The method used for IP_6 analysis was as described by Carlsson et al [7]. The chromatograph consisted of a biocompatible (PEEK) HPLC pump (Waters model 626) equipped with a PA-100 guard column and a CarboPac PA-100 analytical column (Dionex Corp.). The IP_6 was eluted with an isocratic eluent of 80% HCl (1 M) and 20% H_2O. The eluents (0.8 ml/min) were mixed with a ferrous nitrate solution in a post column reaction. IP_6 was analyzed with UV detection at 290 nm (Waters 486, tunable absorbance detector). The run time for each sample was 7 min, and the IP_6 concentration was calculated using a series of external standards between 0.1–0.7 µM IP_6/ml.

Phytase activity definitions

For phytase activity based on analysis of IP_6 concentration, one enzyme activity unit (U) was defined as the amount of enzyme needed to release 1 µM of P_i from IP_6 per minute (equal to degradation of 1 µM IP_6 and formation of 1 µM IP_5).

For phytase activity based on P_i release, one enzyme activity unit (U) was defined as the amount of enzyme needed to release 1 µM of P_i from the assay solution per minute(i.e. phosphate release from all present inositol phosphates).

Identification of phytate degradation products, IP_5–IP_2

The analysis and identification of the IP_6 degradation products IP_5–IP_2, was performed using the same system as for the rapid IP_6 analysis, but with a gradient elution system as described by Skoglund and co-workers [22]. Peak identification was done by analyzing a chemically hydrolyzed sodium phytate sample. The order of the peaks has previously been described by Skoglund et al [22]. Since the detection response for the lower inositol phosphates is lower than for IP_6 (which was used to create the standard curve), theoretical correction factors were used [22].

Table 1. Yeast extract and peptone batches used for phosphate analysis in this study.

Component	Company	Reference number	Lot number	Component concentration (g/L)
Yeast extract 1	Scharlau	07-079	100840	10.35
Yeast extract 2	Scharlau	07-079	102256	11.38
Yeast extract 3	Bacto	210933	3275271	10.07
Peptone	Bacto	211677	3065305	20.41

Standards for phosphate analyses

Standards were prepared from KH_2PO_4 (Sigma) in deionized water at phosphate concentrations of 1, 5, 10, 15 and 20 mg/L. The standards were kept at 4°C, and were used both in P_i-HPIC analysis and colorimetric protocols to calculate phosphate contents of the samples.

Phosphate analysis by P_i-HPIC

The chromatograph consisted of a Dionex GS50 gradient HPLC pump equipped with a PAX-100 OmniPac guard and analytical column (Dionex Corp., Sunnyvale CA) and a anion self-regenerating suppressor (ASRS-I, 4mm) at 100 mA (Dionex Corp.). The phosphate was eluted at a flow rate of 0.8 ml/min, using a gradient elution ranging from 2% to 49% of NaOH (0.2 M in H_2O) with H_2O as counter eluent and a constant 2% isopropanol (50% solution in H_2O). Phosphate was detected using conductivity detection (CD20, Dionex Corp.). Total run time for each sample was 30 min. The phosphate concentration was quantified by integrating the peak using the software Chromeleon (Dionex Corp.).

Colorimetric phosphate analysis methods

The colorimetric phosphate analysis methods evaluated in this study have been previously presented by Peterson [23], Heinonen and Lahti [15], Bae et al [17], together with a water analysis protocol found from both Agilent technologies [24] and Central Pollution Control Board (CPCB) [25]. As a complement we also developed an alternative version of the Peterson method where ANSA was exchanged for ascorbic acid. All solutions presented in this section were prepared in deionized water (18.2 MΩ-cm) if nothing else is stated.

Method by Peterson 1978 [23]

This method had been developed from Fiske and Subbarow, with modifications to achieve increased stability over time and decreased sensitivity to interfering substances. The modifications reportedly resulted in less than 1% change in detection per hour after 30 min of incubation, and showed low impact from interfering substances in the sample [23]. A sample volume of 0.5 ml was mixed with 0.9 ml of 5% (w/v) sodium dodecyl sulfate (SDS) (Sigma-Aldrich) solution. This was followed by addition of 1 ml of 1.25% (w/v) ammonium molybdate (Merck) solution in 2 M HCl (Scharlau). Thereafter, 0.1 ml of 0.025% (w/v) 1-amino-2-naphtol-4-sulfonic acid (ANSA) (Fluka) solution in sodium bisulfite was added followed by mixing before incubating at room temperature (20°C) for 30 min. Absorbance was read at 700 nm.

