Int.J.Curr.Microbiol.App.Sci (2021) *10*(07): 378-385

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 10 Number 07 (2021)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2021.1007.041

Purification of Phytase Enzyme from Four Selected Indian Wheat Varieties (*Triticum aestivum* L.)

Manish Kumar[1, 2] and Sushma[2]

[1]*Department of Biochemistry, Institute of Life Science, Bundelkhand University, Jhansi-284128, India*
[2]*Department of Biochemistry and Biochemical Engineering, Jacob Institute of Biotechnology and Bioengineering, Sam Higginbottom University of Agriculture, Technology and Sciences, Prayagraj-211007, India*

**Corresponding author*

Keywords

Phytase, ammonium sulphate precipitation, dialysis, ion exchange chromatography

Article Info

Accepted:
15 June 2021
Available Online:
10 July 2021

A B S T R A C T

A phytase (*myo*-inositol-hexakisphosphate phosphohydrolase) enzyme was purified and identified from wheat varieties during process. The four wheat varieties, selected on the basis of their higher phytase activity for investigation were DBW-17, HD-2894, HUW-234 and LOK-1. Among the four selected wheat varieties, LOK -1 variety reported the maximum phytase activity in study. The three steps *i.e.* ammonium sulphate precipitation, dialysis and ion exchange chromatography on DEAE-cellulose, Silica gel, Ceralite IR 120, Ceralite IR 400, Dowex 1X8 and Dowex 50X8 column were adopted for phytase partial purification process. Finally, phytase enzyme was purified about 55.18 fold with 17.39% recovery by Dowex 50X8 column from LOK-1 wheat variety in ion exchange chromatography step. Molecular weight of partially purified phytase enzyme of four wheat varieties was found to be monomeric protein as approximately 65kDa mass in SDS-PAGE electrophoresis (denatured phytase) as well as in Native-PAGE electrophoresis (non-denatured phytase) when compared to ladder markers lane. This can have exhibit maximal phytate degrading activity. This enzyme can have good efficiency for industrial applications.

Introduction

Phytase enzyme is instrumental in breakdown of phytic acid thereby releasing inorganic phosphates and myo-inositol as products. The released inorganic phosphate is of importance as it can be used as important mineral for growth and development of cattle, pig, fish and hen *etc*., whereas in soil, this mineral can be used as fertilizer. Further, phytase can be used in paper industries to produce ecofriendly and non toxic papers. Therefore, this enzyme

is of great use. During the last 30 years, phytases have attracted considerable attention from both scientists and entrepreneurs in the areas of nutrition, environmental protection and biotechnology. Phytases represent a subgroup of phosphomonoesterases that are capable of initiating the stepwise dephosphorylation of phytate [*myo*-inositol (1,2,3,4,5,6) hexakisphosphate], the most abundant inositol phosphate in nature. The ability of phytases to hydrolyse phytate is well understood from *in vitro* assays but their activity *in vivo* remains largely unknown. Therefore, some of the enzymes classified as phytases today may not be involved in phytate degradation *in vivo* but may have completely different functions (Greiner, 2006).Phytases are a diverse group of enzymes that encompass a range of sizes, structures and catalytic mechanisms. Based on the catalytic mechanism, phytases can be referred to as histidine acid phytases (HAPhy), ß-propeller phytases (BPPhy), cysteine phytases (CPhy) or purple acid phytases (PAPhy) (Greiner, 2006; Mullaney and Ullah, 2003). Depending on their pH optimum, phytases have been divided into acid and alkaline phytases. Based on the carbon in the *myo*-inositol ring of phytate at which dephosphorylation is initiated, phytases have been divided into 1/3-phytases (E.C. 3.1.3.8), 4/6-phytases (E.C. 3.1.3.26) and 5-phytases (E.C. 3.1.3.72). Today, all phytases used for animal feed application belong to the class of histidine acid phytases (Greiner and Konietzny, 2006). Activity of wheat phytases (EC 3.1.3.26) were first reported in wheat (Posternak and Posternak, 1929), where an aqueous extract of wheat bran was used to investigate optically active myo-inositol polyphosphate esters that were produced during the degradation of phytate (Collatz and Bailey, 1921).

