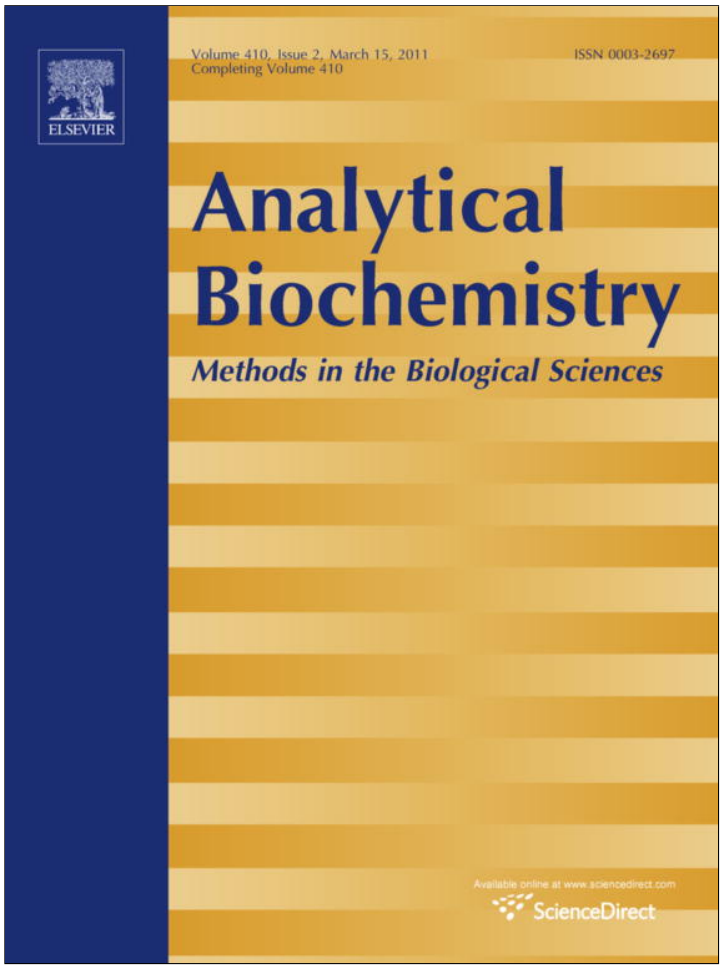

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A simple and fast kinetic assay for phytases using phytic acid–protein complex as substrate

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ABSTRACT

Phytase (EC 3.1.3.–) hydrolyzes phytate (IP_6) present in cereals and grains to release inorganic phosphate (P_i), thereby making it bioavailable. The most commonly used method to assay phytase, developed nearly a century ago, measures the P_i liberated from IP_6. This traditional endpoint assay is time-consuming and well known for its cumbersomeness in addition to requiring extra caution for handling the toxic regents used. This article reports a simple, fast, and nontoxic kinetic method adaptable for high throughput for assaying phytase using IP_6–lysozyme as a substrate. The assay is based on the principle that IP_6 forms stable turbid complexes with positively charged lysozyme in a wide pH range, and hydrolysis of the IP_6 in the complex is accompanied by a decrease in turbidity monitored at 600 nm. The turbidity decrease correlates well to the released P_i from IP_6. This kinetic method was found to be useful in assaying histidine acid phytases, including 3- and 6-phytases, a class representing all commercial phytases, and alkaline β-propeller phytase from *Bacillus* sp. The influences of temperature, pH, phosphate, and other salts on the kinetic assay were examined. All salts, including NaCl, $CaCl_2$, and phosphate, showed a concentration-dependent interference.

Histidine acid phytases constitute an important group of enzymes for feed and grain processing industries due to their high specific activity and wide pH optima for activity [1]. Phytase catalyzes the sequential hydrolysis of phytate (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate [IP_6]),[1] a principal storage form of phosphorus in cereals and legumes, to less phosphorylated *myo*-inositol derivatives with concomitant release of inorganic phosphate (P_i) [1]. Hydrolysis of phytate overcomes a number of its negative effects on human and animal nutrition [2–7] and on the environment [8,9]. It is well established that phytic acid binds positively charged metal ions [10] and biomolecules [11], making them unavailable as nutrients. In grain processing, it is important to add phytase so as to make Ca^{2+} available for α-amylase [12]. Interaction with positively charged dietary proteins leads to the formation of phytate–protein aggregates and precipitates, which decreases their accessibility to proteases, thereby resulting in inefficient protein digestion [13–18]. The decrease in protein solubility results from masking of positive charges on the protein molecules by phytic acid, which changes the isoelectric points of the proteins [11,16].

In vitro studies with phytase reported in the literature have been performed with IP_6 as the substrate, and either the degradation of IP_6 or the amount of P_i released has been followed. This method for assaying phytase might not provide a true picture of the enzyme activity because the phytic acid in vivo does not exist in its free acid or sodium salt form but in the form of protein bodies (globoids). Furthermore, quantitative determination of the IP_6 degradation is tedious and time-consuming [19–21]. It requires the use of chromatographic techniques with multiple devices because neither IP_6, IP_{1-5}, nor P_i can be detected directly by absorbance or fluorescence, which put limitations on developing this technique for high-throughput screening [21–23]. Measurement of P_i released from IP_6 by phytase is an endpoint assay that provides color development proportional to the amount of P_i released, but it requires a suitable choice of enzyme dose and assay time besides the tediousness and carefulness in handling the toxic vanadate and molybdate reagents [24–27]. The color development may vary with the reaction conditions such as pH. Attempts have been made to assay phytase kinetically using *p*-nitrophenyl phosphate or *p*-nitrophenyl pyrophosphate as substrate. However, it is difficult to differentiate whether the yellow color produced on substrate hydrolysis is due to phytase or the action of phosphatases that occur widely in biological materials [28,29]. Moreover, not all phytases show good activity toward these two artificial substrates [30–32].

Due to its importance in animal nutrition, phytase has become one of the most assayed enzymes in feed research and feed

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[1] *Abbreviations used:* IP_6, phytate (phytic acid); IP_x, *myo*-inositol phosphate esters, where *x* denotes the number of phosphate ester bonds; P_i, inorganic phosphate; FTU, phytase activity units; HPLC, high-performance liquid chromatography.