Ascorbic acid method modified from Peterson

As an alternative version to the Peterson protocol, the reducing agent ANSA was exchanged for 0.1 ml of 1 g/L ascorbic acid (Fluka) solution, keeping all other components and procedures the same as described in the original protocol. The absorbance was measured after 30 min of incubation at 700 nm.

Method by Bae et al 1999 [17]

This is a simplified version of a colorimetric phosphomolybdate analysis method, which has been used in several recent papers on phytase analyses [26-28]. A sample volume of 750 µl was mixed with 750 µl of 5% (w/v) trichloroacetic acid (TCA) (Scharlau) solution. From this mixture, 750 µl was transferred to a new tube and mixed with 750 µl ammonium molybdate ferrous sulfate mixture, which had been prepared by mixing 4 volumes of 1.5% (w/v) ammonium molybdate (Merck) in 5.5% sulfuric (Merck) acid with 1 volume of 2.7% (w/v) ferrous sulfate (Fluka) solution. The absorbance was measured at 700 nm.

Method by Heinonen and Lahti 1981 [15]

This method was chosen since it uses the direct detection of the yellow phosphomolybdic acid, without reduction to molybdenum blue. Three stock solutions were prepared: 10 mM ammonium molybdate (Merck), 1 M citric acid (Sigma) and 2.5 M sulfuric acid (Merck). A sample volume of 0.5 ml was mixed with 4 ml freshly prepared acetone-acid-molybdate, which consisted of 1 volume of ammonium molybdate stock solution, 1 volume of sulfuric acid stock solution and 2 volumes of acetone (Sigma-Aldrich). This was followed by vortexing before addition of 0.4 ml citric acid, which complexes with excess molybdate. In case of precipitate formation, samples were centrifuged prior to measuring absorbance, as done by Greiner and Alminger [16]. The absorbance was measured at 355 nm.

Method by CPCB and Agilent [24,25]

This method had been introduced to achieve reproducible analyses of phosphate in water and wastewaters between different labs [25], and it uses the reducing agent stannous chloride. The initial protocol was scaled down to half the working volumes. For the analysis, 1 ml of sample was mixed with 2 ml of 2.5% (w/v) ammonium molybdate (Merck) solution in 5 M sulfuric acid (Merck). This was followed by addition of 0.25 ml of 2.5% (w/v) stannous chloride (Merck) solution in glycerol. Thereafter, deionized water was added up to 50 ml, and the absorbance was measured between 10–12 min of incubation. From scanning of maximum absorbance, the optimum was found at 700 nm, which is why this wavelength was used for all readings.

Spectrophotometer

For detection of color development in original colorimetric assays, a Cary 60 UV-Vis spectrophotometer (Agilent, Victoria, Australia) was used. Since the method by CPCB [25] advises to measure absorbance at 690 nm or 880 nm, an absorbance scanning between 500–1100 nm was done in order to identify the maximum absorbance wavelength for the different protocols. The maximum absorbance was found to be at 700 nm for all colorimetric protocols analyzing molybdenum blue. Therefore, the wavelengths used in this study were 700 nm for analyzing molybdenum blue and 355 nm for analyzing phosphomolybdic acid.

Statistics

The analyses of the different samples was performed in triplicates for each method. The data are presented as the mean values (M.V) of triplicates together with the standard deviation (Std.dev) and the coefficient of variance (C.V) to show the stability within triplicates (see **Table 3**). To visualize the stability of each method used, a mean of the C.V from each sample within a method set was calculated.