Notably, the first phytase was reported in 1907 (Suzuki *et al.,* 1907). The first commercial phytase products were launched into the market in 1991. In 2005 the market volume was in the range of €150 million (Haefner *et al.,* 2005). Even if potential applications of phytase in food processing or the production of pharmaceutical were reported (Greiner and Konietzny, 2006), phytases have been mainly, if not solely, used as an animal feed additive in diets largely for swine (Selle and Ravindran, 2008) and poultry (Selle and Ravindran, 2007), and to some extent for fish (Debnath *et al.,* 2005). 'Ideal' phytases for animal feed applications should fulfil a series of quality criteria. They should be effective in releasing phytate phosphate in the digestive tract, stable. to inactivation by heat from feed processing and storage, as well as cost effective to produce. However, it is important to realise that no single phytase may ever be able to meet all the optimal needs for its commercial application. Thus, screening for phytases with more favourable properties for the commercial application, coupled with engineering phytases to optimise their catalytic and stability features is a rational approach to develop a phytase more suited to animal feed applications. Therefore, it would be of value to be able to predict the efficacy of a phytase in the animal from the current industry standard *in vitro* assay (Engelen *et al.,* 1994).

Materials and Methods

Four locally available wheat varieties were purchased from Alopibagh market, Prayagraj, India to go through the practical aspect of work, which are namely DBW-17, HD-2894, HUW-234 and LOK-1.

Estimation of protein content

0.5g of the wheat samples were homogenized with 10ml of sodium acetate buffer (0.1M, pH 5.0) with mortar and pestle. Centrifuged at 5000rpm for 10min and used the supernatant for protein estimation. Pipette out 0.1ml of

sample and make up volume 1ml with distilled water then added 5ml of mixture (Mixed 50 ml of 2% Sodium carbonate in 0.1N Sodium hydroxide and 1 ml of 0.5% Copper sulphate in 1% Sodium-potassium tartarate, just prior to use), mixed well and incubated at room temperature for 10min. After incubation, 0.5ml of 1N Folin-Ciocalteu's reagent added, again mixed well immediately and incubated at room temperature in dark for 30min. After 30min incubation, absorbance was recorded at 660nm (Lowry *et al.,* 1951).

Phytase assay

0.5g fresh samples of all wheat varieties were separately homogenized in 10ml of sodium acetate buffer (0.1M, pH 5.0). The homogenized samples were centrifuged at 12000g for 5min and supernatants were used for enzyme assay (Senna *et al.,* 2006). The assay mixture consisted of 350µl of sodium acetate buffer (0.1M, pH 5.0) and 100µl of sodium phytate (2mM). This mixture was preincubated for 10min at 40^{o}C and the enzymatic reactions were started by adding 100µl of the crude enzyme to preincubated assay mixture. After incubation at 40^{o}C for 30min, the liberated phosphate was measured by using the ammonium molybdate. For this, to the assay mixture, 1.5ml of a freshly prepared solution of acetone/5N H_2SO_4/10mM ammonium molybdate (2:1:1 v/v/w) and 100µl of 1.0M citric acid were added. Any cloudiness was removed by centrifugation to measurement of the absorbance at 355nm against blank as ammonium molybdate solution (Heinonen and Lahti, 1981).

Partial purification of phytase

The crude enzyme extract was used for an ammonium sulphate precipitation at 0-90% saturation. The precipitate was resuspended in 0.1 M sodium acetate buffer, pH 5.0 and dialyzed against the same diluted buffer.

The dialyzed 80% ammonium sulphate precipitation was loaded onto DEAE-cellulose, Silica gel, Ceralite IR 120, Ceralite IR 400, Dowex 1X8 and Dowex 50X8 columns (1 × 20 cm) equilibrated with 0.1 M sodium acetate buffer, pH 5.0. The column was washed with 50ml same buffer and maximum sample loading volume per run was 5ml and after eluting the fractions of sample. The fractions containing phytate-degrading activity as phytase enzyme.

