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PHYTASE FROM PEARL MILLET: ITS PARTIAL PURIFICATION AND CHARACTERIZATION

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SUMMARY

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is an important food and fodder crop, grown in arid and semi-arid tropical regions. It has great nutritional significance, however, the high amount of phytic acid is considered as antinutritional compound towards its use in food/feed for monogastric animals. Hydrolysis of phytate using phytase enzyme is a promising approach for reducing phytate content. In this study, a pearl millet genotype HC 20 with phytase activity of ~63.3 µg Pi/h/g was used for phytase extraction, partial purification and study of properties of enzyme. Phytase was partially purified by ammonium sulphate precipitation (30-80% saturation) upto 3.8 fold recovering 54.5 % of the activity. The purified preparation showed maximum activity at pH 5.0. Activities of the crude and ammonium sulphate fractions were 3589 µg Pi/h and 1958 µg Pi/h, respectively. The K_m of purified enzyme was 1.51 mM phytate. The purified preparation exhibited optimum activity at 50°C. The enzyme was stable up to 60°C for 30 min. The study revealed the properties of enzyme which might be helpful for its efficient application during food/feed processing for enhanced quality.

Key words : Phytase, phytate, partial purification, characterization, enzyme kinetics

Phytic acid is known as the major source of phosphorus in cereal grains, comprising 1-5% by weight in cereals, legumes, oil seeds and nuts (Vats and Banerjee 2004) and accounting for approximately 65–80% of the total seed phosphorus (Raboy *et al.* 2000, Loewus 2002, Kumar *et al.* 2017). Phytic acid is commonly known as phytate when complexed with positively charged metal ions. During the last several decades, investigators working in the field of nutrition have been focusing their attention on phytate (Kumar *et al.* 2015, Saxena *et al.* 2020).

Pearl millet [*Pennisetum glaucum(L)* R. Br] is grown in the arid and semi-arid tropical regions of Asia and Africa. The crop is grown primarily for grain production but is also valued for its fodder and poultry feed. Pearl millet is grown in areas with very limited rainfall, where other crops are very likely to fail. Therefore, pearl millet is a central component of the food security of the rural poors in dry areas (Arya *et al*., 2013). Pearl millet also contains high content of phytic acid (Kumar 2015). Therefore, reduction of phytate content during food/feed processing might be of immense importance for monogastric animals, which are unable to utilize the phytate-divalent cations complexes in pearl millet based food/feed. *In vitro* studies have shown that phytate–protein complexes are less likely to be digested by proteolytic enzymes (Deshpande and Damodaran 1989, Ravindran *et al.* 1995) and even digestive enzymes, such as pepsin, trypsin, chymotrypsin are inhibited by phytate (Singh and Krikorian 1982, Inagawa 1987). It is evident from the above that the effects of phytic acid are attributed to its ability to form complexes with positively charged minerals and other food components such as proteins, carbohydrates etc. Phytate is not utilized efficiently by monogastric animals due to lack of phytase enzyme. Phytate degrading enzyme, phytase have been studied intensively in recent years (Kumar *et al.* 2014, Kalsi *et al.* 2016, Kaur *et al.* 2017). Phytases are a subgroup of phosphatases with general preference for phytate, which is hydrolyzed in a stepwise manner generating phosphoric acid and myo-inositol phosphates (Kumar *et al.* 2013). Based on the specific consensus sequence, catalytic mechanism and three dimensional structures, so far phytases are therefore classified in four classes, i.e. histidine acid phosphatase (HAPhy) (Kumar *et al.*

2012), cysteine phytase (CPhy) (Kumar and Agrawal 2014), purple acid phosphatase (PAPhy) and beta-propeller phytase (BPPhy) (Kumar *et al.* 2017).

In view of these facts, reducing the phytate content in cereal grains is a desired goal for the genetic improvement of several crops. Several techniques have been made to reduce the phytate content in cereals viz. milling, soaking, sprouting, cooking, fermentation (Gupta *et al.* 2015), and addition of exogenous phytase to flour (Saxena *et al.* 2020). Although effective, these strategies are sometimes associated with negative impacts on crop yield and other parameters of agronomic performance (Raboy 2009). In this context, study of inherent phytase activity and its kinetic characterization from pearl millet might be of great significance for its efficient utilization for crop improvement and quality improvement during food/ feed processing.

