Chapter 4

Characterization and application of solid state Phy III from Aspergillus niger NCIM 563

This chapter discusses the biochemical and molecular characterization and application of phytase in synthesis of hollow silica nanocontainers in ionic liquids. A novel solid state phytase III was isolated from Aspergillus niger NCIM 563 with a procedure involving liquid liquid extraction and column chromatography. It possessed an optimal pH of 5.5 and an optimal temperature of 60°C, and manifested a $K_m$ and $V_{max}$ of 0.156 mM and 220µm/min/mg. Phytase activity was moderately stimulated in presence of 5mM Ca$^{2+}$, Fe$^{2+}$, Fe$^{3+}$, Ba$^{2+}$, Pb$^{2+}$ and inhibited in presence of 1mM Hg$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, Cu$^{2+}$ and Ag$^{2+}$. The protein is a monomer and exhibited a molecular mass of 85kDa in gel filtration and SDS–PAGE. The aqueous two phase extracted phytase exhibited improved thermostability as compared to column chromatography. Phy III exhibited broad substrate specificity but had high affinity for sodium phytate. It was markedly inhibited by N-bromosuccinimide suggesting a possible role of tryptophan in its catalysis. Based on MalDI-LC-MS/MS identification amino acid sequences of the peptides, the enzyme did not show homology with any other known phytases from the literature suggesting its unique nature.

Part of the work presented in this chapter is communicated

1. Introduction

P is an essential nutrient to biological systems. Its requirement is mainly seen in nucleic acids, cell membrane, bones and teeth. It is a main constituent of energy rich compounds such as ATP, ADP, GTP etc and serves as an energy conduit in various metabolic reactions. Despite its importance, P production, utilization and recycling is a slow process due to absence of gaseous phase and thus is therefore well-known as an imperfect cycle [20].

Phytase are a special class of phosphatase that catalyzes the sequential hydrolysis of phytate to less phosphorylated myo-inositol derivatives and inorganic phosphate. Phytic acid (myo-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate) and mixed cation salts of phytic acid, designated as phytates, are a group of organic P (P) compounds found widely in nature [18]. Phytate phosphorous represents approximately 75-80% of total phosphorous in plant seeds and is largely unavailable to monogastric like poultry birds, pigs, fishes and humans due to lack of adequate levels of phytases. The phytic acid in plant derived food acts as an antinutritional factor since it causes mineral deficiency due to chelation of metal ions such as Ca$^{2+}$, Mg$^{2+}$, Zn$^{2+}$ and Fe$^{2+}$ which form complexes with proteins and thus affect their digestion and also inhibit certain digestive enzymes like alpha amylase, trypsin, acid phosphatase and tyrotnsae. Phytic acid excreted in faeces is degraded by soil microorganisms release P in soil which on reaching aquatic bodies that causes eutrophication [15]. Hence dephosphorylation of phytate by phytase is a critical process which overcomes the drawbacks of slow recycling and at the same time eliminating the anti nutritional effects of phytate. They can be produced from sources including plants, animals and microbes. Microbial sources however are promising for their commercial exploitation. Microorganisms produce low levels of phytase and it would be beneficial if these production rates be improved with desirable process features by employing statistical techniques and strain improvement programs.
The available phytase preparations used as feed additives are of fungal origin, produced by recombinant strains under SmF and have shortcomings especially with regard to obtaining diluted product, sensitivity to heat, inactivation under low pH conditions present in the stomach of animals [2] and high product recovery costs. These disadvantages need to be alleviated while at the same time producing phytase with high yield and purity. Due to these limitations, the recovery, yield and purification of phytase represent a technological challenge that needs to be overcome to make the process economically viable for industrial application.

Based on the amino acid residue in the active site, phytate degrading enzymes can be referred to HAP, BPP, CP and PAP [19]. The phosphate residue of phytate is released by phytate degrading organisms at different rates and in different order. During the last few decades phytases have been studied extensively because of great interest in its application as feed additive and environmental protection [25]. Current phytase research and its relationship to a contemporary environmental concern provide insights into budding applications that will promote additional research and development of this key biocatalyst. The potential of phytase in processing of human food and synthesis of lower inositol phosphate thereby improving human health have gained increasingly attention. Physicochemical properties of phytases, namely, broad pH range to survive under varied pH conditions in animal digestive tract, resistance to proteolytic degradation, thermal stability to resist higher temperatures during feed pelleting and substrate specificity, and so on need thorough evaluations to design versatile “second-generation” phytases with wider applicability. Further active research must, therefore, be directed to identify new native phytase proteins from diverse micro flora and plants [27] that would form the basis of creating consensus phytases using genetic and protein engineering approaches.