Determination of molecular weight of partial purified phytase

Molecular weight determination of phytase enzyme is essential step of work followed by SDS - PAGE and Native - PAGE electro-phoresis. The molecular weight of single chain proteins can also be determined by this method (Laemmli, 1970). Native-PAGE is widely use checking protein purity determined by Davis (1964).

Results and Discussion

Purification of phytase showed in Table. 1, Table. 2, Table. 3 and Table. 4. The phytase enzyme sample was precipitated with ammonium sulphate (80% saturation), collected by centrifugation (5000 rpm, 5 min), and resuspended in sodium acetate buffer (0.1 M, pH 5.0) and further use for dialysis.

Ammonium sulphate was removed by dialysis from each sample against the same diluted sodium acetate buffer (0.1 M, pH 5.0) for 15 hrs then purified sample carried out to chromatography.

Table.1 Purification of DBW-17 wheat variety

Purification step	Total protein (mg)	Phytase activity (nM/min)	Specific activity (nM/min/mg)	Fold purification (fold)	Recovery (%)
Crude extract	128	633	4.94	1	100
Salt precipit.	107	565	5.28	1.07	89.26
Dialysis	89	486	5.46	1.1	76.78
DEAE cellulose	67	409	6.1	1.23	64.61
Silica gel	45	314	6.98	1.41	49.6
Ceralite IR 120	27	274	10.15	2.05	43.28
Ceralite IR 400	17	253	14.88	3.01	39.97
Dowex 1X8	8	170	21.25	4.3	26.85
Dowex 50WX8	1.12	93	83.03	16.8	14.69

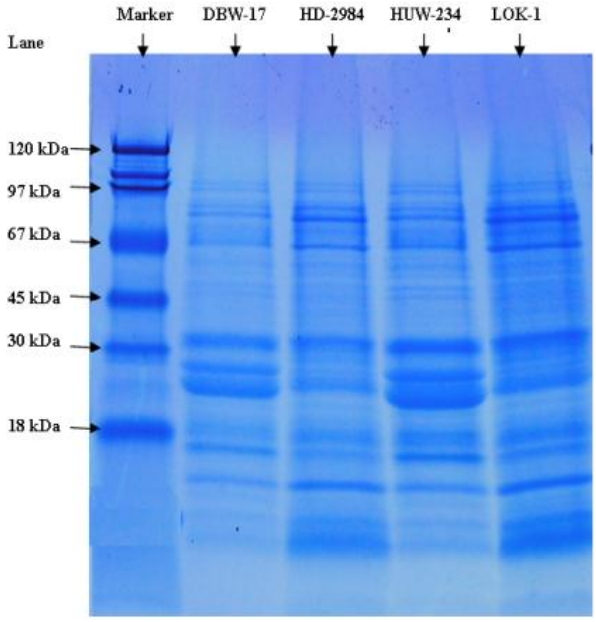
Table.2 Purification of HD-2894 wheat variety

Purification step	Total protein (mg)	Phytase activity (nM/min)	Specific activity (nM/min/mg)	Fold purification (fold)	Recovery (%)
Crude extract	108	641	5.93	1	100
Salt precipit.	90	572	6.35	1.07	89.23
Dialysis	70	493	7.04	1.1	76.91
DEAE cellulose	47	416	8.85	1.49	64.9
Silica gel	26	321	12.35	2.08	50.08
Ceralite IR 120	8	279	34.87	5.88	43.52
Ceralite IR 400	3	238	79.33	13.38	37.13
Dowex 1X8	0.88	217	246.59	41.58	33.85
Dowex 50WX8	0.52	140	269.23	45.4	21.84

