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Purification and characterization of phytase from two enteric bacteria isolated from cow dung

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Abstract

Phytase-producing *Klebsiella* and *Shigella* sp. were isolated from environmental samples based on their ability to degrade phytate and the enzymes were purified and characterized. The enzymes from both sources were intracellular monomeric proteins with the molecular mass of about 40kDa. The enzymes were active in the pH range of 2.0 to 7.5 with pH optima at 5.5. The optimum in vitro temperatures of the enzymes from *Shigella* and *Klebsiella* were 60°C and 55°C respectively. The enzyme from *Shigella* retained about 80% activity up to 75°C. Due to their relatively high specific activity, substrate specificity, good pH profile and thermostability, the enzymes could be interesting candidate for agricultural and feed application.

INTRODUCTION

Phytic acid (myo-inositol 1,2,3,4,5,6 hexakisphosphate) is the main storage form of phosphorus in cereals, legumes, and oilseed crops [13]. Although phytic acid serves as the major source of energy and phosphorus for seed germination, the bound phosphorus is poorly available to monogastric animals. Phytic acid also acts as an antinutritional agent in several ways as it interacts with other food ingredients [4]. Phytase (myo-inositol hexakisphosphate phosphohydrolase) is the enzyme that catalyses the hydrolysis of phytic acid to inorganic monophosphate and lower myo-inositol phosphates, and in some cases to free myo-inositol [5, 16].

The ruminants digest phytic acid through the action of phytases produced by the anaerobic gut fungi and bacteria present in their ruminal microflora. However, monogastric animals such as pigs, poultry and fishes utilize phytate phosphorus poorly because they are deficient in gastrointestinal tract- phytases and therefore an inorganic, non-renewable and expensive mineral supplement is used in diets for swine, poultry and fishes to meet their nutritional requirement of phosphorus. The unutilized phytate phosphorus from plant feeds is excreted as an environmental pollutant in areas of intensive live-stock production [10]. Excessive phosphorus in soil runs off to lakes and the sea, causing eutrophication and also stimulating growth ofvplanktonic vegetation such as algal blooms and aquatic blooms [19, 11] that may produce neurotoxins, injurious to human beings [9, 10]. Therefore, the enzymatic hydrolysis of phytic acid into less-phosphorylated myo-inositol derivatives in the intestine of the monogastric animals is desirable. Attempts to enzymatically hydrolyze phytic acid have been made to improve the nutritional value of feed and to decrease the amount of phosphorus excreted by animals [9, 6]. When used as additives in feed of monogastric animals, phytases not only enhance utilization of phosphate and reduce phosphate output in manures but also increase mineral uptake [3, 19]. Studies are also going on the utilization of phytases in food and industrial purposes [11].

In recent years, bacterial phytases have been isolated, characterized and proposed as potential tools in biotechnology. The highest frequency of phytase-like proteins has been reported in members of the gamma-proteobacteria group. Pure culture studies of ruminal bacteria have demonstrated phytate-degrading activity in various strains belonging to the Enterobacteriaeceae family, such as in *Enterobacter, E.coli, Klebsiella, Citrobacter, Yersinia* etc. Here, we report a comparative study on isolation, purification and characterization of phytases from two enteric bacteria (*Klebsiella* sp. RS4 and *Shigella* sp. W3).

MATERIALS AND METHODS

A. Isolation and selection of phytase-producing bacteria

Bacterial strains were isolated from cow dung. Isolation of pure cultures from different collected samples was done by the simple serial dilution method followed by plating onto phytase screening medium (PSM) at 37°C. Phytase-producing bacteria were screened on the phytase-producing medium (PPM) as described by Kerovuo et al. [5]. On the basis of qualitative and quantitative enzymatic analysis, two potent phytase- producing strains were selected for further studies. Those strains were characterized on the morphological, biochemical, cultural and molecular level.

B. Optimization of phytase production under shake flask cultures

Pure cultures were isolated and screened for phytase production in the PPM at 37°C under shake flask culture and analyzed for intracellular and extracellular phytase activity. The effect of different production parameters, i.e. inoculum age, pH, temperature, simple and complex carbon sources, nitrogen sources, on enzyme production was studied.

C. Phytase activity assays

Enzyme assays were preformed as described by Shimizu [10]. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 µmol of Pi per min under assay conditions. The specific activity was expressed in units of enzyme activity per milligram of protein.

D. Enzyme purification

Phytase enzyme was purified from the phytase-producing bacterial strains by the methods based on solubility as well as standard chromatographic techniques. A combination of two-step ammonium sulfate precipitation, gel filtration and ion exchange chromatographic methods was found to be the best purification method. Unless otherwise indicated, all operations during extraction, separation and purification of the enzyme were carried out at 4°C.

E. Protein estimation and SDS-PAGE analysis

The amount of protein was determined by the method of Bradford [12] using Bovine Serum Albumin as standard. The enzyme fractions from various steps of purification were separated by Sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE), by the method of Laemmli [17].

F. Characterization of the enzyme

The kinetic properties such as K_m, pH optima, temperature optima, thermostability and substrate specificity of the purified enzymes were also compared.

