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Purification and characterization of PAP phytase from Indian wheat variety DBW 17

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Abstract

Phytase (myo-inositol hexakisphosphate phosphohydrolase E.C.3.1.3.8) was isolated and purified from Indian wheat variety DBW 17 using 5-step purification procedure as (NH4)2SO4 fractionation, cold methanol fractionation, DEAE-Trisacryl M chromatography, Ultrogel AcA34 filtration chromatography and SP-Trisacryl M chromatography. Two forms of the enzyme as PHY1 (64 kDa) and PHY2 (62 kDa) were identified. There was 79.0 fold purification of PHY1 and 114.8 fold of PHY2. The optimum pH for PHY1 and PHY2 was 6.0 and 5.5 respectively while optimum temperature 55°C for both the isozymes. The Km value for the hydrolysis of phytate was 123 µM for both the isozymes indicating high specificity for phytate. Wheat phytase can be used in hydrolysis of phytate in food industries for releasing micronutrients and hence increasing their bioavailability.

Keywords: Column chromatography, phytase, phytate, purification, wheat whole meal.

Introduction

Wheat is the main source of world's food energy and nutrition. It contains significant amount of proteins, minerals and vitamins which are essential nutrients for human health (Piironen *et al*., 2009). However, absorption of micronutrients such as Fe and Zn is significantly inhibited in human beings by the presence of substances such as phytic acid (PA) leading to deficiency of these micronutrients (Greiner and Konietzny, 2006). Phytate is also the primary storage form of phosphorus (P) in seeds accounting for up to 85% of the total seed phosphorus. However, monogastric animals such as pig, poultry and fish cannot digest phytic acid and as a result animal feeds are supplemented with phosphorus. Large amount of phytic acid is excreted by these animals leading to accumulation in soil and water and thus cause phosphorus pollution (Brinch-Pedersen *et al*., 2002). Therefore, degradation of phytate present in the grain is needed to overcome the problem of micronutrient deficiency in humans and monogastric animals.

Phytate is degraded by phytase enzyme present in the seed. Significant positive correlation has been reported between native phytase activity and phosphorus utilization (Oloffs *et al*., 2000) in broilers and micronutrient bioavailability in humans (Sandberg and Andersson, 1998) and rat (Lopez *et al*., 2003). Phytases (myo-inositol hexakisphosphate phosphorylase, EC 3.1.3.8) represent a subgroup of phosphatases which are capable of initiating the stepwise dephosphorylation of phytate. Although microbial phytases have been used in food processing, cereal phytases can be alternative due to their higher acceptance among consumers and their assumed lower allergenic potential (Baur *et al*., 2002). In humans, phytic acid breakdown in the stomach and the intestine is influenced mainly by the dietary phytases (Sandberg and Andersson, 1998) which are active in the human stomach. In addition, there are reports indicating improved bread making quality by higher phytase levels during fermentation (Haros *et al*., 2001). In plants the activity varies considerably, from below 100 FTU kg−1 in maize up to approximately 1200 FTU kg−1 in wheat and 5000 FTU kg−1 in rye (Eeckhout, 1994). Recently large variations have been reported in phytase activity in wheat by Ram *et al*., 2010.

Two types of phytases namely purple acid phosphatase phytases (PAPhys) and multiple inositol phosphate phosphatases (MINPP) have been reported in wheat. Based on enzyme kinetics, two types of PAPhys (encoded by the TaPAPhy a and TaPAPhy b genes) were found more prominent by Dionisio *et al*., 2011. TaPAPhy a and TaPAPhy b are synthesized mainly during seed development and germination, respectively. They further localized TaPAPhy in the globoids present in protein storage vacuoles in the aleurone layer (Dionisio *et al*., 2011). However, there are few reports of kinetic properties of phytase in wheat (Nagai and Funahashi, 1962, 1963; Lim and Tate, 1971, 1973; Nakano *et al*., 1999). Hence characterization of phytase in wheat can lead to better understanding of the role of phytase in improving nutritional quality. Therefore, the major objective of this work was to isolate and purify wheat PAP phytases in order to study their kinetic properties in wheat.