Table.3 Purification of HUW-234 wheat variety

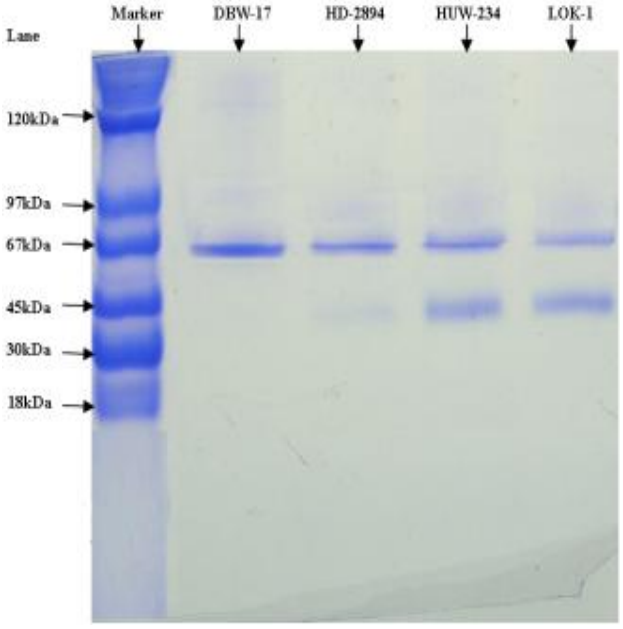
Purification step	Total protein (mg)	Phytase activity (nM/min)	Specific activity (nM/min/mg)	Fold purification (fold)	Recovery (%)
Crude extract	102	623	6.1	1	100
Salt precipit	90	590	6.55	1.07	94.7
Dialysis	75	505	6.73	1.1	81.05
DEAE cellulose	57	430	7.54	1.24	69.02
Silica gel	34	353	10.38	1.7	56.66
Ceralite IR 120	12	258	21.5	3.51	41.41
Ceralite IR 400	10	217	21.7	3.56	34.83
Dowex 1X8	4	197	49	8.03	31.62
Dowex 50WX8	0.9	117	130	21.31	18.78

Fig.1 Protein profile of selected four wheat varieties



{Lane 1 - Marker (120kDa - β-galactosidase; 97kDa – Phosphorylase B; 67kDa – Bovine albumin; 45kDa – Ovalbumin; 30kDa – Carbonic anhydrase; 18kDa – Myoglobin); Lane 2 – Protein extract from DBW-17; Lane 3 - Protein extract from HD-2894; Lane 4 - Protein extract from HUW 234; Lane 5 – Protein extract from LOK 1**}**

Fig.2 SDS-PAGE of selected four wheat varieties

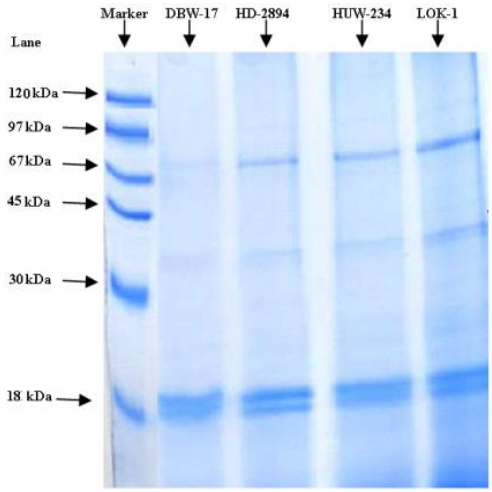


{Lane 1- Marker (120kDa - β-galactosidase; 97kDa – Phosphorylase B; 67kDa – Bovine albumin; 45kDa – Ovalbumin; 30kDa – Carbonic anhydrase; 18kDa – Myoglobin); Lane 2 – enzyme extract from DBW-17; Lane 3- enzyme extract from HD-2894; Lane 4 – enzyme extract from HUW 234; Lane 5 – enzyme extract from LOK-1**}**