MATERIALS AND METHODS

Pearl millet sample and Extraction of phytase enzyme

In present study, the pearl millet genotype HC 20 was procured from Bajra Section, Department of Genetics and Plant Breeding and used for phytase extraction, partial purification and study of properties of enzyme. The extraction of phytase from pearl millet flour was carried out according to Verma *et al.* (2011) with minor modifications. Briefly, 2 g flour was suspended in 10 ml of 0.2 M sodium acetate buffer (pH 5.4) for 2 h at 0-4°C. The contents were shaken occasionally. The homogenate was centrifuged at 10000 rpm for 20 min in a refrigerated centrifuge. The supernatant was carefully filtered through four layers of cheese cloth and used as crude enzyme preparation for determining enzyme activities. For preparing extract for purification, 40 g flour was suspended in 250 ml of buffer. Flour was suspended overnight. Other conditions were similar as mentioned earlier for preparing the extract.

Estimation of phytase activity

The activity of phytase and acid phosphatase were determined by measuring the increase in inorganic phosphate by the method of Lolas and Markakis (1977). Briefly, the reaction mixture contained 0.5 ml of 0.2 M sodium acetate buffer (pH 5.4), 0.2 ml of 10 mM sodium phytate and 0.2 ml of enzyme extract with a 0.9 ml total volume of the reaction mixture. Reaction was initiated with addition of sodium phytate. Incubation was done at 37°C for 20 h. Incubation period for determining the activity in partially purified enzyme preparation was 2 h. Reaction was terminated with addition of 1 ml of 20% (w/v) TCA. Contents were centrifuged at 5000 rpm for 5 min and 40 µl supernatant was collected for determining the concentration of Pi in accordance to procedure of Chen *et al.* (1956). The enzyme activity was expressed as µg Pi/h/g.

Protein estimation in enzyme extract

The estimation of protein concentration (mg) in enzyme preparation was carried out by the method of Bradford (1976). To 1 ml of suitably diluted protein sample, 5 ml of Bradford reagent was added and mixed well. Absorbance was recorded at 595 nm on a spectrophotometer. The amount of protein per ml of sample was calculated from a standard curve. Specific activity of enzyme was calculated as enzyme activity/ protein concentration.

Partial purification of phytase using $(NH_4)_2SO_4$ fractionation

As explained earlier, the crude extract was added with solid $(NH_4)_2SO_4$ slowly with constant stirring so as to bring the saturation to 30%. The stirring was continued for another few min after addition of total amount of $(NH_4)_2SO_4$ and the solution were kept in a refrigerator for at least 6 h for complete precipitation of proteins. The contents were centrifuged at 10000 rpm for 25 min. The precipitates were discarded as it had negligible activity of phytase. The resultant supernatant was brought to 80% saturation with solid $(NH_4)_2SO_4$. The precipitates were collected by centrifugation as before and dissolved in 15 ml of 0.2M sodium acetate buffer (pH 5.4). The resulting solution was dialyzed against the same buffer for 24 h. The dialysis buffer was changed thrice every 4 h and collected the dialyzed fraction. Precipitates, if any, were removed from dialyzed samples by centrifugation at 5000 rpm at 0-4°C. The supernatant recovered after centrifugation was used for characterizing the enzyme.

Kinetic characterization of phytase

The optimum pH value for phytase was determined by assaying enzyme activity using acetate buffer of pH values ranging from 3.5 to 5.5. The

enzyme activity was measured as mentioned earlier.

The optimum temperature for the enzyme was determined by measuring the enzyme activity at temperatures ranging from 20 to 60°C. Except the enzyme, all constituents of the reaction mixture were maintained at appropriate temperature in a water bath before starting the reaction. Enzyme activity was determined and thermostability of the partially purified enzyme was determined by measuring the residual enzyme activity after incubating 0.2 ml of the enzyme for 30 min at temperature ranging from 20 to 60 °C in a water bath.

For determination of K_m for sodium phytate as substrate, the activity of the partially purified enzyme preparation was measured using sodium phytate as the substrate with range of concentration varying from 0.11 to 2.25 mM. The K_m value was determined by using the Line Weaver-Burk reciprocal plot method.

Statistical analysis

The data were statistically analyzed in factorial CRD for calculating CD using software 'Statistical Package for Agriculture Scientists', OPSTAT (available online at www.hau.ernet.in). The data presented represents mean of replicates.