Materials and Methods

Homogeneous seed of a wheat variety (DBW17) grown at Directorate of Wheat Research, Karnal during 2010-11, was used for the isolation and purification of phytase. Phytase was extracted from 500 g of whole meal flour (milled by Cyclotec, Tecator, 0.5 mm sieve) using 5000 ml of extraction buffer (50 mM acetate buffer, pH 5.3). The samples were soaked in extraction buffer over night at 4°C and centrifuged at 4000 X g for 30 minutes. The 1 ml of the supernatant (crude extract) was used for enzyme assay and the remaining extract was used for purification of the enzyme using following methodologies.

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Purification of phytase

The purification procedure was adapted from that described by Laboure *et al*., 1993 and Nakano *et al*., 1999. All the steps were performed at 4°C. The crude enzyme was adjusted to 40% $(NH4)_2S04$ saturation. After centrifugation at 15000 X g for 20 min, the supernatant was adjusted to 70% $(NH4)_2SO4$ saturation. The 40-70% saturated $(NH4)_2SO4$ pellet was solubilized in about 50 ml of extraction buffer and extensively dialyzed against 50 mM sodium acetate buffer, pH 5.3. The precipitate formed during dialysis was removed by centrifugation at 15000 g for 20 min and checked for phytase levels. There was no appreciable phytase activity in the precipitate.

Cold methanol up to 45% (v/v) concentration was added to the supernatant and centrifuged at 15000 g for 20 min. The supernatant was brought up to 65% (v/v) concentration of methanol. The 45-65% concentrated pellet was solubilized in minimum amount of extraction buffer and extensively dialyzed against 20 mM Tris acetate buffer pH 7.5.

The dialyzed fraction was loaded on to a DEAE Trisacryl M (IBF) anionic-exchanger column (3x7 cm), equilibrated with 20 mM Tris acetate buffer pH 7.5, at a flow rate of 30 ml/h. The column was washed with 100 ml of loading buffer, and eluted with a linear gradient of 100 ml of same buffer in mixing chamber and 100 ml of buffer containing 0.3 M NaCl in the reservoir. The fractions (3 ml) containing phytase activity were pooled and concentrated by addition of $(NH4)_2SO4$ to 65% saturation. After centrifugation at 15000 g for 30 min, the pellet was solubilized in 4-5 ml of 100 mM sodium acetate buffer pH 4.8/0.25 M KCI and dialyzed against the same buffer.

The dialyzed fraction was then loaded on to an Ultrogel AcA34 (IBF) column (1.4x120 cm) and eluted (3 ml fractions) with the same buffer at a flow rate of 13 ml/h. The fractions containing phytase activity were pooled, added to 10% glycerol and dialysed against 50 mM sodium acetate buffer (pH 4.8), containing 10% glycerol. All the subsequent operations were done in the presence of 10% glycerol to stabilize enzyme activity.

Further purification of the dialyzed fraction was obtained on a SP-Trisacryl M (IBF) cationic-exchanger column (0.8x4 cm) equilibrated with the loading buffer (50 mM sodium acetate buffer pH 4.8/10% glycerol). After washing with 30 ml of the same buffer, the column was eluted with a linear gradient from 0.05-0.4 M NaCl in the same buffer (50 ml) at a flow rate of 30 ml/h. The active fractions were pooled, concentrated by saturating with 70% $(NH4)_2SO4$ dialyzed against the same buffer without NaCl.

Protein concentration in the extracts was determined by Folins-Lawry method using BSA (Sigma) as the standard.

Enzyme assay

The enzyme assay was performed as per the method of Ram *et al*., 2010. 0.2 ml of the enzyme solution was used in determining phytase activity. 0.2 ml of 1% (w/v) sodium phytate (phytic acid sodium salt hydrate from rice) supplied by Sigma (P8810) was added into 0.2 ml of enzyme solution extracted above and incubated for 30 min at 50°C. The reaction was stopped by adding 0.4 ml of absolute trichloroacetic acid (room temperature). The mixture was centrifuged at 10,000 X g for 30 min and 0.2 ml of the supernatant fraction was mixed with 1.8 ml of distilled water and 2 ml of fresh colour reagent and incubated at 50°C for 15 min. The colour reagent was prepared by mixing three volumes of 1 M sulfuric acid, one volume of 2.5% ammonium molybdate and one volume of 10% ascorbic acid (w/v). Series of diluted potassium phosphate solutions were used as standards. The absorbance of each reaction mixture was taken at 820 nm and the enzyme activity calculated. One unit of phytase is defined as the amount of enzyme required to release 1 µmol of inorganic P/min from sodium phytate at 50°C.