RESULTS AND DISCUSSION

Six strains that can solubilize sodium phytate and form peripheral halo zone on phytase screening agar medium around colonies were isolated form cow dung. By plate screening, RS4 and W3 were found to be the most potent strains as phytase producers. Quantitative screening methods done by phytase assay also supported the same fact. The phytase activity assay was carried out using media supernatant as well as sonicated fractions showing that phytase production in both the isolated strains is intracellular. RS4 produced maximum amount of phytase after 24h of incubation, while W3 showed maximum

phytase activity at 72h (Fig.1a). 16S ribosomal DNA analysis revealed that RS4 was a representative of *Klebsiella* sp. showing close sequence homology to *Klebsiella pneumoniae* and W3 was a representative of *Shigella* sp. (GenBank accession number FR745402 and FR682761 respectively). Morphological and biochemical characterization were also done for both the strains. For optimization of culture condition, the parameters tested were carbon sources such as 1% glucose, maltose, sucrose, mannose, lactose, galactose or starch and 0.5% glucose + 0.5% mannose and nitrogen sources, both organic and inorganic, such as ammonium sulphate, sodium nitrate, ammonium nitrate, peptone, tryptone, casein, beef extract and yeast extract. Result of the experiments showed the best carbon source for both the strains was glucose while in presence of ammonium sulphate they showed highest phytase activity (Fig.1b & c).

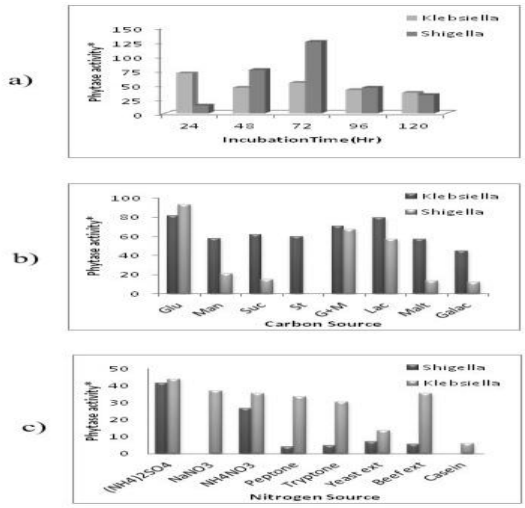

Fig. 1. Optimization of phytase production for both *Klebsiella* sp. RS4 and *Shigella* sp W3 with respect to (a) Incubation time (b) Carbon sources (c) Nitrogen sources

The *Klebsiella* phytase was purified 101 fold from the cell extract with specific activity of 598 U mg-1 (Table 2) for sodium phytate hydrolysis and the 133 fold purified enzyme from *Shigella* sp. W3 had specific activity of 780 U mg-1 protein (Table 1).

Table 1. Purification of phytase from *Shigella* sp.W3

Purification steps	Total Proteins (mg)	Total hytase Activity (U*)	Sp. Activity#	Fold purification	% Yield
Crude	19.2	112.00	5.83	1	100
(NH4)2SO4 30-70%	4.4	76.00	17.27	2.96	67.80
CM cellulose	0.40	65.00	162.5	27.87	58.0
Biogel P-100	0.06	46.80	780	133.7	41.0

*1U= 1µmol of Pi released min-1under assay conditions; # U mg-1protein

Both the purified enzymes gave a single protein band on SDS-PAGE (Fig. 2), indicating that they were probably single chain proteins or homomultimeric proteins of apparent molecular mass of about 45kDa and 43 kDa, respectively.

Phytases from both *Klebsiella* sp. RS4 and *Shigella* sp. W3 was found to have pH optima at pH 5.5 when assayed at 37°C (Fig.3b).

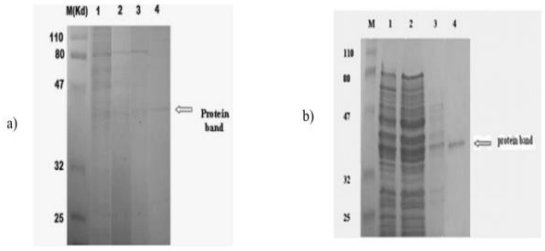

Fig. 2. SDS-PAGE analysis of purification of phytase enzyme from (a) *Klebsiella* sp. RS4 and (b) *Shigella* sp. W3. (a) Lane M- Protein molecular weight marker, Lane 2- Crude extract, Lane 3- 30-70% ammonium sulphate fraction, Lane 4- DEAE Sephacel eluate, Lane 4-CM Cellulose eluate (b) Lane M-Protein molecular weight marker, Lane 2-Crude extract, Lane 3-30-70% ammonium sulphate fraction, Lane 4- CM Cellulose eluate, Lane 4-P100 eluate.