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industrial analysis laboratories as well as in feed mills. Research laboratories need to screen protein-engineered phytase variants with improved performances, especially heat stability and pepsin resistance, whereas breeders need a phytase assay to be sure that the phytase added to their feed is still active so that they can save the addition of calcium phosphate. However, due to the toxic chemicals or the specific equipment needed for the phytase assay, routine phytase assay has not been done outside a well-equipped laboratory. Thus, it is clear that there is a need for a simple and faster alternative method for assaying phytases. Our preliminary tests have shown that phytases catalyze the hydrolysis of protein/peptide–phytate complexes and, to a lesser extent, complexes with calcium ion, releasing P_i and simultaneously reducing the turbidity of the substrate solution. In the current study, the use of IP_6–lysozyme complex as substrate to determine phytase activity kinetically by monitoring the turbidity reduction was established and compared with that of the traditional method. The developed method was used for the determination of activities of different bacterial and fungal phytases over a wide range of pH values.

Materials and methods

Enzymes and chemicals

The bacterial phytases (EC 3.1.3.26) used in this study were *Escherichia coli* phytase at 10,000 phytase activity units (FTU)/g (Phyzyme XP, Danisco, Brabrand, Denmark), which is here referred as *E. coli* phytase variant 1, *E. coli* phytase at 2000 FTU/g (OptiPhos, JBS United, Sheridan, IN, USA), which is here referred to as *E. coli* phytase variant 2, and *Bacillus* sp. MD2 phytase at 163.5 FTU/ml, which was cloned and expressed in our laboratory [33]. The fungal phytases used in this study were *Aspergillus niger* phytase (EC 3.1.3.8) at 5000 FTU/g (Natuphos, BASF Animal Nutrition, Germany) and *Peniophora lycii* phytase (EC 3.1.3.26) at 10,000 FTU/g (Ronozyme P-(CT), DSM Nutritional Products Europe, Switzerland). Sodium phytate (IP_6) and chicken egg white lysozyme (EC 3.2.1.17) were purchased from Sigma–Aldrich, and other chemicals of analytical grade were obtained from Merck.

Phytase extraction and activity determination

All four commercial phytases were obtained in granulated form and were extracted as described below except the *Peniophora* phytase, which was first ground in a mortar and pestle to improve the extraction. The granulated enzymes or the ground powder of *Peniophora* phytase (200–500 mg) were dispersed in 50 ml of MilliQ water in 100-ml beakers and stirred using a magnetic stirrer at room temperature (22 °C) for 30 min. The suspensions were then left standing overnight (16–18 h) at 5 °C to allow the particles to settle down. The supernatant was then withdrawn gently using Pasteur pipettes and diluted with MilliQ water to a final enzyme concentration of 20 FTU/ml based on the FTU values provided by the manufacturers. The standard phytase solutions were stored as stock enzyme solutions at −20 °C for subsequent experiments. Phytase from animal feed (12.5 g) was extracted with MilliQ water (37.5 ml), and supernatant was obtained by centrifugation and assayed for phytase activity by using IP_6 and IP_6–lysozyme complex as substrate, respectively.

The activity of the different commercial bacterial and fungal phytases was determined based on the amount of P_i released from IP_6 in 0.25 M acetate buffer (pH 5.5) at 37 °C [24,25]. The activity of *Bacillus* sp. MD2 phytase was assayed in 0.1 M Tris–HCl (pH 7.0). The enzyme stock solutions were diluted to approximately 0.03 FTU/ml in 0.25 M acetate buffer (pH 5.5) for histidine acid phytases or in 0.1 M Tris–HCl buffer (pH 7.0) containing 1 mM $CaCl_2$ and 0.01% (v/v) Tween 20 for *Bacillus* sp. MD2 phytase. Then 2 ml of 7.5 mM IP_6 in 0.25 M acetate buffer (pH 5.5) was added to 1 ml of the enzyme solution preincubated at 37 °C for 5 min. The reaction was performed for 60 min, followed by the addition of 2 ml of freshly prepared stop reagent containing a 1.5:1.5:1 mixture of 10% ammonium heptamolybdate/0.24% ammonium vanadate/65% nitric acid. Subsequently, the reaction mixtures were centrifuged at 8000*g* at room temperature for 10 min before the absorbance of the supernatant at 415 nm was recorded. A P_i calibration curve was made by treating standard P_i solutions of 0–4.0 mM KH_2PO_4 without added phytase under the same conditions as described above. All samples were assayed in triplicates. The phytase activity was calculated from the calibration curve of absorbance (OD_{415}) versus P_i concentration (in mM). One unit of phytase activity (FTU) was defined as the amount of phytase that releases 1 μmol of P_i per minute under the assay conditions. The activity values obtained for the five phytases were used to design all subsequent enzymatic reactions.

Preparation of phytate–substrate complex

Phytate–protein substrate complexes were prepared in 96-well flat-bottomed plates in a total volume of 120 μl. Here 60 μl of a specific buffer, 12 μl of 25 mg/ml lysozyme, 12 μl of 3 mM IP_6, and 36 μl of MilliQ water were added to each well and mixed at 1400 rpm on an Eppendorf thermomixer (Comfort MTP model) for 2 min at room temperature to form a homogeneous IP_6–lysozyme complex.

Other substrate complexes (IP_6–soy protein, IP_6–lysine, and IP_6–Ca^{2+}) were prepared in the same way as IP_6–lysozyme by varying the final concentration of IP_6 and the corresponding ligands (soy protein, lysine, and calcium, respectively) to get the desired turbidity.

Kinetic measurement of turbidity reduction of substrate complex catalyzed by phytases

The substrate complexes prepared as described above were preheated at 37 °C with vigorous shaking (1400 rpm) for 2 min prior to the addition of 1–12 μl of phytase to reach the desired final enzyme concentration (in FTU). The reaction was carried out at 37 °C for 20–60 min using a microplate reader (PowerWave$_x$, BioTek Instruments, Winooski, VT, USA). The reaction was monitored at 600 nm, and data were collected every 30 s. Before each reading, the microplate was shaken at full scale for 5 s. Reactions with MilliQ water instead of phytase samples were used as controls for each enzymatic reaction. All samples were assayed in triplicates. Activity of each phytase sample was calculated as the rate of turbidity reduction of the substrate complex (in milli-optical density at 600 nm [mOD_{600}] per minute).

