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Purification, Characterization of Phytase Enzyme from *Lactobacillus acidophilus* Bacteria and Determination of It's Some Kinetic Properties

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Phytases (myo-inositol hexakisphosphatephos phohydrolase, EC 3.1.3.8) catalyze the release of phosphate from phytates. Many of the cereal grains, legumes and oilseeds store phosphorus in phytate form. Phytases are used in many areas such as in food industry, in preparation of myo-inositol phosphates, in the paper industry and in soil improvement. In this study, isolation and characterization of phytase enzyme from *Lactobacillus* spp. (ATCC) strain was studied. Phytase production from bacterial strains was determined by zone production formed around colonies after 48 hours of incubation at 30 °C in MRS medium. The phytase enzyme that was partially purified by precipitation of ammonium sulphate from *Lactobacillus acidophilus* bacteria extracellularly was put into liquid culture medium, and its optimum pH and optimum temperature values were measured. Optimum activity of the enzyme derived from *Lactobacillus acidophilus* bacterium was at 30 °C and pH 5.0. It was observed that *Lactobacillus acidophilus*'s extracellular enzyme maintains its 90% of activity at 10-100 °C for 120 minutes. Effects of certain metal ions on activity of phytase enzyme derived from *Lactobacillus acidophilus* were also investigated. Of these, $CuCl_2$, $MnCl_2$ and $CoCl_2$ inhibited enzyme activity significantly. And, $FeCl_2$ has increased enzyme activity by 164%. Based on these results, the phytase enzyme of *Lactobacillus acidophilus* is considered suitable for use in many industrial areas, in fertilizer and food industries in particular, due to its thermal stability and resistance against metal ions.

Key words: Purification, Characterization, Phytase, *Lactobacillus acidophilus*.

Phytic acid is myo-inositol 1,2,3,4,5,6-hexa-dihydrogen phosphate[1]. A mixture of potassium, magnesium and calcium salts of phytic acid is defined as phytate. Phytic acid is the main phosphorus source in cereals, legumes and oilseeds. Phytic acid molecule has a high level of phosphorus content (28.2%) and has the potential to make chelates with minerals[2,3]. They form insoluble complex salts by these metal cations (Zn^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+} and Fe^{2+}), and form compounds that have gradually decreasing insolubility again in that order. And as a result of this, they reduce the digestibility of protein and amino acids[4,5].

Phytases (myo-inositol hexakis phosphate phosphohydrolas, EC 3.1.3.8) are enzymes that catalyze hydrolysis reaction in transformation of phytic acid (myo-inositol hexaphosphate) to myo-inositol phosphate, inorganic monophosphate and free myo-inositol[6]. Phytase enzyme was first found in rice bran[7]and in

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the blood of calves[8]. Then the presence of phytase enzyme in plants, yeast, fungi and microorganisms was identified. Phytases are used in many fields such as in food and feed industry, in the preparation of myo-inositol phosphates, in the paper industry, in soil improvement and in removing environmental contamination.

Today, bacterial phytases have more alternative uses than other phytase sources, due to their features such as commercial substrate specificity, resistance against metal ions, thermal stability, proteolysis-resistance and high catalytic activity[9]. Phytase enzyme was purified and characterized from several microbial sources, such as *Pseudomanas* spp.[10], *Bacillus subtilis*[11], *B. subtattoilis*[12], *B. amyloliquefaciens*[13], *Eschericia coli*[14], *Klebsiella aerogenes*[15], *K. terrigena*[16], *K. oxytoca*[17], *Enterobacter* spp.[18].

Lactic acid bacteria (LAB) are gram-positive, facultative anaerobic, catalase-negative, immobile (with one or two exceptions), cytochrome-free bacteria that do not produce spores except *Sporolactobacillusinulinus*, and produce lactic acid as an end product during carbohydrate fermentation. These bacteria are almost never present in water and soil, however, their various genus and species can be seen in milk, dairy products, dairy farms, plants, plant waste, and intestinal systems of humans, animals and other living organisms; and these bacteria are non-toxic[19]. In our study, *L. acidophilus*, one of the lactic acid bacteria was used. *L. acidophilus* bacteria are not toxic and used in the food industry quite safely.

The aim of this study was to produce phytase enzyme extracellularly from *L. acidophilus* and study its certain kinetic properties.