The immobilization of technologically important phytase onto solid supports is an area of extensive research because of their widespread applications in biomedicine as therapeutic peptide
loaded nanovehicles and in various industries as reusable biocatalysts [1, 10, 21]. However, one of the major challenges associated with enzyme immobilization is the retention of enzymatic activity after their immobilization onto a substrate. Previous studies have indicated that the enzyme activity can be preserved in ionic liquids (ILs) because of their unique solvent properties. Bioscience is among the most interesting areas where ILs are just beginning to play an important role, with demonstrated applications in enzyme stabilization, protein crystallization, and biofuel cells. The unique solvent properties of ILs have led to the exploration of different biocatalytic reactions in ILs [28-30] as well as self-assembly of nonbiological amphiphiles (e.g., surfactants, lipids, block copolymers, etc.). However, there are few reports exploring the potential of ILs as designer solvents for self-assembly of amphiphilic biomacromolecules [22].

We have earlier reported high levels of phytase by solid state fermentation using statistical techniques that involve a combination of PBD and BBD [5]. The present investigation deals with characterization and immobilization of SSF phytase (Phy III) in silica hollow nanocontainers.

2. Materials and Methods

2.1. Chemicals

Phytic acid sodium salt was purchased from Sigma Chemical Company, St Louise, MO, USA. All other chemicals used were of analytical grade and obtained from leading manufacturers including BDH, Sigma and Glaxo. SDS–PAGE and gel filtration markers, Coomassie Brilliant Blue R-250 and Bromophenol Blue were purchased from Sigma Chemical Company, USA. Sephacryl S-300, Phenyl-Sepharose CL-4B were obtained from Sigma.

2.2 Fungi and inoculum preparation

*A. niger* NCIM 563 was used in the present study from NCIM Resource Center, Pune, India. The stock cultures were maintained on Potato Dextrose Agar (PDA) slants and stored at 4°C.
Spores from 7 days old PDA slant were harvested using sterile distilled water containing 0.01% Tween 80 to obtain 5x10^7 spores/ml and used as inoculum for solid state fermentation.

2.3. Phytase production and extraction

The SSF medium contained 10g of wheat bran in 250ml Erlenmeyer flask plus glucose 3g, dextrin 1.25g, sodium nitrate 0.2g, magnesium sulphate 0.3g moistened with 20ml distilled water and sterilized by autoclaving at 121°C for 30min. On cooling fermentation medium was inoculated with 1% spore suspension of A. niger NCIM 563 and incubated for 4 days at 30°C. Enzyme production was expressed as enzyme activity IU/g DMB. Phytase extraction from koji was done as mentioned and the specific activity of crude enzyme was approximately up to 5U/mg of protein. The crude extract was stored at 4°C and used as and when required for the experiments.

2.4. Analytical methods

Phytase measurements were carried out at 50°C. The reaction mixture consisted of 3mM sodium phytate buffered with 100 mM acetate buffer (pH 5.5). Enzymatic reactions were started by the addition of 50ml of enzyme solution. After 30min at 50°C, the liberated inorganic phosphate was measured by a modification of the ammonium molybdate method [9]. A freshly prepared solution of acetone: 5N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v/v) and 400µl 1M citric acid was added to the assay mixture. Absorbance was measured at 370 nm. One unit of phytase activity (U) was expressed as the amount of enzyme that liberates 1µm P/min under standard assay conditions. Each experiment was carried out in triplicate and the values reported are the mean of three such experiments in which a maximum of 3–5% variability was observed. Concentration of protein was determined using Lowry method using bovine serum albumin as standard [17].
2.5. Purification of Phy III

Purification of phytase was done by aqueous two phase extraction system as described earlier [6]. The system consisted of combination of polyethylene glycol (PEG) 6000 and 8000 (10.5%) and sodium citrate (20.5%) for partitioning of phytase.

2.6 Gel permeation chromatography

The molecular mass of purified enzyme was estimated by gel filtration. The gel filtration column of Sephacryl S-200 column equilibrated with 50mM sodium acetate buffer, pH 5.5 was calibrated using gel filtration standard molecular mass markers; cytochrome c (12.4kDa), bovine serum albumin (66kDa), alcohol dehydrogenase (150kDa), b-amylase (200kDa), apoferritin (443kDa) and thyroglobulin (669kDa).