RESULTS AND DISCUSSION

Phytase activity and partial purification

Phytase purification is a significant step for determining its kinetic parameters and its efficient applications (Singh *et al.* 2011, Saxena *et al.* 2020, Singh *et al.* 2020). The phytase activity in pearl millet genotype HC 20 was obtained as 63.3 µg Pi/h/g. The acid phosphatase activity was determined as 10170 µg Pi/h/g and the ratio of Acid phosphatase to phytase activity was 161. This magnitude of activity was lower than that of the enzymes isolated from other cereals (Lolas and Markakis 1977, Greiner *et al.* 1998). The enzyme was partially purified from flour of pearl millet variety HC 20 by ammonium sulphate fractionation. The total supernatant recovered after centrifugation and filtration of the homogenate was 200 ml. It contained 450 mg protein and showed enzyme activity equivalent to 3589 µg Pi/h/0.2ml (Table 1). Thus specific activity of the enzyme in crude extract was 7.9 µg Pi/h/0.2ml**.** The total magnitude of activity was taken as 100% recovery for further calculations. The fraction obtained with 30-80 % saturation of $(NH_4)_2SO_4$ retained most of the enzyme activity. This fraction was dialyzed for 24 h against 0.2 M sodium acetate buffer (pH 5.4) and substantive activity (54.5%) was recovered in a final volume of 19 ml of the buffer. This primary purification step resulted in 3.8 fold purification of phytase from crude enzyme extract with specific activity of 30.5 µg Pi/h/0.2ml. This fraction was used for characterization of the enzyme. Ammonium sulphate up to 80% saturation has been used for precipitating the enzyme from barley, maize root and navy beans (Lolas and Markakis 1977, Hubel and Beck 1996, Greiner *et al.* 1998). Higher recovery of phytase yield during ammonium sulphate precipitation based purification was reported by Verma *et al.* (2011).

Kinetic characterization of phytase

The optimum temperature of the partially purified phytase was determined at specified temperature ranging between 20 and 60°C. It was revealed that the activity of phytase increased rapidly with increasing temperature from 20°C and reached to a maximum value of 331 ng Pi/h/0.2ml at 50°C (Fig. 1). The activity started declining gradually with rise in temperature beyond 50°C. At 60°C phytase lost about 36.8% of activity recorded at 50°C. Thus the partially purified enzyme showed maximum activity at 50°C. The thermal stability profile is presented as residual activity after pre incubation of purified preparation of phytase for 30 min at the specified temperature (Fig. 2). The partially purified enzyme

Table 1
Summary of partial purification of phytase by ammonium sulphate fractionation from flour of pearl millet variety HC 20

S. No.	Fraction	Volume (ml)	Total activity (µg Pi/h)	Protein (mg)	Specific activity (µg Pi/h/ mg protein)	Fold purification	Recovery (%)
1.	Crude Extract	200	3589	450	7.9	-	100
2.	Ammonium Sulphate fraction (30-80% saturation)	19	1958	64	30.5	3.8	54.5

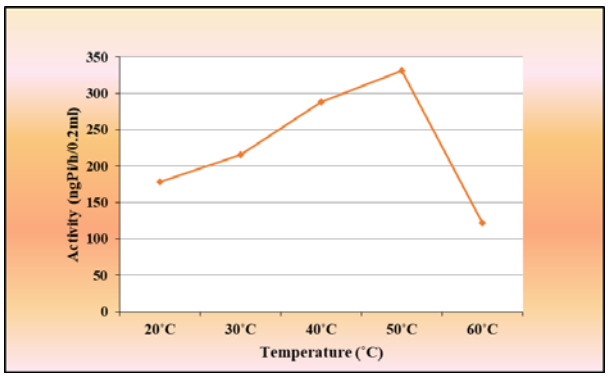

Fig. 1. Effect of temperature on the activity of partially purified phytase.

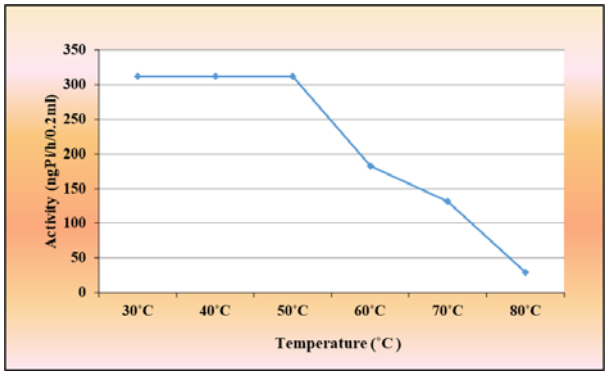

Fig. 2. Effect of temperature on the stability of partially purified phytase.

retained maximum activity when kept at a temperature ranging from 20 to 50°C, thereafter the activity declined sharply. At 80°C the residual enzyme activity was 10% of original activity. The optimum temperature for maximum phytase activity was reported as 55°C for the phytase isoenzymes from wheat flour (Verma *et al.* 2011) and barley seedlings (Greiner *et al.* 2000). The optimal temperature of phytase from faba bean, lentil and kidney bean was 50°C, while of pea phytase it was 45°C (Vashishth *et al.* 2017). Lower optimum temperature of 37°C has been reported for phytases from wheat bran (Nagai and Funahashi 1962), and 40°C for rice bean phytase (Belho *et al.* 2016). Pearl millet enzyme retained maximum activity when kept at a temperature ranging from 20 to 50°C, thereafter the activity declined sharply. At 80°C, 10% of residual activity was obtained.