MATERIALS AND METHODS

Microorganism strains and medium used

The lactic acid bacteria *Lactobacillus acidophilus* used in the study are provided by the University of Ataturk, Department of Food Engineering, Laboratory of Microbiology. Bacteria seeded into MRS agar first, and activated by incubating for 48 hours at 26 to 30 °C. Later, a sample taken from a single colony was added to MRS Broth and allowed to incubate for 48 hours again. Then extracellularly produced homogeneous enzyme solution was used in the enzyme studies. During this time, liquid cultures were allowed to stand in the refrigerator (+4 °C).

Ammonium Sulphate Precipitation for Phytase Enzyme

After centrifugation of bacteria broth at +4 °C at 9000 xg for 10 minutes, supernatant and the precipitate was separated. The precipitate was discarded after centrifugation and the supernatant fraction was subjected to ammonium sulfate precipitation. In order to determine the precipitation range of phytase enzyme, ammonium sulphate precipitation was performed at 0-20%, 20-40%, 40-60% and 60-80% saturations. Precipitates were dissolved in small amount of 0.1 M Tris-HCl buffer (pH 5.5), and phytase activity was determined both in the precipitate and supernatant[20]. Active fractions were dialyzed against the same buffer. Active fractions were pooled and allowed to stand at 4 °C.

Measuring enzyme activity

Enzyme activity was determined by using Na-phytate. In short, 0.1 ml enzyme solution and 250 µL Na-fitate were mixed in a vortex and incubated for 10 minutes at 37 °C. Then, the reaction was stopped by adding 500 µL 10%TCA to the medium, and after incubating for 5 mins at 90 °C, a 500 µL coloring solution was added and allowed to stand for 15 minutes, then it was centrifuged for 5 minutes at 3000 xg. After centrifugation, absorbant change in the samples was measured spectrophotometrically (PG Instrument T80 Spectrophotometer) at 700 nm against blind sample.

Determining Optimum pH Value of Enzyme

To determine the optimum pH of the phytase enzyme, Sodium Acetate (pH 2.0-6.0), Tris (pH 7.0-9.0), and Carbonate (pH 10-11) buffers were used to prepare substrate solutions that contain 2 mM sodium phytate at different pH values (pH 2.0-11). And, in order to determine activity, 100 µL enzyme and 250 µL substrate were mixed in a vortex and let it reacted for 10 minutes in a water bath at 37 °C. To stop the reaction, 500 µL 10% TCA was added. After removing tubes from the water bath, 500 µL coloring solution was added, allowed to stand for 15 minutes, and centrifuged for 5 minutes at 3200 rpm. After centrifugation, samples were measured in a spectrophotometer at 700 nm against blank.

Determining Optimum pH Value of Enzyme

To determine the optimum pH of the pure phytase enzyme in the range of pH 2.0-11.0, different buffers (Sodium Acetate (pH 2.0-6.0), Tris

(pH 7.0-9.0), and Carbonate (pH 10-11)) were used and optimum pH-value for Na-phytate substrate was determined spectrophotometrically by using the above-mentioned standard method.

Determining Stable pH Value of Enzyme

Na-phytate substrate was used in the range of pH 2-11 for activity measurement, in order to determine pH stability of the phytase enzyme purified from *Lactobacillus acidophilus*. For this purpose, the buffer solutions mentioned above and enzyme solutions that were adjusted for each pH values were put into the reaction medium and enzyme activity was determined for 10 hours at certain intervals and the pH stability of the enzyme was determined.

Determining Optimum Temperature Value of Enzyme

For determination of optimum temperature of enzyme activity, the above-mentioned standard activity assay was used for activity measurements at 10 °C intervals ranging from 10 to 100 °C.

Determining Stable Temperature Value of Enzyme

In order to determine temperature stability of the phytase enzyme purified from *Lactobacillus acidophilus*, the reaction mixture was allowed to stand at temperature values ranging from 10 to 100 °C, and activity measurement was performed at every 15 minutes for 2 hours.

Determining protein content

Bradford method was used to determine the protein amount in the enzyme solutions obtained via purification procedures. This method is based on reading the change in absorbance of the amplitude of the complex formed by Coomassie Brilliant Blue G-250 with proteins, in acidic medium at 595 nm. The sensitivity of this method is 1 to 100 µg[21].