P_i analysis by Konelab

To measure P_i released from the substrate complex in the reactions described above, the reactions were stopped at different time intervals by the addition of 30 μl of 2.5 N HCl. Microplates having the reactants were centrifuged at 8000*g* for 10 min, and 100 μl of the clear supernatants were transferred manually to 0.5-ml sample cups for P_i analysis by Konelab Analyzer according to the instructions from the manufacturer (Thermo Scientific, Germany). All samples were measured in triplicates.

HPLC analysis of inositol phosphate esters

The substrate IP_6 and its degradation products (IP_{1-5}) from the phytase-catalyzed reactions were analyzed on a Dionex DX-500 ion chromatograph system (Sunnyvale, CA, USA) with a Dionex CarboPac PA-100 column (4 × 250 mm) and a Dionex knitted coil (75 μl) for postcolumn reaction. The reactions were stopped by

the addition of 30 µl of 2.5 N HCl at a specific time, and the solutions were filtered through 96-well filter membranes (Pall Life Sciences, Deland, FL, USA) with a 0.45-µm pore size before being injected into the high-performance liquid chromatography (HPLC) column with an injection volume of 100 µl. Separation was performed by a linear gradient of 1–92% of 1 N HCl at a flow rate of 1 ml/min. The eluant was mixed in the postcolumn mixing chamber with 0.1% $Fe(NO_3)_3{\cdot}9H_2O$ in 2% $HClO_4$ [23] for detection of the inositol phosphate esters. Samples were analyzed in triplicates.

Results and discussion

Effect of phytic acid and lysozyme concentration on turbidity of phytic acid–lysozyme complex

Most proteins of plant origin, such as those derived from soybean, peanut, cottonseed, and rapeseed, have their isoelectric points (p*I* values) in the acidic range (~ pH 4.0). Hence, the solution of IP_6 complex with these proteins develops turbidity when the pH is lower than their p*I* [34,35]. For example, the solution of IP_6–soy protein complex is turbid only when the pH of the solution is decreased below pH 4.0. On the other hand, chicken egg white lysozyme has a p*I* of approximately 11.0 [36] and is very stable in the acidic pH range. IP_6–lysozyme complex was found to show turbidity and stability in a wide pH range from 2.5 to 8.5.

Fig. 1 shows that the turbidity of IP_6–lysozyme complex increases with increase in the concentrations of IP_6 and lysozyme at pH 4.0. At approximately 0.5 mM IP_6 and 0.35 mM lysozyme, the turbidity levels off. The highest turbidity of the solution is seen at an IP_6/lysozyme molar ratio higher than 1.5:1. The ratio of 0.3 mM IP_6/0.23 mM lysozyme (~2.5 mg lysozyme/ml) was subsequently chosen to prepare IP_6–lysozyme complex for use as substrate for various phytase assays because it showed high turbidity (OD_{600} > 1) and the reduction in turbidity of the complex was linearly related to the hydrolysis of the IP_6 in the complex. Changes in pH, temperature, and salt concentration were subsequently examined for their impact on the turbidity and stability of IP_6–lysozyme complex, as shown below.

Factors affecting turbidity and stability of phytic acid–lysozyme complex

Table 1 shows the half-life of IP_6–lysozyme complex based on its turbidity at different temperatures and pH values. Between pH 2.5 and 5.5, the complex was stable for more than 10 days at room temperature (22–25 °C) and for 2 months at 5–8 °C without any significant reduction in turbidity. However, at higher temperatures (> 30 °C), the stability of the complex was reduced dramatically, particularly at pH 5.5 and above, due to the instability of lysozyme under these conditions. It was observed that denatured proteins, including lysozyme, did not form stable complexes with IP_6 partly because the proteins themselves precipitate on denaturation.

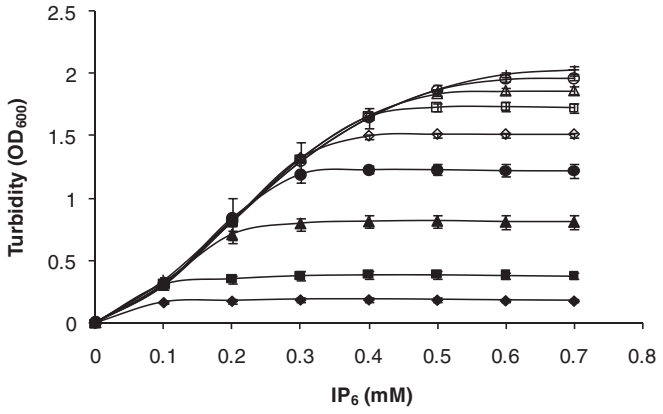

Fig.1. Effect of IP_6 and lysozyme concentration on the turbidity of IP_6–lysozyme complex solution. The complex was prepared in 50 mM sodium acetate buffer (pH 4.0) containing 0–0.7 mM IP_6 and lysozyme at different concentrations: 0.03 mM (♦), 0.06 mM (■), 0.12 mM (▲), 0.17 mM (●), 0.23 mM (◇), 0.29 mM (□), 0.35 mM (Δ), 0.41 mM (○), and 0.46 mM (+) in a total volume of 120 µl. Experimental details are described in Materials and methods.

Table 1
Stability of IP_6–lysozyme complex at various pH values and temperatures.

Temperature	2.5 ⩽ pH < 3.5	3.5 ⩽ pH < 5.5	⩾pH 5.5
45 °C	10 min	>50 min	6–12 min
37 °C	75 min	110–170 min	10–70 min
30 °C	>1.5 h	Stable for 60 min	>90 min
Room temperature (20–25 °C)	16 days	40–45 days	12–13 days
Refrigeration (5–8 °C)	84 days	No change in 2 months	>2 months

Note: IP_6–lysozyme complex (0.3:0.23 mM) was prepared in a total volume of 120 µl in 50 mM glycine–HCl (pH 2.5–3.5), 50 mM sodium acetate (pH 3.5–5.5), and 50 mM Tris–maleate (pH 5.5–8.5) containing 0.3 mM IP_6 and 0.23 mM lysozyme. IP_6–lysozyme complexes at different pH values were incubated at different temperatures, and their turbidity was followed. Data were reported as half-life ($t_{1/2}$) of the turbidity reduction of IP_6–lysozyme complex.