2.7 Electrophoretic techniques, Zymogram analysis and mass spectrometry

Native PAGE (8%) was performed in a vertical gel apparatus at pH 7.5. Samples containing approximately 5-10µg of protein were applied to the gel and electrophoresis was carried out at 200V for 3-4h. Protein bands were visualized by silver staining (0.4% w/v) [7].

The subunit molecular mass was determined by SDS–PAGE in a vertical slab gel apparatus at pH 8.3 using Sigma high molecular mass markers [14]. The samples were boiled for 5 min with the presence of 1% SDS, 80mM 2-mercaptoethanol, 100mM Tris–HCl buffer (pH 6.8) and 15%glycerol and loaded onto a 12% SDS polyacrylamide gel. After electrophoresis, the gels were stained with silver staining protocol. Protein bands were detected either by Coomassie Brilliant Blue R-250 (0.2% w/v) or by silver staining (0.2% w/v).

Non-denaturing electrophoresis was carried out in the same manner, but with omission of SDS from the gel running and loading buffers, and the sample was not pre-treated under
denaturing conditions. Staining was carried out using Blue Coomassie R-250 or by zymogram analysis. The latter entailed overlay of the gel with molten agar (1.5% w/v) in 200 mM sodium acetate buffer, pH 5.5, containing phytic acid (0.15% w/v). After overlay solidification, the gel was incubated at 55°C for 12h, followed by flooding with calcium chloride (10% w/v). Bands of phytase activity were detected as translucent zones against an opaque background [3].

Mass spectral analysis was performed on a Voyager-De-STR (Applied Biosystems) MALDI-TOF. A nitrogen laser (337 nm) was used for desorption and ionization. Spectra were acquired in the range of 10–100 kDa, in linear mode with delayed ion extraction and with an accelerating voltage of 25 kV. The low mass ion gate was set at 4,500 Da. All the analyses were performed in four replications. The instrument was calibrated with myoglobin and bovine serum albumin. In-gel tryptic digestion and analysis of peptides was done accordingly [4].

2.8 Effect of pH and temperature on phytase activity and stability

The optimum pH was determined by measuring the activity between pH 2.0-10.0 using 200 mM buffers; glycine-HCl (pH 2.0-3.0), sodium acetate (pH 4.0-6.0), Tris-HCl (pH 7.0-8.0) and glycine-NaOH (pH 9.0-10.0) at 50°C. Stability assay was performed by incubating the partially purified enzyme at 30°C for 24h in 50 mM buffers of different pH values glycine-HCl (pH 2.0-3.0), sodium acetate (pH 4.0-6.0), Tris-HCl (pH 7.0-8.0) and glycine-NaOH (pH 9.0-10.0). The residual activity was then assayed using under standard assay conditions considering enzyme activity at zero time as 100%. The optimum temperature was determined over temperature range 45-60°C. The thermal stability was studied up to 60°C and the residual enzyme activity was determined using standard assay conditions and compared with the control without incubation.
2.9 Hydrolysis of soybean meal phytate in simulated gastric fluid

One gram soybean meal was dissolved in 9 ml simulated gastric fluid (SGF) [0.25 M glycine-HCl containing 2.0 mg/ml NaCl and 3.2 mg/ml pepsin] and pH was adjusted with HCl or NaOH to a final pH of 1.5, 2.0, 2.5, 3.5, 5.5 or 6.5. The solutions were incubated with agitation at 37°C for 30min, and pH was adjusted to the corresponding values again. Then 1ml partially purified enzyme was added to the solutions and incubated by agitation at 37°C for 60min. The amount of released P was determined by modified ammonium molybdate method as described in Section 2.5.

2.10 Effect of metal ions, inhibitors, detergents and organic solvents on phytase activity

The effect of various metal ions (1, 5 mM), sulfurhydryl compounds (1, 5 mM), chelating agents (1, 5 mM), detergents (0.1, 0.5%) and organic solvents (10%) on the enzyme activity by incorporating these into the reaction mixture.

2.11 Substrate specificity

Substrate specificity was examined by measuring the phytase activity with different phosphorylated substrates in place of sodium phytate. Enzyme activity was determined after incubating the enzyme with 3mM of each substrate under standard assay conditions.

