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Nanoparticle assisted activity optimization and characterization of a bacterial phytase immobilized on single layer graphene oxide☆

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ABSTRACT

A thermostable phytase is a need of the hour towards prevention of environmental pollution by accumulation of phosphate. In this study, an extracellular phytase was purified from a strain of *Bacillus subtilis* obtained from cattle sheds in 24 Parganas (Kolkata, West Bengal). The native enzyme showed optimum activity at 42 °C and pH 8.5. In presence of magnesium nanoparticle (MgN), the graphene oxide (GO) immobilized enzyme showed 2.40 fold increased activity at 42 °C and more than 20 fold activity increment at 85 °C. The GO-MgN-phy complex (phytase coupled to MgN immobilized on graphene oxide) showed a decrease in K_m by 3.29 folds at 42 °C and 6 folds at 85 °C. Again, the enzyme system showed 3 fold and 5.5 fold increase in V_{max} at 42 °C and 85 °C respectively than the untreated enzyme. Compared to the untreated enzyme, energy of activation (E_a) was lowered by 2.5 and 6 folds repectively at 42 °C and 85 °C for GO-MgN-phy. At 85 °C, the half life ($t_{1/2}$) for GO-MgN-phy was 29.5 times than that of untreated enzyme with energy of dissociation (E_d) being 1.45 fold higher than the untreated enzyme. Enzymatic activity of GO-MgN-phy was retained by 86.6%when tested for storage stability at 4 °C after 140 days. This nanoparticle assisted immobilization technique employed in developing a thermostable phytase enzyme could withstand high temperatures during the pelleting of animal feeds. The increased activity of the enzyme would be beneficial to breakdown phytic acid during storage of feeds.

1. Introduction

The enzyme phytase (EC 3.1.3.8) is a phosphatase which catalyzes a hydrolysis reaction in which myo-inositol hexaphosphate is converted to myo-inositol phosphate with concomitant release of inorganic phosphate and myo-inositol. Monogastric animals cannot digest the phytate form of phosphorus which eventually results in environmental pollution by accumulation of phosphorus. This is encountered in regions of extensive livestock production. The importance of phytase application lies in the fact that it can diminish the amount of inorganic phosphate in animal diets thereby improving bioavailability of minerals, proteins and vital amines (Haefner et al., 2005; Cowieson and Adeola, 2005). Supplementing poultry and swine feeds with phytases is a routine practice nowadays to facilitate phosphorus digestion which simultaneously prevents phosphorus pollution in the environment (Selle and Ravindran, 2007). Certain myo-inositol phosphates derived from phytate is reported to be metabolically beneficial (Ohkawa et al., 1984; Potter, 1995; Vucenik and Shamsuddin, 2003). The bacterial phytases scores over fungal phytases due to their increased catalytic efficiency and specificity towards substrate.

Of late, in order to explore the immense potential offered by the enzymes, a host of synthetic organic molecules, proteins and peptides have come into being. Moreover, commercial applications of immobilized enzymes have made a headstart due to their catalytic efficiency under adverse conditions of pH and/or temperature. A range of nanocomposites and nanomaterials of various forms and sizes are being currently used as substrates to regulate enzyme activity. Among the nanomaterials used; gold (Sahoo et al., 2011; Yang et al., 2012; Thompson et al., 2011) alumina (Yang et al., 2008), silica (Lei et al., 2002; Luckarift et al., 2004; Lee et al., 2009) calcium nanoparticles (Dutta et al., 2013, 2014) are reported to yield satisfactory results in the process of enzyme immobilization.

The immobilization of enzymes on nanoparticles is reported to enhance enzyme stability and activity which is reflected in repeated use of the enzyme. Moreover, the nanomaterials are easily separated from the reaction mixture and their chaperone like property prevents the possibility of protein/product contamination. Bio-engineering involving graphene has gained headway in recent past with promising results w.r.t its stereoselectivity and biosolubility (Wang et al., 2011). Graphene oxide (GO), owing to its varied flexibility in biological

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applications as nanosupport has found its use in production of biocatalysts, biosensors, and drug delivery vehicles (Kuila et al., 2011). Graphene oxide nanosupport provide large surface area for enzyme immobilization and maintains its integrity in different aqueous suspensions (Zhao et al., 2014; Zhang et al., 2010, 2012).

GO possesses a single layered Sp2 hybridized structure which is 2 dimensional in nature. Owing to its unique chemical and physical properties, it can be used for immobilization of organic biomacromolecules. The use of glutaraldehyde as a cross linker for enzyme immobilization on graphene was found to confer increased structural stability to GO-enzyme complex which reflected in its increased activity and multiple usage (Sun et al., 2014). In this paper we investigated the altered enzyme activity of an extracellular phytase obtained from *Bacillus subtilis* upon supplementation of magnesium nanoparticles (MgN). The native enzyme showed maximum activity at 42 °C and pH 7.5. However upon supplementation with MgN, the MgN-enzyme system showed increased stability and activity at temperature as high as 85 °C withstanding pH of 9.2. Increased half-life upon approach of temperature extreme, reciprocal relationship between K_m and V_{max}, decreased E_a and increased E_d were salient features of the GO-MgN-phy system at 85 °C. This thermostability and increased activity of this phytase-NP system will be extremely beneficial and environment friendly to the industries involved in production of animal-feeds.