RESULTS

Phytase and total inositol phosphatase activity determinations

The enzymatic assay samples taken at 0 min, 15 min, 30 min and 60 min were used for determination of phytase activity based on analysis

of released P_i using selected phosphate analysis methods (**Fig. 1**). **Table 2** further presents the determined phytase activities at 15 and 30 min of enzymatic assay, calculated based on analysis of IP_6 concentration and based on analysis of P_i using selected methods. Activity determination based on analysis of IP_6 concentration resulted in an activity of 152 mU/ml and 99 mU/ml for the first 15 and 30 min respectively. These data was used as reference for comparison with activities calculated from P_i analysis. As seen in **Table 2**, the activities determined from P_i analysis were from 181 to 386 mU/ml for the first 15 min of assay, depending on the method used. This corresponds to between 181–386% higher activities compared to determination based on IP_6 concentration. The different phosphate analysis methods also detected rather different levels of released phosphate in the same sample, therefore also yielding variations in apparent activity. The most extreme case is seen for the 15 min assay sample, where almost twice as much released phosphate–and twice as high activity–was detected using the Heinonen and Lahti method compared to that of the CPCB/Agilent method.

Table 2. Phytase activity determinations (mU/ml) based on seven different methods, and comparison of the determined activities based on analysis of P_i concentration (using different analysis methods) versus the activity determined based on analysis of IP_6 concentration (set as 100%).

Method	15 min assay sample (mU/ml)	Comparison of determined activity at 15 min (%)	30 min assay sample (mU/ml)	Comparison of determined activity at 30 min (%)
IP_6-HPIC	152	100	99	100
P_i-HPIC	316	208	286	290
Peterson	308	202	287	290
Ascorbic acid	335	220	293	297
Bae et al	447	294	350	354
Heinonen & Lahti	586	386	376	380
CPCB / Agilent	275	181	239	242

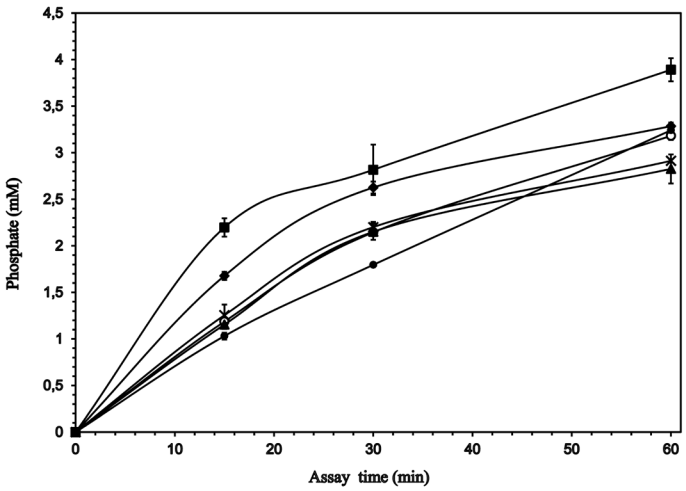

Figure 1. Detection of phosphate release (mM) at 0, 15, 30 and 60 min during enzymatic assay, determined based on various phosphate analysis methods. The methods used were P_i-HPIC (o), Peterson (▲), Ascorbic acid version of Peterson (×), Bae et al (♦), Heinonen and Lahti (■) and CPCB / Agilent (●).

Figure 2 presents the IP_6 degradation process during 60 min of enzymatic assay, presenting the formation and degradation of lower inositol phosphates down to IP_2. Analysis of IP_1 was not possible on the system used. As seen from **Figure 2**, already during the first 15 min of the assay, IP_5, IP_4 and IP_3 are being formed as products from the degradation of IP_6, IP_5 and IP_4 respectively. This clearly shows that the released P_i at 15 min of assay originates from degradation of several lower inositol phosphates in addition to IP_6.

During the time between 15 and 30 min of the assay, degradation of IP_5 and formation of IP_4–IP_2 is seen (**Fig. 2**). The curves for the intermediate inositol phosphates (IP_5–IP_2) correspond to the net content of the respectively inositol phosphate, i.e. resulting from the combined formation and degradation which occur simultaneously during the assay as long as there is higher inositol phosphates available as substrates.