The pH dependence of the turbidity of IP_6–lysozyme complex is further shown in Fig. 2. As expected, the turbidity was high (OD_{600} = 1–1.2) in the acidic region (pH 3.5–5.5), and it started to decrease around neutral pH and became constant at the lowest level between pH 7.0 and 8.5, corresponding to approximately 30% of the highest turbidity value.

From Fig. 2, one can further see the effect of the common salts NaCl and $CaCl_2$, which may be present in assay buffers and biomaterial extracts, on the turbidity of IP_6–lysozyme complex. It can be seen that the turbidity of IP_6–lysozyme complex was quite stable at NaCl concentrations below 15 mM in the acidic region (pH 3.0–5.5) (Fig. 2A). Increase in NaCl concentration to 30 and 45 mM resulted in significant decrease in turbidity except at pH 3.5. The solution of IP_6–lysozyme complex became clear in the presence of 100 mM NaCl at all pH values in the range of 3.5–8.5.

Investigation of the effect of increasing concentrations of calcium salt at varying pH values showed that the turbidity of IP_6–lysozyme complex was stable at pH 3.0–4.0 at less than 3 mM $CaCl_2$, but it decreased dramatically at higher $CaCl_2$ concentrations, especially at pH values higher than 4.5 (Fig. 2B). It is well known that divalent and trivalent metal ions, such as Zn^{2+}, Ca^{2+}, and Fe^{3+}, have strong interactions with IP_6 [10]. Fig. 3 shows that ethylenediaminetetraacetic acid (EDTA in the form of sodium salt), the commonly used chelator in buffers, was not able to restore the high turbidity in the presence of Ca^{2+}, and at concentrations higher than 10 mM EDTA itself also contributed to reducing the turbidity of the solution. Due to the higher affinity to calcium or iron, IP_6 has been used as an alternative chelating agent in therapy for calcium urolithiasis [37] and for inhibiting *Vibrio vulnificus* on septicemia-induced mice [38,39].

P_i is the main product of the phytase-catalyzed reaction, and it can potentially compete with phytate to bind to the positive-charged lysozyme. In the standard kinetic assay of phytase having phytate at a concentration of 0.3 mM, the final P_i can be 1.5 mM when all phytate molecules are converted to P_i and *myo*-inositol monophosphate. It was found that at pH 3.5 in 50 mM glycine–HCl, the turbidity of IP_6–lysozyme complex was not affected at P_i concentrations up to 50 mM and the turbidity disappeared at 80 mM P_i (Fig. 4). At pH 3.5–8.5, P_i up to 5 mM did not interfere with the turbidity of IP_6–lysozyme complex (data not shown).

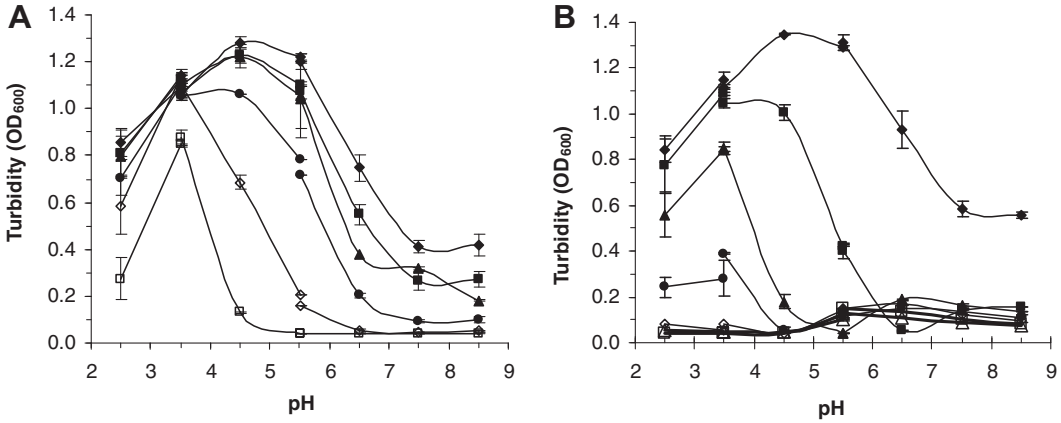

Fig.2. Effect of pH and concentrations of NaCl (A) and $CaCl_2$ (B) on the turbidity of IP_6–lysozyme complex (in a ratio of 0.3:0.23 mM) in 50 mM glycine–HCl buffer (pH 2.5–3.5), 50 mM sodium acetate buffer (pH 3.5–5.5), and 50 mM Tris–maleate buffer (pH 5.5–8.5) containing 0.3 mM IP_6, 0.23 mM (2.5 mg/ml) lysozyme, and either NaCl at different concentrations [0 mM (♦), 5 mM (■), 10 mM (▲), 15 mM (●), 30 mM (◇), and 45 mM (□)] (A) or $CaCl_2$ at different concentrations [0 mM (♦), 1 mM (■), 3 mM (▲), 5 mM (●), 7 mM (◇), 10 mM (□), and 15 mM (Δ)] (B) in a total volume of 120 μl. Experimental details are described in Materials and methods.

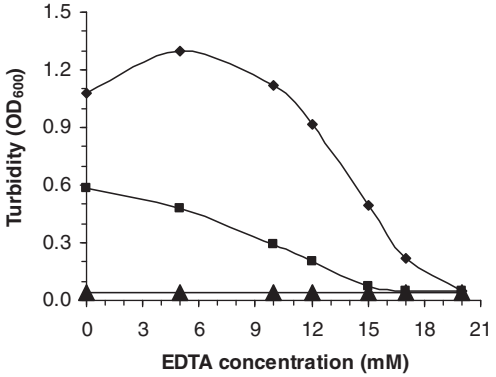

Fig.3. Effect of EDTA and $CaCl_2$ concentrations on the turbidity of IP_6–lysozyme complex (0.3:0.23 mM) in 50 mM glycine–HCl (pH 3.5) containing 0.3 mM IP_6, 0.23 mM lysozyme, and $CaCl_2$ at different concentrations: 0 mM (♦), 5 mM (■), and 10 mM (▲) in a total volume of 120 μl.