Gel electrophoresis and Acid Phosphatase Activity Staining

Native gel electrophoresis was carried out with 10% gel. Activity staining of the purified phytase was performed with 1-naphthyl phosphate (2 mM) as the substrate in sodium acetate buffer (50 mM, pH 5.0, 5 mM $MgC1_2$,) and Fast Garnet GBC (1.5 mM, Sigma) as the dye in the dark. The reaction was terminated by fixing the gel with methanol: acetic acid: water (45:10:45, v/v) (Hubel, 1996). Sodium Dodecyl Sulfate PAGE was carried out using 12.5% gel as per Laemmli, (1970). Gel was stained with silver staining method.

Results

Purification of phytase

Phytase was purified from whole meal of wheat in a sequential 5-step procedure including ammonium sulfate precipitation, cold methanol precipitation, anion and cation-exchange chromatography and size exclusion chromatography. Ammonium sulfate (40-70%) saturation and methanol (45-65%) precipitation yielded a protein mixture. The protein mixture separated by DEAE-Trisacryl M column showed phytase activity from fraction no. 37-50 (Fig. 1a). Enzyme separated through Ultrogel AcA34 gel-filtration showed phytase activity from fraction no. 26-37 (Fig. 1b). Purification through SP-Trisacryl M column showed phytase activity from fraction no. 21-31 (Fig. 1c). The details of the purification steps and the purification fold levels are given in Table 1. There was 79.04 fold purification of PHY1 and 114.75 fold of PHY 2. The recovery of PHY1 was 0.024% and that of PHY2 was 0.017 %.

Table 1. Summary of purification of phytase from whole meal of wheat variety DBW 17

S.No.	Steps	Volume (ml)	Activity (Units)	Protein (mg/kg)	Specific activity (Units/mg)	Purification (fold)	Yield of Activity. (%)
1	Crude enzyme	5000.0	738.9	119475.0	0.03	1.0	100.0
2	40-70%(NH4)2SO4 fraction	264.0	608.3	4248	0.14	29.4	82.3
3	45-65% Methanol fraction	50.0	135.7	740.8	0.18	47.2	18.4
4	DEAE Tris acryl column	70.0	27.3	94.1	0.29	47.0	3.7
5	AcA34 filtration	72.0	22.8	50.2	0.46	73.6	3.1
6	SP- Trisacryl column PHY 1	1.00	0.18	0.36	0.49	79.0	0.02
7	SP- Trisacryl column PHY2	1.00	0.12	0.17	0.71	114.6	0.02