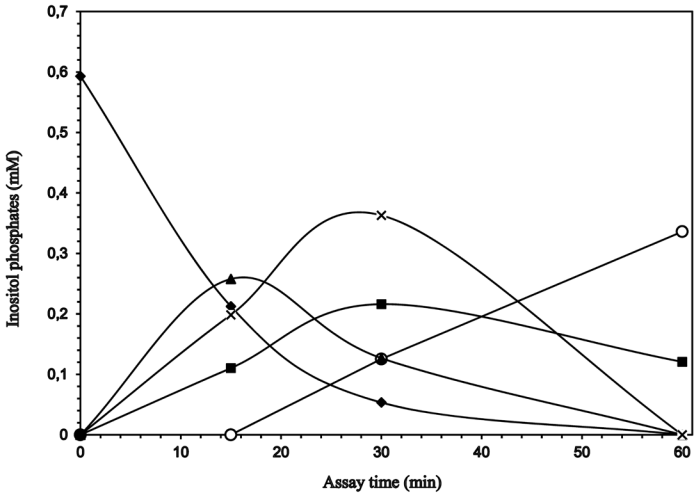

Figure 2. Detection of inositol phosphatases (IP_6 to IP_2) at 0, 15, 30 and 60 min during enzymatic assay, analyzed by HPIC. The degradation process from IP_6 down to IP_2 is presented; IP_1 was not detectable using this system. The curves correspond to IP_6 (♦), DL-Ins(1,2,4,5,6)P_5 (▲), DL-Ins(1,2,5,6)P_4 (×), IP_3 (■) and IP_2 (o).

Phosphate analysis by P_i-HPIC compared to selected colorimetric methods

Table 3 presents the results from phosphate analysis of eight different samples using selected methods. Each sample was measured in triplicate, from which the mean value (M.V), standard deviation (Std. dev) and coefficient of variation (C.V) was calculated. The last row in **Table 3** shows the mean C.V for each separate method. The mean C.V reflects the collected deviations for all samples analyzed within one method. The phosphate content in the 0 min assay samples resulted in undetectable phosphate levels (n.d).

Table 3. Phosphate analysis results for eight different samples analyzed in triplicates using six different phosphate analysis methods.

Sample		P_i-HPIC	Peterson	Peterson Ascorbic acid	Bae et al	Heinonen & Lahti	CPCB / Agilent
Yeast extract 1	M.V (mg/L)	149.20	144.19	140.13	183.54	145.43	106.33
	Std.dev (mg/L)	6.60	6.67	5.25	9.50	7.66	20.82
	C.V (%)	4.4	4.6	3.7	5.2	5.3	19.6
Yeast extract 2	M.V (mg/L)	113.30	101.86	96.55	157.91	103.93	56.94
	Std.dev (mg/L)	4.73	5.49	4.62	14.77	6.21	7.56
	C.V (%)	3.9	5.4	4.8	9.4	6.0	13.3
Yeast extract 3	M.V (mg/L)	187.32	186.95	179.79	222.26	220.83	144.91
	Std.dev (mg/L)	2.73	1.09	1.76	3.27	37.02	19.64
	C.V (%)	1.5	0.6	1.0	1.5	16.8	13.6
Peptone	M.V (mg/L)	37.64	37.57	38.08	57.62	39.17	40.40
	Std.dev (mg/L)	0.88	1.03	2.48	1.01	1.95	7.19
	C.V (%)	2.3	2.7	6.5	1.7	5.0	17.8
Assay sample 0 min	M.V (mg/L)						
	Std.dev (mg/L)	n.d	n.d	n.d	n.d	n.d	n.d
	C.V (%)						
Assay sample 15 min	M.V (mg/L)	90.11	87.64	95.33	127.43	166.92	78.28
	Std.dev (mg/L)	0.43	3.39	8.69	3.34	7.40	2.82
	C.V (%)	0.5	3.9	9.1	2.6	4.4	3.6
Assay sample 30 min	M.V (mg/L)	162.98	163.41	167.20	199.49	213.97	136.41
	Std.dev (mg/L)	0.87	6.56	4.35	4.87	20.58	0.51
	C.V (%)	0.5	4.0	2.6	2.4	9.6	0.4
Assay sample 60 min	M.V (mg/L)	241.67	214.72	221.26	249.58	109.71	246.25
	Std.dev (mg/L)	5.01	11.87	1.39	0.43	8.62	6.27
	C.V (%)	2.1	5.5	0.6	0.2	7.9	2.5
Mean C.V. (%) for each method		2.2	3.8	4.0	3.3	7.9	10.1