2.12 Enzyme kinetics

The kinetic rate constants, $V_{\text{max}}$ and $K_m$ were determined with sodium phytate (0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 mM) as substrate using Lineweaver–Burk plot. Data obtained were analyzed by creating: (i) a plot of initial velocities versus substrate concentrations ($V_0$ versus $[S]$); and (ii) a reciprocal plot of $V_0$ and $[S]$ to make a Lineweaver-Burk plot for calculation of $K_m$ and $V_{\text{max}}$ [16].
2.13 Peptide sequencing

The enzyme was sent to Sutton Bonington Proteomics Facility, United Kingdom for MALDI-LC-MS/MS peptide sequence analysis. In-gel tryptic digestion was performed and the digested mixture was subjected to MALTI-TOF and LC-MS/MS for peptide mass fingerprinting and peptide sequencing. The data thus obtained was searched against the public database Swiss-Prot using the MS/MSIONS search tool on the MASCOT web site.

2.14 Synthesis of Hollow silica spheres using phytase enzyme as a template-

*Ionic-Liquid-Mediated Synthesis of Phytase Capsules* - 0.5mL volume=490µL of the respective ionic liquid (IL) 1-butyl-3-methylimidazoliun tetrafluoroborate ([BMIM][BF4] +10µL purified phytase enzyme (1mg/ml dissolved in water), thus achieving a final enzyme concentration of 20µg/ml in the reaction. The IL-phytase mixture was incubated at 37°C (24h with gentle reciprocal shaking, after which samples were centrifuged (14000rpm), followed by washing with deionized water and acetonitrile to remove the viscous IL. Phytase capsules thus obtained were further analyzed by TEM.

*Ionic-Liquid-Mediated Synthesis of Silica Nanospheres* - To obtain silica nanospheres, 20mM TEOS (silica precursor, tetraethyl orthosilicate) stock solution was prepared in the respective ILs. TEOS stock solution (500µL, 20mM) in ILs was added to 500µL of the reaction mixture containing IL and phytase capsules. The 1mL reaction contents were incubated at 37°C (24h under stirring conditions, during which all reactions involving phytase became turbid, indicating TEOS hydrolysis. Samples were centrifuged at 14000rpm, followed by washing with deionized water and acetonitrile to remove the viscous IL. Silica nanostructures thus obtained were further analyzed by TEM.
3. Results and discussion

3.1 Purification of Phytase

The enzyme extracted from the SSF koji was subjected to aqueous two phase extraction resulting in one-sided partitioning of phytase in bottom phase with recovery of 98.5% and purification factor 2.5 within 3hrs.

3.2 Determination of molecular mass

Purification to homogeneity was confirmed by SDS-PAGE analysis and a single band was also observed on nondenaturing electrophoretic gels. The identity of this band on the non-denaturing gels as phytase was confirmed by zymogram analysis (Fig. 1). The subunit molecular mass of the purified phytase was estimated to be 85kDa by SDS-PAGE (Table 1).
### Table 1 Biochemical properties of Phy III from *A. niger* NCIM 563

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH</strong></td>
<td></td>
</tr>
<tr>
<td>Optimum</td>
<td>5.6</td>
</tr>
<tr>
<td>Stability</td>
<td>2.0-9.5</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td></td>
</tr>
<tr>
<td>Optimum</td>
<td>60°C</td>
</tr>
<tr>
<td>Stability</td>
<td>20% residual activity at 60°C after 1 hr</td>
</tr>
<tr>
<td><strong>Molecular mass</strong></td>
<td></td>
</tr>
<tr>
<td>Gel filtration</td>
<td>85kDa</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>85kDa</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>50.58kDa</td>
</tr>
<tr>
<td><strong>Effect of metal ions</strong></td>
<td></td>
</tr>
<tr>
<td>Stimulated (5mM)</td>
<td>Ca^{2+}, Fe^{2+}, Fe^{3+}, Ba^{2+}, Pb^{2}</td>
</tr>
<tr>
<td>Inhibited (1mM)</td>
<td>Hg^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Ag^{2+}</td>
</tr>
<tr>
<td><strong>Kinetic constants</strong></td>
<td></td>
</tr>
<tr>
<td>$K_m$</td>
<td>0.156 mM</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>220µm/min/mg</td>
</tr>
</tbody>
</table>