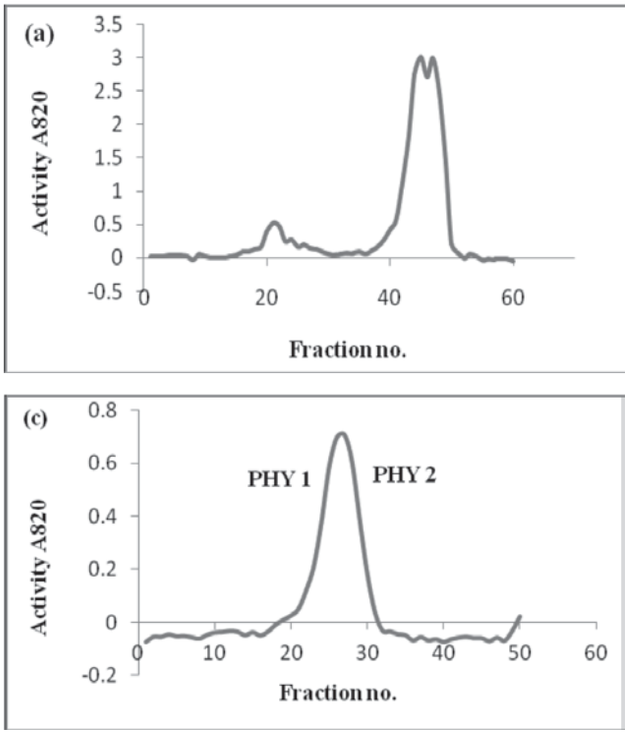

Fig. 1. (a) DEAE-Trisacryl M column chromatography of dialysed 45-65% methanol fraction of the extract. The fractions containing phytase activity (37-50) were taken for further purification. (b) AcA34 gel-filtration column chromatography of the eluted fraction containing phytase activity from DEAE-Trisacryl M column. Fractions containing phytase activities (26-37) were used for further purification.(c) SPTrisacryl M column chromatography of the fraction containing phytase activity from AcA34 gel-filtration column. The fractions containing phytase activities (21-31) used for kinetic analysis and gel electrophoresis.

The molecular mass of the purified enzyme estimated on SDS- PAGE was approximately 64 kDa of PHY1 and 62 kDa of PHY2 (Fig. 4). Activity staining of the purified phytase run on Native PAGE showed the presence of dark-brown color band on the gel. This indicated that the purified enzyme belonged to acid phosphatase group.

Effects of pH on enzyme activity and stability

Phytase activity was assayed over a pH range from 4.0-6.5 using three different buffer systems of 50 mM concentration such as acetate HCl (pH 3.0-4.0), sodium acetate (pH 4.5-5.5), and tris-maleate (pH 6.0-7.0). The phytase was stable in the pH range from 4.0-6.5, whereas below pH 4.0 and above pH 6.5 a rapid decline in activity was observed. The optimum pH for PHY1 and PHY2 was 6.0 and 5.5 respectively (Fig. 2a and b).

Temperature optimum and stability

Phytase activity was assayed over a range of temperatures to determine the optimum temperature for hydrolysis of phytate by the enzyme. The enzyme was pre-incubated at temperatures ranging from 40°C-65°C for 15 min in sodium acetate buffer pH 5.3. The optimum temperature for both PHY1 and PHY2 was 55°C (Fig. 3a and b) and there was fast decline in activity above and below 55°C.

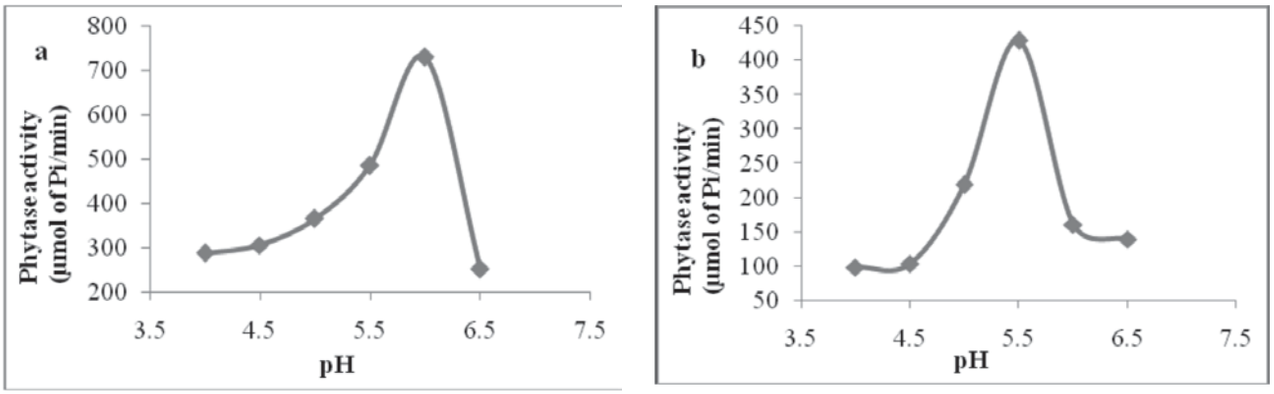

Fig. 2. Effect of pH on phytase activity. The purified phytase was assayed for activity at different pH range from pH 3.0 to pH 7.0. (a) The pH activity curve of PHY1 showing peak activity at pH 6.0 and (b) activity curve of PHY2 showing peak activity at pH 5.0.