Table.4 Purification of LOK-1 wheat variety

Purification step	Total protein (mg)	Phytase activity (nM/min)	Specific activity (nM/min/mg)	Fold purification (fold)	Recovery (%)
Crude extract	130	644	4.95	1	100
Salt precipit	110	576	5.24	1.05	89.44
Dialysis	74	497	6.72	1.36	77.17
DEAE cellulose	51	422	8.27	1.67	65.52
Silica gel	29	345	11.90	2.40	53.57
Ceralite IR 120	7	250	35.71	7.21	38.81
Ceralite IR 400	2	209	104.5	21.11	32.45
Dowex 1X8	1.1	188	170.9	34.52	29.19
Dowex 50WX8	0.41	112	273.17	55.18	17.39

Fig.3 Native-PAGE of selected four wheat varieties



{Lane 1- Marker (120kDa - β-galactosidase; 97kDa – Phosphorylase B; 67kDa – Bovine albumin; 45kDa – Ovalbumin; 30kDa – Carbonic anhydrase; 18kDa – Myoglobin); Lane 2 – enzyme extract from DBW-17; Lane 3 - enzyme extract from HD-2894; Lane 4 – enzyme extract from HUW 234; Lane 5 – enzyme extract from LOK-1**}**

In case of DEAE-Cellulose column, LOK-1 wheat variety reported 1.67-fold purification with 65.52% recovery followed by DBW-17 (1.23-fold; 64.61% recovery), HD-2894 (1.49-fold; 64.9% recovery) and HUW-234 (1.24-fold; 69.02% recovery). Scott and Loewus (1986) reported that DEAE-Cellulose have 0.54 U enzyme activity and specific activity 0.066 U/mg but in case of Dowex 50Wx8 column, LOK-1 wheat variety reported 55.18-fold purification with 17.39% recovery followed by DBW-17 (16.8-fold; 14.69% recovery), HD-2894 (45.4-fold; 21.84% recovery) and HUW-234 (21.31-fold; 18.78% recovery). The molecular weight of the purified phytase was determined by SDS-PAGE with 10% separating gel. Protein markers (120kDa- β-galactosidase; 97kDa – Phosphorylase B; 67kDa – Bovine Albumin; 45kDa – Ovalbumin; 30kDa – Carbonic Anhydrase; 18kDa - Myoglobin) were used in lane-1 and lane 2 to 5 defined phytase

molecular weight 65kDa isolated from DBW-17, HD-2894, HUW-234 and LOK-1, respectively. The molecular weight of phytase bands from four selected wheat variety was 65kDa in partially purified extract as shown in Fig. 2 (SDS-PAGE) and Fig. 3 (Native-PAGE). Similarly, Bohn *et al.*, (2007) reported that the molecular weight of phytase from wheat crude extract of wheat was 65kDa while lower molecular weight 47kDa was reported by Lim and Tate (1973) and 66kDa to 68kDa by Nakano *et al.*, (1999) in wheat bran. Protein content of four selected wheat varieties by SDS-PAGE is shown in Fig. 1.

On basis of the results obtained in the present study, it seems that among the four wheat varieties (DBW-17, HD-2894, HUW-234 and LOK-1), LOK-1 variety have maximum specific activity (273.17 nM/min/g) with 55.18-fold purification and 17.39% recovery during 8-step purification compared to DBW-17, HD-2894 and HUW-234 with 65 kDa molecular mass of wheat phytase determined by SDS-PAGE. Application of phytase in food industry seems to be a gifted approach nutritionally and economically.

Acknowledgement

Authos are thankful to Head of Department of Biochemistry and Biochemical Engineering and Dean of Jacob Institute of Biotechnology & Bioengineering, Sam Higginbottom University of Agriculture Technology and Sciences, Prayagraj for providing all necessary facilities to conduct the experiment in research laboratory.

References

Bohn Lisbeth, Meyer Anne S. and Rasmussen Søren K. 2008. Phytate: impact on environment and human nutrition- a challenge for molecular breeding. *Journal of Zhejiang University science B*, 9(3): 165-191.