M.V, mean value of triplicates; Std.dev, standard deviation between triplicates; C.V, coefficient of variation for the triplicates; Mean C.V, mean C.V for each phosphate analysis method; n.d, not detectable

The low standard deviations found from P_i-HPIC analysis led us to choose this method as reference for comparison of the colorimetric methods tested in this study. The Peterson method [23] showed phosphate levels in good agreement with those detected by P_i-HPIC analysis. The Peterson method also yielded low standard deviations in all analyzed samples, resulting in a low mean C.V at 3.8%. The modified Peterson method where ANSA had been exchanged for ascorbic acid showed results close to those of the Peterson and P_i-HPIC analyses, with low standard deviations and a low mean coefficient of variation for the method at 4.0%. The two versions of the Peterson method also showed strong stability during prolonged incubation times (data not shown), which is in agreement with what Peterson concluded about his method [23]. The other three investigated methods yielded phosphate values with less good agreement to the P_i-HPIC analysis results, and in some cases yielding very large standard deviations, up to almost 20% of the detected phosphate content. The Bae et al method [17] gave relatively low C.V within triplicates, and a mean C.V of 3.3% for the method. However, this method consistently detected higher phosphate contents for all analyzed samples, compared to the P_i-HPIC method. Both the method by Heinonen and Lahti and by CPCB/Agilent [24,25] yielded inconsistent phosphate analysis results in our samples, accompanied with large C.V's.

DISCUSSION

Phytases are enzymes able to readily catalyze the degradation of IP_6 during release of inorganic phosphate (P_i) and formation of lower inositol phosphates. Phytase activity on the other hand is the activity by which IP_6 is being hydrolyzed to form IP_5 and inorganic phosphate. Several studies on phytase expression and phytase activity are based on analysis of released P_i for determination of the phytase activity. As mentioned, there are some possible pitfalls when using released P_i as measure of phytase activity; presence of non-phytase phosphatases, presence of additional sources of phosphate in the extract and/or assay substrate, and the fact that the products of IP_6 degradation are also substrates for the enzyme. In **Figure 2** it is seen that both IP_4 and IP_3 are formed during the first 15 min of assay, which is a result from the degradation of (and simultaneous P_i release from) IP_5 and IP_4 respectively.

Enzymatic activity determinations based on phosphate release are better referred to as total inositol phosphatase activity in assays where IP_6 is the only substrate and phytase is the only enzyme, since it reflects the degradation of the total mixed inositol phosphatase pool catalyzed by phytase during the assay. When there are other phosphate sources in addition to IP_6 present as substrates, and when the extract to be assayed may contain non-phytase phosphatases, the term total phosphatase activity better reflects the activity assessed, since it includes the phosphate released from all possible sources by all phosphatases during the assay. The term phytase activity reflects the enzymatic activity on IP_6 degradation only, which by definition is performed only by phytases, and which is assessed by analyzing IP_6 concentration.

Degradation of lower inositol phosphates may be practically important from a nutritional point of view since IP_5, IP_4 and IP_3 also interfere with mineral absorption from the diet [29,30] and also for the feed industry, where release of phosphate is the main issue. To allow reliable comparisons of the enzymatic activities between different samples and from different studies, it is important to specify what type of enzymatic activity that is determined. The results in our study show that phytase activity determination based on using different methods are not comparable and can give great variations in the result.

Phytase activity determination based on released P_i results in activities up to 386% higher than from IP_6 data. Further, analysis of the IP_6 degradation products IP_5–IP_2 (**Fig. 2**) revealed that after 15 min of enzymatic assay, the inositol phosphates IP_5, IP_4 and IP_3 had been formed via the degradation of IP_6, IP_5 and IP_4 respectively. This shows that the detected P_i release is a result of degradation from both IP_6 and lower IPs, and the activity corresponds to the total inositol phosphatase activity.