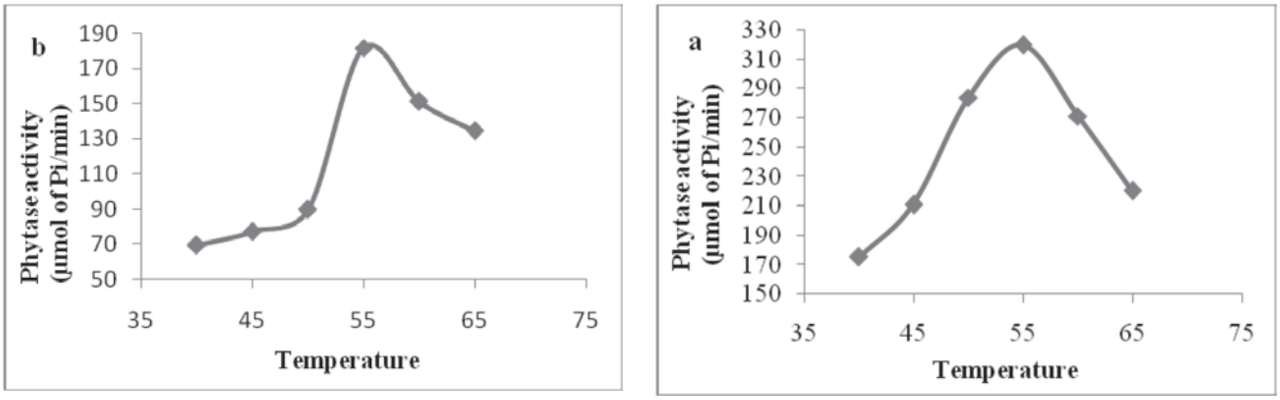

Fig. 3. Effect of temperature on phytase activity. The phytase activity was carried out at a temperature ranging from 35-70°C. Both PHY1 (a) and PHY2 (b) showed temperature optimum at 55°C.

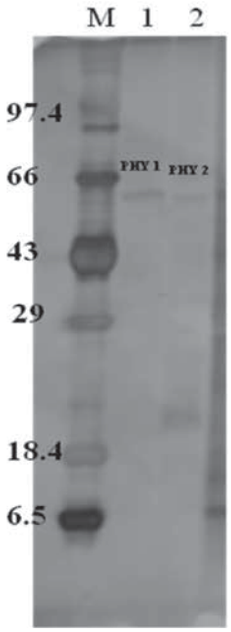

Fig. 4. SDS-PAGE of phytase from wheat variety DBW17. M, molecular marker Lanes: 1, PHY1; 2, PHY2; The gel was stained by silver staining method. The molecular mass of PHY1 and PHY2 appears to be 64 and 62 kDa, respectively.

Substrate specificity

The rate of enzyme activity of purified wheat whole meal phytase on several phosphorylated compounds, -Napthyl P, G-6-P, AMP, ADP, ATP and phytic acid were tested. The phosphate compounds were dissolved in 50 mM sodium acetate buffer pH 5.3. The relative rate of hydrolysis is summarized in Table 2. The enzyme showed broad substrate specificity and the highest activity (5.6 fold) was observed with ATP by both the enzymes, PHY1 and PHY2. The Km values for phytate were calculated as 123 µM for both PHY1 and PHY2 by Lineweaver-Burk plots 1934.

Effect of metal ions on the phytase activity

Effects of metal ions on the enzyme activity were examined using sodium phytate as a substrate in the presence of each metal ion (Table 3). Salts of different metal ions used were $ZnSO_4$, $CuSO_4$, $CaCl_2$, KCl, $MgCl_2$ and $FeCl_3$. Ca and Mg ions showed positive effect on both the enzymes. PHY1 activity increased by 1.2 times, while PHY2 by 2.5 and 3.2 times, respectively. Both PHY1 and PHY2 were strongly inhibited by $ZnSO_4$ and $FeCl_2$,. KCl had inhibitory effect on PHY 1 while it enhanced activity of PHY 2.