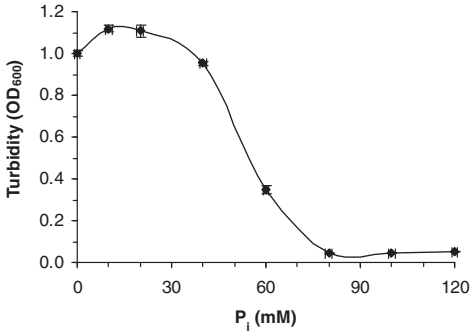

Fig.4. Effect of phosphate concentration on the turbidity of IP_6–lysozyme complex (0.3:0.23 mM) in 50 mM glycine–HCl (pH 3.5) containing 0.3 mM IP_6, 0.23 mM lysozyme, and different concentrations of KH_2PO_4 in a total volume of 120 μl.

The above results suggest that in spite of the variation in the turbidity with varying testing conditions, the IP_6–lysozyme complex can be an ideal substrate for determining phytase activity by following the decrease in turbidity provided that negative controls (without phytase or with denatured phytase) for each enzymatic reaction are run simultaneously. Under the assay conditions, neither the bacterial nor fungal phytases themselves in the range of 0.1–2.8 FTU/ml reaction mixture caused detectable turbidity with IP_6.

Comparison of five microbial phytases for their activity toward IP_6 and various IP_6–ligand complexes

Table 2 shows that all five phytases of bacterial or fungal origin were able to hydrolyze the IP_6 in the IP_6–protein complexes, as indicated by P_i release determined by Konelab analysis. It should be noted that the *E. coli* phytases (*E. coli* phytase variants 1 and 2) hydrolyzed IP_6, as well as IP_6–lysozyme and IP_6–soy protein, several fold faster than the two fungal phytases (Table 2), in line with literature data showing that *E. coli* phytase has higher activity at pH 3.0 than its fungal counterparts [40]. The activity of a phytase at approximately pH 3.0 is an important criterion for its efficiency as a feed enzyme for animal nutrition. Phytases from *P. lycii* and *Bacillus* sp. MD2 showed the least hydrolysis of the three substrates under these assay conditions. In contrast to the *A. niger* phytase, all four phytases showed 1.3- to 2.3-fold higher activity toward IP_6–lysozyme and IP_6–soy protein than IP_6 based on the P_i released. The difference in the substrate preference of these five phytases could not be related to the ester bonds that are first hydrolyzed because phytases from *E. coli* and *P. lycii* initiate their hydrolysis reaction by attacking the ester bond at position 6 in the phytate molecule [41,42], whereas *A. niger* phytase hydrolyzes initially at position 3 [43]. *Bacillus* phytase is a hybrid 3/6-phytase, which initially attacks the phosphate groups at position 3 and/or position 6 [43] or any position of phosphate [44] in phytate molecules. The observations with the *E. coli* phytase shown in Table 2 were further supported by analysis of the reaction products by HPLC that separated IP_6 and its degradation products IP_5, IP_4, IP_3, and IP_2 [22]. It was confirmed that the *E. coli* phytases showed higher activity in degrading IP_6 in IP_6–lysozyme complex than IP_6 in the form of sodium phytate (data not shown). The exact reason why IP_6–protein may be a much better substrate for the *E. coli* phytases remains an enigma and needs to be examined by ^{31}P NMR (nuclear magnetic resonance). On the other hand, because IP_6 can be bound with seed proteins in plant seeds and with food and feed proteins in the upper parts of the digestive tracts of monogastric animals having an acidic environment, the efficient hydrolysis of IP_6 in IP_6–protein complex under conditions close to in vivo is a prerequisite for a good feed phytase.

The *E. coli* phytase variant 1 and *A. niger* phytase as representatives of bacterial and fungal 6- and 3-phytases, respectively, were chosen to further examine their activity toward IP_6–lysine

Table 2
Activity of different commercial phytases on IP_6–lysozyme and IP_6–soy protein complexes as compared with IP_6 as substrate.

Phytase	Relative activity (%)		
	IP_6–soy protein	IP_6–lysozyme	IP_6–Na^+
Escherichia coli phytase variant 1	164.3	229.0	100.0
Escherichia coli phytase variant 2	137.8	151.8	102.7
Aspergillus niger phytase	31.8	23.1	37.0
Peniophora lycii phytase	24.5	13.0	9.8

Note: The assay was carried out in a total volume of 120 μl in 50 mM glycine–HCl (pH 3.0) at 37 °C for the five different phytases added at a dose of 0.1 FTU/ml. The reaction rate in terms of P_i release (in μmol P_i/ml/min) was measured by stopping the reaction at different time intervals and analyzing P_i on Konelab. Activity of *E. coli* phytase variant 1 (0.096 μmol P_i/ml/min) on IP_6–Na^+ was set at 100%. Activities of the phytases on the other substrates are reported relative to the activity of *E. coli* phytase variant 1 on IP_6–Na^+.