2. Materials and methods

2.1. Reagents

The magnesium oxide nanoparticle water dispersion, (MgO, 99.95%, 50 nm, 20 wt% in water) (Stock #: US7018) was supplied by US Research Nanomaterials. Eurofins supplied primers for PCR. Graphene Oxide (SLGO) was purchased from mkNANO (80% carbon, 20% Oxygen. Flake size: 0.5–5 μm. Thickness: Single atomic layer > 80% Colour: Bright yellow Purity: Carbon contents over 80%). Sigma Aldrich supplied agar powder, glutaraldehyde and all other reagents required for subsequent media preparation. Protein markers were obtained from Fermentas, a Germany based organization. All other requisite chemicals for silver staining and bacterial culture media preparation were obtained from Sigma Aldrich.

2.2. Identification and isolation of enzyme secreting bacterial strains

Soil samples were collected physically from cattle sheds in 24 Pargana's (Kolkata, West Bengal) which lies in the outskirts of the city. Approximately 2.5 g of soil sample was added to sterile 0.85% saline water. It was incubated at 50 °C overnight. The soil suspension was then subjected to serial dilution and inoculated on nutrient agar plates. The inoculated plates were incubated at 42 °C for 36 h. For screening of phytase production, the strains were maintained in nutrient agar plates containing glucose 0.5%, peptone 1%, beef extract 0.5%; $MgSO_4$ 0.1%;$FeSO_4{\cdot}7H_2O$ 0.01%, $MnSO_4{\cdot}7H_2O$ 0.02%, $CaCl_2$ 0.1%, agar 1.5% sodium phytate 0.1% and (pH 7.5). The colonies exhibiting translucent zones were selected and streaked onto fresh nutrient agar plates and were maintained at 42 °C and pH 7.5.

2.3. Ribotyping studies

We performed 16S rDNA gene sequencing as previously described by the author (Dutta et al., 2013, 2014). Finally, the nucleotide sequence was deposited in GenBank.

2.4. Method of phytase purification

Phytase enzyme was purified from an initial 150 ml culture of NPHY02. After centrifugation at 10000 r.p.m for 25 min the cell free supernatant was precipitated with (0–30; 30–80%) saturation of ammonium sulfate followed by dialysis. All subsequent steps of purification procedure were performed at 4 °C. The dialysed proteins were loaded onto a DEAE-Sepharose CL-6B column pre-equilibrated with 20 mM Tris–HCl buffer (pH 7.5) and allowed to equilibrate overnight. After washing the column with 20 mM Tris–HCl buffer (pH 7.5), a 60 ml increasing discontinuous gradient (0–200 mM) of NaCl dissolved in Tris–HCl buffer (pH 7.5) was applied to the column. The fractions showing phytase activity were loaded onto a glass column packed with Sephadex G-100 (bed volume 30 ml, 0.9×13 cm) and equilibrated with Tris–HCl buffer (pH 7.5). The fractions showing phytase activity were further loaded on Sephadex G-75 and the positive 1 ml fractions (showing phytase activity) were run on 12% SDS PAGE. The amount of protein that was loaded on SDS–PAGE gel lanes was 0.65 mg/ml with specific activity of 1193.2 Unit/mg. In order to determine whether the phytase enzyme was pure and free from contamination, zymography study of the enzyme was performed. Zymograms were prepared by soaking the gels first in 1% Triton X-100 for 1 h at room temperature and then in 20 mM Tris buffer (pH 7.5) for 1 h at 42 °C. Phytase activity was detected by incubating the gels for 16 h in a 20 mM Tris buffer (pH 7.5) containing 0.4% (w/v) sodium phytate. Activity bands were visualized by immersing the gel in a 2% (w/v) aqueous cobalt chloride solution. After a 5 min incubation at room temperature the cobalt chloride solution was replaced with a freshly prepared solution containing equal volumes of a 6.25% (w/v) aqueous ammonium molybdate solution and 0.42% (w/v) ammonium vanadate solution. Phytase activity was evident from the zone of clearing in an opaque background corresponding to the purified enzyme fraction in SDS-PAGE.

2.5. Enzyme assay for phytase

Phytase activity was assayed by mixing 500 μl of enzyme solution with 1.0 ml of 2 mM sodium phytate in 20 mM Tris–HCl buffer (pH 7.5) supplemented with 2 mM $MgCl_2$. This reaction was carried out at 42 °C for 25 min, followed by stopping the reaction by the addition of 0.9 ml of 5% trichloroacetic acid. The liberated inorganic orthophosphate (Pi) was measured by spectrometer at 700 nm with concomitant production of phosphomolybdate on addition of 2.0 ml of colour reagent. The preparation of colour reagent was done by homogenous mixing of four volumes of 2.0% ammonium molybdate solution in 5.5% H_2SO_4 and one volume of 2.7% $FeSO_4$ solution. One unit of phytase activity was defined as to liberate 1 μmol of phosphate per minute under the assay condition.

2.6. Measurement of Mg concentration by Atomic Absorption Spectra (AAS)

The Mg content of MgN and 1 mM $MgCl_2$, was measured using AAS technique (Analyst 200, Perkin Elmer). Standard Mg ion solution was provided by Perkin Elmer. MgN of different dilutions (0.005–0.035 M) were prepared and their concentration was determined by comparison of data with the standard solutions provided by Perkin Elmer.

2.7. MgN effecting phytase activity

Purified phytase enzyme (100 μl in volume with concentration of 0.65 mg/ml with specific activity of 1193.2 Unit/mg) was incubated with MgN concentrations of (2.0–18.0)μg/ml. Enzyme activity for each fraction was measured by the process described above.

2.8. Effect of temperature and pH on activity of MgN-phy and untreated phytase

The optimum temperature of MgN-phy (phytase enzyme coupled to MgN) and untreated phytase were determined by carrying out the standard enzyme assay in Tris–HCl buffer (pH 7.5) at increasing