Determining the Effects of Certain Metal Ions on Enzyme Activity

Effects of certain metal ions on enzyme activity were investigated. For this purpose, effects of certain metal ions such as Cu^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+} and Zn^{2+} on enzyme activity at 0.5 and 1 mM concentrations were investigated[20].

RESULTS AND DISCUSSION

In this study, phytase enzyme of *Lactobacillus acidophilus*, which is a LAB that have an industrial significance, was purified partially and its certain kinetic properties were studied.

Lactobacillus acidophilus is forced to produce phytase enzyme extracellularly in a liquid medium. Enzyme crude extract was determined to have an activity of 163 EU/ml, using Na-phytate

Table 1. The purification process of phytase from *Lactobacillus acidophilus*

	Volume (mL)	Activity (EU/mL)	Total activity EU %	Protein amount (mg/mL)	Specific activity (EU/mg)	Purification fold
Crude Extract	50	163,1	8155, 100	5.85	27.88	-
Amonium sulphate precipitation (%60-80)	50	45,3	2265 27.8	0.28	161.79	5.80

Table 2. The effect of some chemical compounds on phytase?activity

Chemical Compounds	Concentration (mM)	Relative Activity (%)	Concentration (mM)	Relative Activity (%)
None	-	100 ± 0.0	-	100 ± 0.0
Ca^{2+}	0.5	60.9	1	50.3
Fe^{2+}	0.5	123.7	1	179.6
Zn^{2+}	0.5	84.1	1	65.8
Co^{2+}	0.5	64.1	1	63.6
Cu^{2+}	0.5	40.6	1	30.7
Mn^{2+}	0.5	55.5	1	23.6

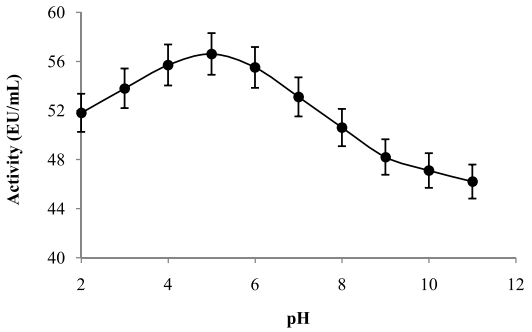

Fig. 1. The effect of pH on the activity of purified phytase from *Lactobacillus acidophilus*

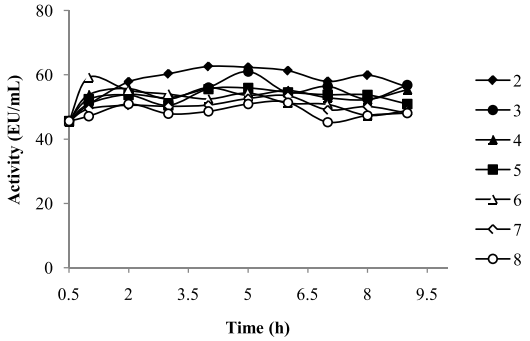

Fig. 2. Effect of pH on the activity of the purifed phytase from *Lactobacillus acidophilus*

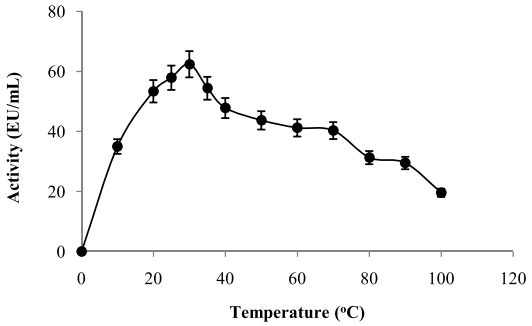

Fig. 3. Effect of temperatures on the activity of purifed phytase from *Lactobacillus acidophilus*

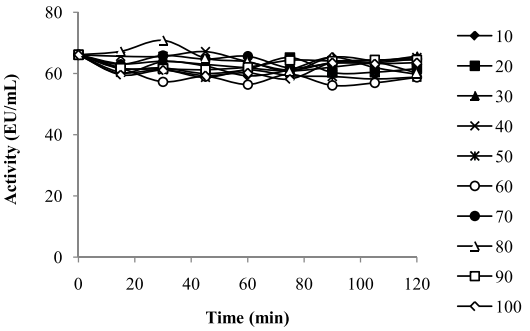

Fig. 4. Effect of temperatures on the stability of the purifed phytase from *Lactobacillus acidophilus*

substrate. In the literature, Anastasio et al.[22] have found the extracellular phytase activities of *Enterococcus faecium* A86 and *Lactobacillus plantarum* H5 strains as 0.74 U/ml and 0.71 U/ml respectively. In our findings, activity of phytase enzyme produced by L. *acidophilus* bacteria was very high.