Table 2. Effect of the substrate on relative activity of phytase from wheat variety DBW 17

S.No.	Substrate	Relative activity (%) PHY 1	PHY 2
1	Phytate	100.0	100.0
2	Napthyl P	79.3	70.5
3	G-6-P	47.6	69.4
4	AMP	34.7	64.0
5	ADP	162.5	392.7
6	ATP	560.1	566.4

Table 3. Effect of metal ions on the relative activity of phytase from wheat variety DBW 17

S.No	Metal ion	Relative Activity (%) PHY 1	PHY 2
1	Control	100.0	100.0
2	ZnSO4	15.0	0.0
3	CuSO4	26.5	66.5
4	CaCl2	122.2	315.9
5	KCl	82.4	189.0
6	MgCl2	120.7	245.9
7	FeCl3	40.3	0.0

Discussion

There was 79.04 fold purification of PHY1 and 114.75 fold of PHY2. Laboure *et al*., 1993 utilizing the similar columns reported 73 fold purification in maize. However, Nakano *et al*., 1999 reported 260.5 fold purification for PHY1 and 288.4 for PHY2 from wheat bran phytase purified through DEAE-TOYOPERAL, CM-TOYOPEARL and Sephacryl S-200 HR. This showed that different columns give different purification levels depending upon the method used.

The molecular mass of the purified phytase was estimated to be around 64 kDa and 62 kDa for PHY1 and PHY2, respectively. Other reports indicated molecular mass of phytase varying from 60-66 kDa (Brejnholt *et al*., 2011) and 66-68 kDa (Nakano *et al*., 1999) in wheat. However, Laboure *et al*., 1993 reported phytase as dimer of 38 kDa subunit purified from maize.

Two main types of plant phytases have been identified, acidic phytase with pH optimum around 5.0 and alkaline phytases with pH optimum around 8.0. The first group includes seeds of Soybean (Gibson and Ullah, 1988), maize (Laboure *et al*., 1993), barley (Greiner, 2000), wheat bran (Nakano *et al*., 1999) pollen of Lilium longiflorum (Lin *et al*., 1987) and Petunia hybrid (Jackson and Linskens, 1982). The second group includes seeds of Phaseolus vulgaris, Pisum sativum (Scott and Loewus, 1986) and pollen of Typha lattifolia (Hara *et al*., 1985). The phytases from wheat whole meal, PHY1 and PHY2 belong to the first group, since their optimum pH were 6.0 and 5.5, respectively.

The optimum temperature for PHY1 and PHY2 was 55°C. Different reports showed different optimum temperature ranging from 37-55°C for wheat phytase activity (Viveros *et al*., 2000; Lopez *et al*., 2003 and Okot-Kotber *et al*., 2003). Nakano *et al*., 1999 reported the optimum temperature for PHY1 as 45°C and that of PHY2 as 50°C.

The enzyme exhibited broad range of activity against various substrates. Other reports also indicated broad range of phytase activity with respect to different substrates (Konietzny *et al*., 1995; Nakano *et al*., 1999). PHY1 and PHY2 showed highest activity on ATP (5.6 fold) as compared to phytate as the substrate. The apparent Km of the wheat whole meal phytase for phytate is 123 µM. Km values for phytate of various phytases have been reported varying from 2.64-570 µM (Greiner, 1998; Konietzny *et al*., 1995; Laboure *et al*., 1999; Nagai and Funahashi, 1962)

Different metal ions were tested for their effect on phytase activity. Mg2+ and Ca2+ increased 1.2 fold activity of PHY1 and 2.45 and 3.15 fold activity of PHY2, while Nakano *et al*. 1999 reported inhibitory effect of Mg and Ca on both PHY1 and PHY2. There was strong inhibitory effect of Fe2+ and Zn2+ on both the phytases supporting earlier reports of by Nakano *et al*., (1999); Greiner, (2000); Nagai and Funahashi, (1962).

In this investigation, kinetic properties of phytases exhibited that wheat phytases are active at acidic conditions and showed specificity towards phytate. This showed that the phytase from wheat may have role in enhancing hydrolysis of phytate and release of micronutrients. The information is useful in utilization of wheat phytases in food industry for increasing bioavailability of calcium, iron, and zinc. Since plant phytases have more acceptance than microbial phytases, wheat phytase may be more useful in human nutrition and monogestric animals.

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