Collatz, F. and Bailey, C. 1921. The activity of phytase as determined by the specific conductivity of phytin-phytase solutions. *Journal of Industrial and Engineering Chemistry*, 13(4): 317-321.

Davis, B. J. 1964. Disc electrophoresis-II: Method and application to human serum proteins. *Annals of the New York Academy of Sciences*, 121: 404-427.

Debnath, D., Sahu, N. P., Pal, A. K., Baruah, K., Yengkokpam, S. and Mukherjee, S. C. 2005a. Present scenario and future prospects of phytase in aquafeed – review. *Asian-Australian Journal of Animal Science*. 18: 1800-1812.

Engelen, A. J., van der Heeft, F. C., Randsdorp, P. H. G. and Smit, E. L. C. 1994. Simple and rapid determination of phytase activity. *Journal of AOAC International*. 77: 760-764.

Greiner, R. 2006. Phytate-degrading enzymes: Regulation of synthesis in microorganisms and plants. In: Turner, B.L., Richardson, A.E. and Mullaney, E. J. (eds.) *Inositol Phosphates: Linking Agriculture and Environment*, CAB International, London, England. pp. 78-96.

Greiner, R. and Konietzny, U. 2006. Phytase for food applications. *Food Technology and Biotechnology*. 44: 125-140.

Haefner, S., Knietsch, A., Scholten, E., Braun, J., Lohscheidt, M. and Zelder, O. 2005. Biotechnological production and applications of phytases. *Applied Microbiology and Biotechnology*. 68: 588-597.

Heinonen, J. K. and Lahti, R. J. 1981. A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic phosphatase. *Analytical Biochemistry*, 113(2): 313-

317.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4.*Nature*, 227: 680-685.

Lim, P. E. and Tate, M. E. 1973. Phytases: 2-Properties of phytase fractions F_1 and F_2 from wheat bran and myo-inositol phosphates produced by fraction F_2. *Biochimicaet Biophysica Acta* , 302(2): 316-328.

Lowry, O. H., Rosebrough, N. J., Farr, A. l. and Randall, R. J. 1951.Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*,193(1): 265-275.

Mullaney, E. J. and Ullah, A. H. J. 2003. The term phytase comprises several different classes of enzymes. *Biochemical and Biophysical Research Communications.* 312: 179-184.

Nakano, T., Joh, T. T., Okumoto, E. and Hayakama, T. 1999. Purification and characterization of phytase from bran of *Triticum aestivum* L. cv. *Nourin #61Food Science and Technology Research*, 5(1): 18-23.

Posternak, S. and Posternak, T. 1929. Sur la configuration de l'inosite inactive. *Helvetica Chimica Acta*, 12(1): 1165–1181.

Scott, J. J. and Loewus, F. A. 1986. A calcium-activated phytase from pollen of *Lillium Longiflorum.Plant physiology*, 82(1): 333-335.

Selle, P.H. and Ravindran, V. 2007. Microbial phytase in poultry nutrition. *Animal Feed Science and Technology*. 135: 1-41.

Selle, P. H. and Ravindran, V. 2008. Phytate-degrading enzymes in pig nutrition. *Livestock Science.* 115: 99-122.

Senna, R., Simonina, V., Silva-Neto, M. A. C. and Fialho, E. 2006. Induction of acid phosphatase activity during germination of maize (*Zea mays*) seeds *Plant Physiology and Biochemistry*, 44(7-9): 467–473.

Suzuki, U., Yoshimura, K. and Takaishi, M. 1907. Über ein enzym 'Phytase' das anhydro -oxy- methylen diphospho-rsaure spalter. *Journal of the College of Agriculture*, 7(5): 03–12

How to cite this article:

Manish Kumar and Sushma. 2021. Purification of Phytase Enzyme from Four Selected Indian Wheat Varieties (*Triticum aestivum* L.). *Int.J.Curr.Microbiol.App.Sci.* 10(07): 378-385.
doi: https://doi.org/10.20546/ijcmas.2021.1007.041