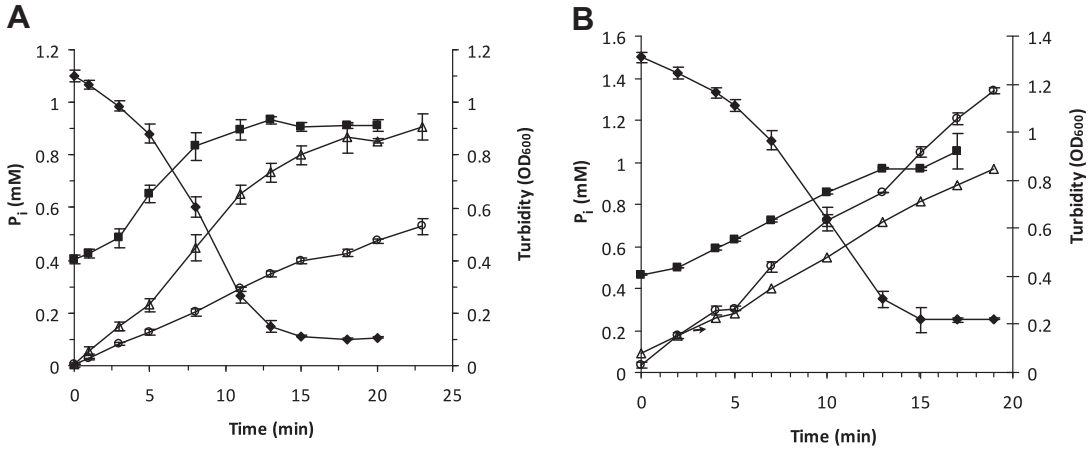

Fig.5. Activity of *E. coli* phytase variant 1 (0.1 FTU/ml) (A) and *A. niger* phytase (0.5 FTU/ml) (B) with IP_6–lysozyme substrate based on turbidity reduction (♦) and P_i released (■), with IP_6 based on P_i released (Δ), and with IP_6–lysine complex based on P_i released (○). The three substrates were prepared in 50 mM glycine–HCl (pH 3.5) containing 0.3 mM IP_6 (for IP_6 substrate) and either 0.23 mM lysozyme (for IP_6–lysozyme substrate) or 23 mM lysine (for IP_6–lysine substrate) in a total volume of 120 μl. Reactions were performed at 37 °C for 25 min with continuous mixing. Turbidity of the reactions was determined every 30 s. For P_i determination, the reactions were stopped by adding 30 μl of 2.5 M HCl and centrifuged, and the supernatants were analyzed for P_i on Konelab.

complex. Lysine as a positively charged amino acid and a food and feed additive was chosen because it can potentially complex with IP_6 in vivo in the digestive tract. IP_6–lysine complex, unlike IP_6–lysozyme complex, was not turbid at all pH values between 3.5 and 8.5. Hence, its role as a substrate complex with IP_6 for phytase needed to be evaluated by the release of P_i. Fig. 5A shows that whereas hydrolysis of IP_6–lysozyme complex by *E. coli* phytase variant 1 was faster compared with that of IP_6, in line with the results shown in Table 2, hydrolysis of IP_6–lysine complex was slower. In contrast, *A. niger* phytase did not show much difference in its initial reaction rates with the two substrates IP_6–lysine complex and IP_6 (Fig. 5B). It should be noted in Fig. 5A and B that the *A. niger* phytase was dosed five times higher than the *E. coli* phytase considering its lower activity at pH 3.5.

Besides IP_6–protein complexes, IP_6–Ca^{2+} complex can also develop turbidity and, therefore, can be used for the assay of phytases by following the decrease in turbidity. However, the turbidity of IP_6–Ca^{2+} complex was low (OD_{600} < 0.2), especially at pH values lower than pH 5.5. IP_6–Ca^{2+} complex could, however, be used as an alternative substrate to IP_6–lysozyme for the assay of neutral and alkaline phytases (data not shown).

Relationships between turbidity decrease and phosphate release

If the phytase activity assay based on the turbidity reduction of IP_6–lysozyme complex catalyzed by 3- and 6-phytases can reflect the hydrolysis of IP_6, it is essential that it be validated by the well-established method measuring P_i release. Fig. 6 shows that turbidity reduction (OD_{600}) of IP_6–lysozyme in the kinetic assay developed here correlated well to P_i released from the same enzymatic reaction using both *E. coli* phytase (Fig. 6A and B) and *A. niger* phytase (Fig. 6D and E). Phytase activity in these assays could, thus, be measured in mOD/min, which is equal to the slope of turbidity reduction in the linear range (OD_{600} = 0.1–0.9). The reaction rate in mOD/min could be converted to the common unit of phytase activity (FTU/ml) by correlating it to the P_i released (Fig. 6C and F). In Fig. 6C and F, it is estimated that every 1 μmol of Pi released per minute based on the results from Konelab analysis is related to a 3.03 ± 0.27-unit OD decrease at 600 nm/min.

Effect of substrate and phytase concentration

The optimal substrate concentration was found to be 0.3 mM IP_6 and 0.23 mM lysozyme, which was used in all assays in this study. With lower substrate concentrations, the turbidity was low and made the linear range of the reaction shorter, whereas with high substrate concentrations, the turbidity decrease was not linear with the substrate concentration. It should be noted that the kinetic assay of phytase developed here is based on the monitoring of substrate hydrolysis instead of product formation as in most enzyme assays. This is also one of the reasons why one cannot use high substrate concentrations. Hence, this kinetic method was found to be not