Phytase enzyme was partially purified by ammonium sulfate precipitation of the crude extract. For this purpose, ammonium sulphate precipitation was performed at 0-20%, 20-40%, 40-60% and 60-80% saturations in order to determine the precipitation range of phytase enzyme. It was determined that enzyme is precipitated in 60-80% range. The enzyme was 5.8-fold purified by this precipitation from *L. acidophilus* by 27.8 yield (Table 1).

The enzyme activity was measured in the range of pH 2.0-11.0, in order to determine optimum pH of the phytase enzyme purified from *Lactobacillus acidophilus*. 10 mM acetate buffer was used for the pH 2.0-6.0 range, Tris/HCl buffer was used for the pH 6.0-9.0 range, and carbonate buffer was used for the pH 9.0-11.0 range to measure enzyme activity spectrophotometrically. According to the results, the optimum pH of the phytase enzyme purified from *Lactobacillus acidophilus* was found as pH 5 (Fig. 1). After performing activity measurements for 10 hours periodically in order to determine stable pH values of pure phytase enzyme, it was found that different pH values do not alter enzyme activity much and enzyme was stable at different pH values (Fig. 2). Optimum activities of the phytase obtained from the *L. plantarum* NRRL B-4496, *L. pentosus* CECT4023 and *L. sanfranciscensis* CB1 were pH 5.5, 5.0 and 4.0 respectively[23,24]. And, it was observed that optimum pH-value of phytase derived from *Lactobacillus acidophilus* supports the findings in the studies.

The activity was measured in the range of 10-100 °C, in order to determine optimum temperature of the phytase enzyme obtained from *Lactobacillus acidophilus* (Fig 3). According to

the results, the optimum temperature of the phytase enzyme from *Lactobacillus plantarum* was 30 °C. Pure phytase enzyme was incubated for 2 hours at 10-100 °C, and its temperature stability was found by activity measurements at certain intervals. For phytase enzyme, it was observed after 2 hours that the enzyme was stable at the applied temperature values and maintains its 90% of activity (Fig 4).

Effects of certain metal ions such as Cu^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+} and Zn^{2+} on the activity of phytase enzyme purified from *L. acidophilus* were also studied. It was determined that Cu^{2+}, Mn^{2+} and Ca^{2+} inhibit pure phytase enzyme activity, whereas Fe^{2+} activates the enzyme (Table 2).

Yoon *et al.*[18] have found that *Enterobacter* spp., which is an extracelluar phytase producer, was inhibited by the addition of 4.1 mM Zn^{2+}, Ba^{2+}, Cu^{2+}, Al^{3+} and Ethylenediaminetetra-acetic acid (EDTA). Yanke *et al.*[25] have determined that 5 mM concentration of Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} and Hg^{2+} inhibits phytase enzyme of *Selenomonas ruminantum*, whereas Pb^{2+} activates the enzyme.

In a study conducted byShimizu[12], it was found that the activity of extracellular phytase enzyme purified from *B. subitilis* (natto) N-77 strains is inhibited by addition of EDTA, Zn^{2+}, Cd^{2+}, Ba^{2+}, Cu^{2+}, Fe^{2+} and Al^{3+}.

In another study, it was found that the activity of extracellular phytase enzyme obtained from Bacillus spp. KHU-10 is inhibited by EDTA and metal ions such as Ba^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, $Hg^{2+}$and Mn^{2+} [26]. These findings in literature are in line with the findings in our study.

CONCLUSION

According to the results we obtained, it was revealed that the phytase enzyme purified partially from the *L. acidophilus* (ATTC) bacteria is extremely resistant against pH and temperature changes and certain metal ions, and maintains its activity for a long time. Consequently, it was concluded that it would be appropriate to use the resulting phytase enzyme in food, feed, paper, environmental industries and in soil improvement by enabling its conversion to myo inositol derivatives through hydrolysis of phytic acid found in grains and legumes.

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