For determining optimum pH, activity of the partially purified enzyme was assayed using sodium acetate buffers in pH range of 3.5 to 5.5. Results presented in the **Fig. 3** revealed that optimum pH for obtaining maximum activity of HC 20 pearl millet phytase was 5.0. The phytase activity increased gradually from 108 ng Pi/hr/0.2ml at pH 3.5 to 321 ng Pi/hr/0.2ml at pH 5.0. The activity started decreasing

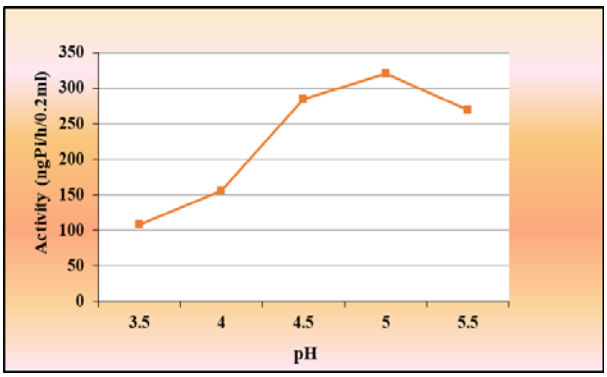

Fig. 3. Effect of pH on the activity of partially purified phytase.

rapidly thereafter with gradual increase in pH till 5.5. The optimum pH of pearl millet phytase was similar to wheat bran phytase (Nagai and Funahashi 1962), but lower than phytase 1 (pH 6.0) and phytase 2 (pH 5.5), from wheat flour (Verma *et al.* 2011) and barley seedlings phytase having (pH 6) (Greiner *et al.* 2000). Phytase purified from rice bean had optimum pH as 4.0 (Belho *et al.* 2016).

The data given in Fig. 4 represents the effect of varying concentration of sodium phytate in the range from 0.11 mM to 2.25 mM on activity of phytase. Activity of phytase increased with the increasing concentration of phytate until the concentration was raised to 1.375 mM and further increase in concentration of phytate had no effect on the initial velocity. Thus the enzyme showed a typical hyperbolic response with increasing concentration of substrate. The partially purified enzyme displayed Michaelis - Menten kinetics for phytate. The K_m value calculated from the Lineweaver-Burk plot was found as 1.51 mM sodium phytate (Fig. 5). This value was higher than the values reported for the enzyme purified from cereals, grains and pulses. The K_m value of 1.25 mM was reported for phytase from *Enterobacter cloacae* PSB-45 (Kalsi *et al.* 2016).

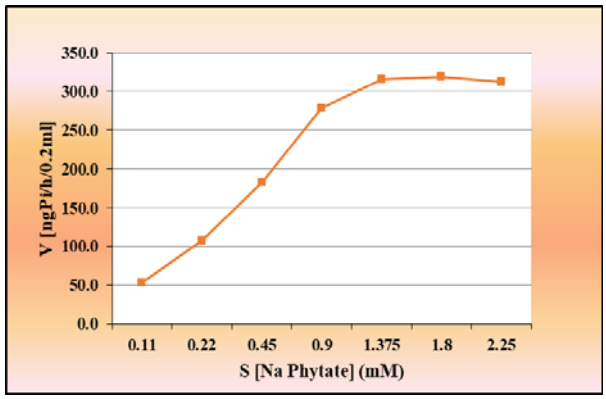

Fig. 4. Effect of concentration of sodium phytate on the activity of the partially purified phytase.

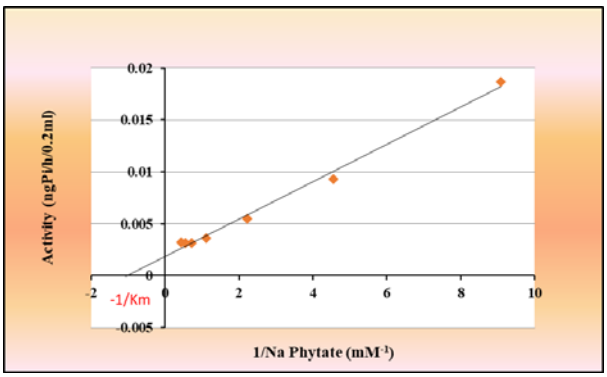

Fig. 5. Lineweaver-Burk Plot showing Km value for partially purified phytase as a function of sodium phytate.

CONCLUSION

The study revealed phytase activity from pearl millet flour with concomitant characterization of kinetic properties of phytase after its partial purification. Ammonium sulphate fractionation was successfully applied for recovery of more than half of enzyme activity after 3.8 fold purification. The kinetic properties of HC 20 pearl millet phytase are in accordance with reported phytases and these may be utilized for quality improvement of processing based pearl millet products.

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