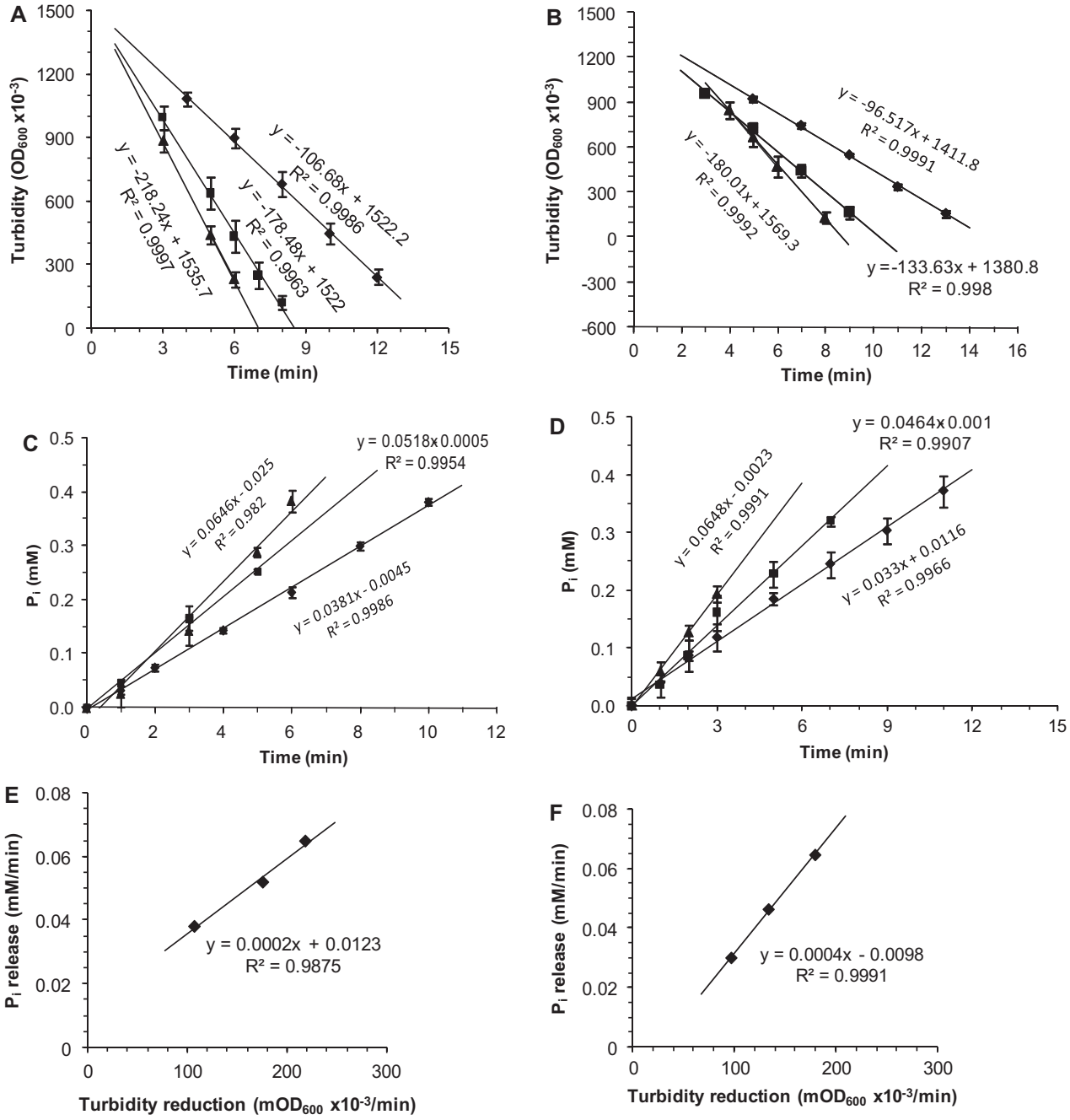

Fig.6. Relationship between turbidity reduction and phosphate release during reaction with different concentrations of phytases—(A–C), *E. coli* phytase variant 1, 0.1 FTU/ml (♦), 0.2 FTU/ml (■), and 0.3 FTU/ml (▲); (D–F) *A. niger* phytase, 0.1 FTU/ml (♦), 0.15 FTU/ml (■), and 0.2 FTU/ml (▲)—in 50 mM acetate buffer (pH 5.5) at 37 °C with a total reaction volume of 120 μl. Turbidity is shown in panels A and D; P_i released is shown in panels B and E, and the relationship between turbidity reduction (mOD/min) and P_i release (in mM/min) is shown in panels C and F.

useful for the estimation of K_m and V_{max} of phytases just like the conventional colorimetric assay of phytases. The suitable method of estimation of K_m and V_{max} is by HPLC [22,23].

At an assay pH of 3.0–3.5, an assay temperature of 30 °C, and a reaction time of 20–60 min, the suitable phytase concentration for *E. coli* and *A. niger* phytases was in the range of 0.1–0.8 FTU/ml reaction mixture. The phytase activity of some cereals, such as rye, triticale, wheat, and barley, is in the range of 500–5000 FTU/kg [45], and the microbes, *Aspergillus* spp., *E. coli*, and *Bacillus* sp. are reported to have a phytase activity of 0.1–1.8 FTU/ml broth [33,41,46]. In the current study, feed samples containing corn and soy flour and *E. coli* phytase variant 1 at 400–2300 FTU/kg could be assayed by the kinetic method, whereas with a phytase level at 200 FTU/kg or lower in the feed the kinetic method was not suitable because the incubation needed to be overnight.

pH profiles of five microbial phytases using phytic acid–lysozyme complex as substrate

To evaluate the usefulness of the kinetic method developed, it is also important to test this method in a wide pH range that is relevant for phytases. Fig. 7 shows the activities of the five microbial phytases as a function of pH in the range of pH 2.0–8.5 using IP_6–lysozyme complex as the substrate. In general, the pH profiles