Table 2. Purification of phytase from *Klebsiella* sp. RS4

Purification steps	Total Proteins (mg)	Total hytase Activity (U*)	Sp. Activity#	Fold purification	% Yield
Crude	19.89	117.00	5.88	1	100
(NH4)2SO4 30-70%	8.26	82.00	9.93	1.69	70.09
CM cellulose	0.41	45.60	111.22	19.01	38.97
Biogel P-100	0.057	34.10	598.25	101.74	29.14

*1U= 1µmol of Pi released min-1under assay conditions; # U mg-1protein

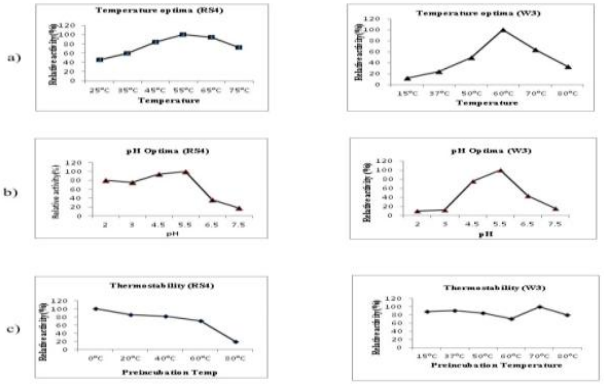

Fig. 3 Effect of pH and temperature on activity of purified phytases. (a) Effect of pH on phytase activity. The effect of pH was determined in 100 mM glycine (pH 2.5 & 3.5), 100 mM sodium acetate (pH 4.5 & pH 5.5), 100 mM phosphate (pH 6.5) Tris-Hcl (pH 7.5) buffer at 37°C (b) Profile of phytase activity at optimum pH and at temperature ranging from 20° to 80°C (c) Effect of temperature on stability of phytase. Purified enzyme preparation was incubated at indicated temperature for 30 min and enzyme activity was determined under standard conditions as described in Materials and method section.

Previously reported pH optima for phytases varied from 2.2 to 8.0 [2, 7]. Most microbial phytases and especially those of fungal origin have pH optima between 4.5 and 5.6. In contrast to most fungal phytases, *A. fumigatus* phytase has activity over a broad pH range (i.e., at least 80% of maximum activity between pH 4.0 and 7.3). Some bacterial phytases and especially those from *Bacillus* have pH optima at 6.5 - 7.5 [5, 20]. The pH optima of plant seed phytases range from 4.0 to

7.5, but most fall between 4.0 and 5.6. Two alkaline plant phytases having pH optima around 8.0 have been described from legume seeds [7] and lily pollen [hara]. Optimal temperatures of most phytases vary from 37 to 77°C [1]. Phytases from both *Klebsiella sp.* RS4 and *Shigella sp.* W3 fall into this range with their temperature optima being 55°C and 60°C respectively (Fig.3a). When preincubated at temperatures 20 to 80°C for 30 min, *Shigella* phytase was stable even at 80°C retaining almost 80% of its activity, while *Klebsiella* phytase lost its activity above 60°C (Fig.3c). This was considered to be relatively stable compared to most bacterial phytases. However, a phytase from *A. fumigatus* was found to be more thermostable, with only 10% loss in activity was found when it was exposed at 100°C for 20 minutes [pasamontes]. Phytases can be divided into two classes based on specificity: i.e., those with broad substrate specificity (e.g., *Aspergillus fumigatus*, *Emericella nidulans* and *Myceliophthora thermophila*) and those with rather high specificity for phytic acid (e.g., *A. niger*, *A. terreus* and *E. coli*) [12, 15]. Most belong to the first group, as did *Klebsiella* sp. RS4 phytase, because it exhibited relatively high activity toward a wide spectrum of substrates (Fig.4). Even substrates such as ADP, ATP, p-nitrophenyl phosphate, fructose 6-phosphate and glucose 6-phosphate that are not structurally similar to phytic acid were hydrolysed by this enzyme. However, phytase from *Shigella sp.* W3 differed from the former in having strict specificity for phytic acid and least activity with other phosphorylated substrates (Fig.4).

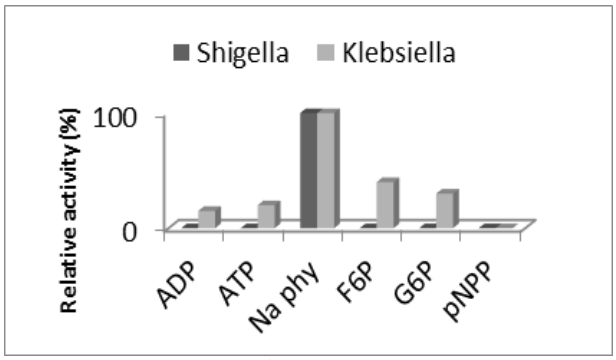

Fig. 4. Relative activity of *Klebsiella* sp. RS4 and *Shigella* sp. W3 phytases towards different substrates.

Phytases are of great interest for biotechnological applications, in particular for the reduction of phytate content in feed and food. The enzymes from *Klebsiella* sp. RS4 and *Shigella* sp. W3 have shown relatively high specific activity, substrate specificity, good pH profile, proteases insensitivity, thermostability. The superior biochemical properties suggest that the enzymes have a great potential as commercial phytase sources.

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