Colorimetric phosphate analysis methods are however very useful both for determination of total inositol phosphatase activity, as well as for other purposes such as assessing water quality or determining phosphate levels in biological samples. The second aim of this study was to compare selected colorimetric phosphate analysis methods with phosphate analysis on P_i-HPIC. We hypothesized that the chemicals used in some of the selected colorimetric analysis methods may hydrolyze labile bound phosphate from phosphorylated compounds in the sample, possibly yielding higher detected phosphate levels than those detected by P_i-HPIC.

The Peterson method showed phosphate levels close to those detected by P_i-HPIC analysis, together with low C.V in all analyzed samples. The modified Peterson method where ANSA had been exchanged for ascorbic acid showed similar results, also with generally low C.V for the analyzed samples, and a low mean C.V for the method. The additional observations of maintained stability during prolonged incubation times, indicates good applicability of those methods for larger sample sets. Further, our developed version of the Peterson method successfully excluded the hazardous and laborious preparation of the ANSA solution, yet with maintained analysis performance. In a study by Sanikommu and co-workers [11], the authors found that phosphate determination by the Fiske-Subbarow method was negatively influenced by presence of phytic acid. However in our study, the Peterson method (which was developed from Fiske-Subbarow [14]) applied on samples containing phytic acid did not show such effect; the results correlated well with the P_i-HPIC results. The two main differences between the method used by Sanikommu et al [11] and the Peterson method is that the acid concentration is about twice as high in the Sanikommu method as the concentration used in the Peterson protocol, and that the Peterson method includes SDS in the sample preparation. As we have seen for the other methods tested in our study, e.g. by Bae et al [17], higher acid concentrations may yield higher phosphate response, possibly from hydrolyzing weakly bound phosphates from organic P-compounds present.

The method presented by Bae et al [17] detected consistently higher phosphate content in all tested samples compared to P_i-HPIC. In this protocol, the first step was to make a 1:1 mix of the sample with 5% TCA solution. The relatively large addition of acid may potentially have induced the hydrolysis of labile phosphate groups in the samples, causing the higher detection of released phosphate. The Bae et al method yielded relatively low standard deviations, with a mean C.V of 3.3%, indicating good reproducibility and stability.

The method by Heinonen and Lahti [15] and the method by CPCB/Agilent [24,25] both showed higher C.V for several of our analyzed samples, resulting in higher mean C.V at 7.9% and 10.1% respectively. The results showed less agreement with the P_i-HPIC method, which was especially the case when using the CPCB/Agilent analysis for the yeast extract and peptone solutions. In the yeast extract and peptone samples, phosphate may be found in several different forms, which possibly makes those samples too complex for this specific analysis method. In addition, the precipitate formation in some samples of the Heinonen and Lahti analysis method made it necessary to include a centrifugation step prior to spectrophotometric analysis, which makes this method slightly more laborious than the other methods tested.

To conclude, this study provides evidence that phytase activity determination based on P_i analysis generates higher activities and induce the risk of detecting false positive results, as compared to determination based on analysis of IP_6 concentration. The activity determination based on P_i release reflects the total inositol phosphatase activity, involving P_i release also from lower inositol phosphates, or the total phosphatase activity when assessing P_i released from other present phosphate sources and/or by the action of non-phytase phosphatases present in the enzyme preparation. Phytase activity on the other hand is measuring the enzymatic activity on IP_6, and is best addressed by directly analyzing IP_6 concentration. Those terminological distinctions are of importance when i) comparing phytase activity data between different studies, and ii) when investigating novel phytases and phytase sources to avoid false positive results.

Although P_i analysis may not be suitable for phytase activity determination, the analysis methods are still useful for other purposes. Our comparison of the selected colorimetric phosphate analysis methods with P_i-HPIC analysis revealed some variations in detected P_i levels depending on the method used. The Peterson method [23], and our developed version thereof, yielded the most satisfying results compared to P_i-HPIC analysis.

Acknowledgements

The Swedish Research Council Formas (Grant nr 222-2012-1064) is gratefully acknowledged for financial support.

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