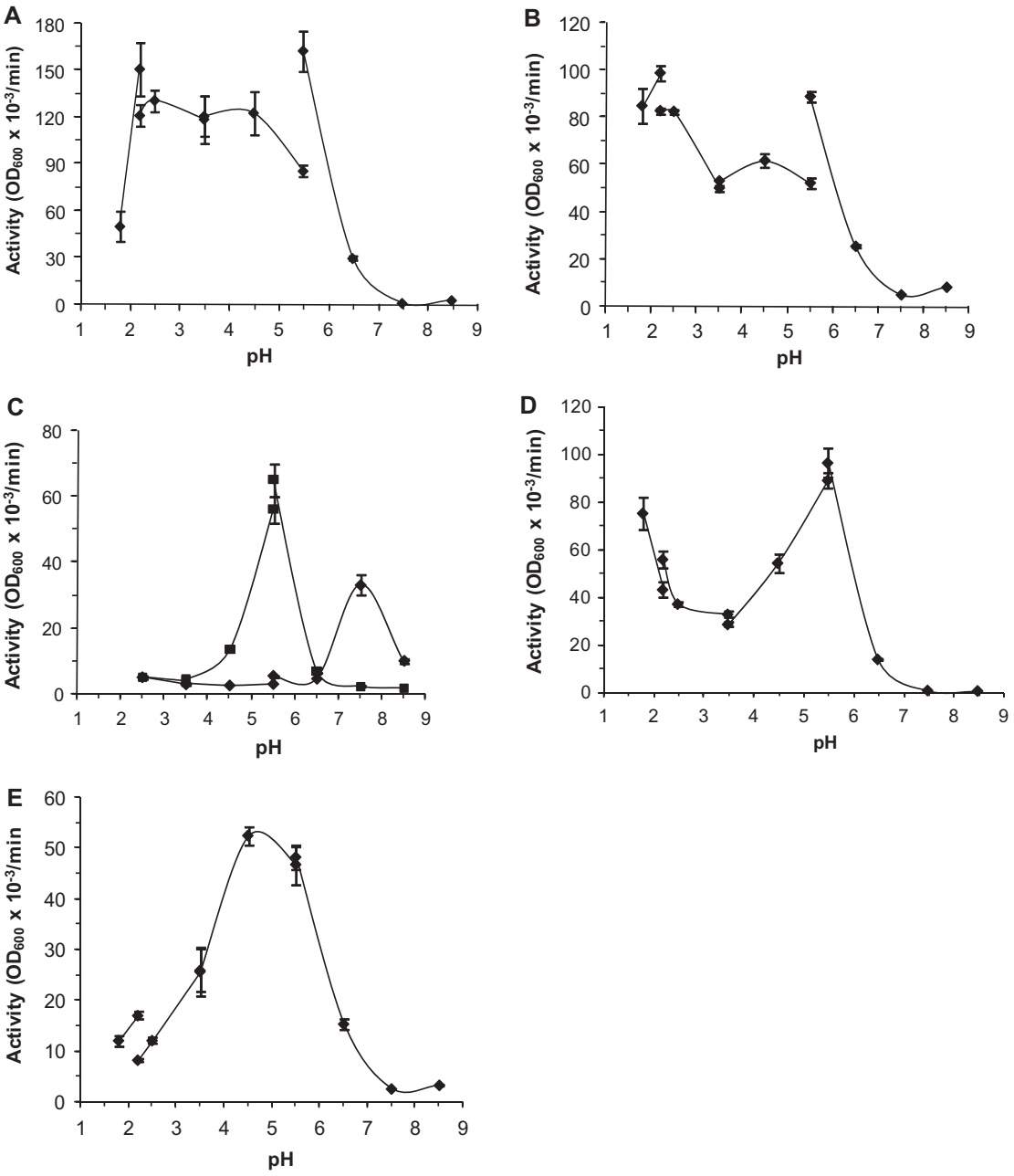

Fig.7. pH profiles of *E. coli* phytase variant 1 (A), *E. coli* phytase variant 2 (B), *Bacillus* phytase (C), *A. niger* phytase (D), and *Peniophora lycii* phytase (E) on IP_6–lysozyme substrate complex. Reactions were carried out in 50 mM potassium–HCl (pH 1.5–2.5), 50 mM glycine–HCl (pH 2.5–3.5), 50 mM sodium acetate (pH 3.5–5.5), and 50 mM Tris–maleate (pH 5.5–8.5) containing 0.3 mM IP_6 and 0.23 mM lysozyme in a total volume of 120 μl at 37 °C. The enzyme dose for each reaction was 0.1 FTU/ml based on P_i released from IP_6 in conventional phytase activity assay. In panel C, enzymatic reactions were performed without $CaCl_2$ (♦) and with 1 mM $CaCl_2$ (■).

obtained by the kinetic method (Fig. 7) agree well with those reported in the literature using IP_6 as substrate [31,32,37,40–42,47]. From Fig. 7A and B, one can see that the two *E. coli* phytases showed similar pH profiles with IP_6–lysozyme complex as with IP_6 alone [41,47] except for an extended pH optimum to the acidic region (pH 2.0–5.5). This could mean that IP_6–lysozyme complex as a substrate makes the phytase more stable, and therefore active in the acidic region. It should be noted that in Fig. 7A and B, the activity measured varied greatly even at the same pH due to the different buffers used having different ionic strengths that, in turn, affect the turbidity of the system. Using IP_6–lysozyme complex as substrate, it was observed that *Bacillus* sp. MD2 phytase, a calcium-dependent enzyme [31,32], showed a shift of 2.0 pH units in its pH optimum toward the acidic region in the presence of 1 mM $CaCl_2$ (Fig. 7C). *Aspergillus niger* phytase showed two optimal peaks of activity at pH 5.5 and 2.0 (Fig. 7D) as with IP_6 except that the second pH optimal peak shifted 0.5 unit toward the acidic region (pH 2.0 instead of pH 2.5) compared with IP_6 alone as substrate [37]. The pH profile of *Peniophora* phytase showed one optimum around pH 4.0–5.0, in accordance with that reported previously for the enzyme with IP_6 [42]. The broadening of pH optimum in the case of *E. coli* phytases and the *A. niger* phytase, as well as the shift of pH optimum in the case of *Bacillus* phytase, are obviously advantageous considering the pH of the digestive tract in monogastric animals, which is generally in the range of pH 2.5–6.0.

Conclusions

IP_6 and lysozyme complex at a concentration of 0.3 and 0.23 mM, respectively, forms a turbid solution and can be used

for the kinetic assay of the activity of phytases by monitoring the decrease in absorbance. The decease in turbidity correlates well with the release of P_i. The method is useful for assaying histidine acid phytases, represented by all commercial phytases, and β-propeller phytase tested in this study. Other IP_6–ligand complexes, including IP_6–soy protein, IP_6–lysine, and IP_6–Ca^{2+}, were also investigated as substrates, but they were less suitable than IP_6–lysozyme complex. Compared with the conventional endpoint colorimetric method based on P_i measurement, the kinetic assay described here is simple, fast, safe, adaptable to high-throughput screening, and more close to the in vivo physiological conditions, making it more suitable for use in phytase protein engineering, feed mills, and industrial analysis laboratories to estimate the phytase activity before use in feed and food applications.

The usefulness of this method has been further demonstrated in studying the pH profiles of five different phytases. The main limitation of this method is that the enzymatic reaction needs to be mixed well before measurements to avoid precipitation of IP_6–lysozyme complex, which can lead to greater deviation in measurement. The linear range for turbidity at OD_{600} (0.1–0.9) is quite narrow compared with the linear range for the colorimetric assay of P_i release. Because this kinetic method is based on the monitoring of substrate consumption instead of product formation as in most enzyme assays, it is not suitable for the estimation of K_m and V_{max} of phytases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2010.10.034.

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