This molecular mass is similar to that of the purified phytase (phy A) from *A. ficuum* NRRL 3135, and falls well within the molecular mass range of previously purified phytases (38–200kDa) [8, 26]. The molecular masses of the native enzyme were determined to be 87 kDa on a calibrated 16/60 Sephacryl S-200 HR column with elution position being measured by determination of enzyme activity. These suggest that Phy III is a monomer of 85kDa, however, MALDI data revealed the molecular weight of Phy III to be 50.58 kDa (Table 1 and Fig. 1). This could be because of the doubly charged species of phytase during ionization process of MALDI-TOF spectrometry [13].
3.3 Characterization of phytase from A. niger NCIM 563

The highest phytase activity was observed at pH 6.0. The enzyme retained ≈75% activity over a wide pH range, 2.0-9.5 (Table 1). The pH optima and pH stability profile of phytase determines its ability to act efficiently in crop and stomach of digestive tract of poultry. Fungal phytase acts efficiently in stomach (pH 2.0-5.0) and need reactivation to maintain activity in crop (pH 4.0-5.0) in digestive tract of poultry and bacterial phytases act vice versa. The catalytic efficiency of A. niger NCIM 563 phytase will be more in both in crop and stomach of poultry because it retains activity over a wide pH range (2.0-9.5) and will not require reactivation.

The maximum of phytase activity was at 55°C and declined thereafter (Table 1). Phytases from various Aspergilli show optimum temperature in the temperature range 40-65°C. Thermostability is prerequisite for the successful application of phytase in animal feed because of
exposure at 60–90°C during pelleting process for a few minutes. The poor thermostability of existing phytase enzymes is still a major concern for animal feed applications. Phytase from *A. niger* is thermo-labile and exhibits 80% activity at 60°C after 5min and 20% activity after 60min. It is assumed that the fermented koji be dried and then used in animal feed. In practice, the step of drying at high temperature and the presence of proteases lowers the phytase activity in the dried product. The improvement in thermostability using aqueous two phase extraction has been reported (Refer Section 2.5) and it exhibits 4 times better thermostability profile at 60°C as compared to chromatography purified process and is therefore likely to withstand the high temperature required for product formulation.

Phytase activity was moderately stimulated in presence of 5mM Ca^{2+}, Fe^{2+}, Fe^{3+}, Ba^{2+}, Pb^{2+} and inhibited in presence of 1mM Hg^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+} and Ag^{2+} (Table 1). The enzyme retained 63% and 43% activity in presence of Zn^{2+} and Cu^{2+} in contrast to most phytate-degrading enzymes that are greatly inhibited by Cu^{2+} and Zn^{2+}. Actually the influence of zinc, iron, copper and calcium is potentially significant from an applied perspective with respect to phytase in animal feed. Retention of phytase activity in presence of Pb^{2+} (125% for 5mM) and Ag^{2+} (50% for 1mM) provides an opportunity for phytate hydrolysis in soils contaminated with heavy metals.
Table 2 Effect of various solvents, detergents and inhibitors on Phy III activity

<table>
<thead>
<tr>
<th>Effect</th>
<th>Relative enzyme activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Organic solvents</td>
<td></td>
</tr>
<tr>
<td>Acetone 10% v/v</td>
<td>80.9</td>
</tr>
<tr>
<td>Isoamyl alcohol</td>
<td>110.4</td>
</tr>
<tr>
<td>Ethanol</td>
<td>94.5</td>
</tr>
<tr>
<td>Hexane</td>
<td>105.6</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>98.7</td>
</tr>
<tr>
<td>Glycerol</td>
<td>80</td>
</tr>
<tr>
<td>Toluene</td>
<td>114.7</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>102.5</td>
</tr>
<tr>
<td>Detergents 0.1%</td>
<td>104.3</td>
</tr>
<tr>
<td>Tween 20 0.5%</td>
<td>115</td>
</tr>
<tr>
<td>Tween 80</td>
<td>103.8</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>100.5</td>
</tr>
<tr>
<td>SDS</td>
<td>18.9</td>
</tr>
<tr>
<td>Inhibitors 1Mm</td>
<td>102</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>100.4</td>
</tr>
<tr>
<td>N-ethylmaleimide</td>
<td>100</td>
</tr>
<tr>
<td>N-bromosuccinimide</td>
<td>0</td>
</tr>
<tr>
<td>PMSF</td>
<td>70.5</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>100</td>
</tr>
</tbody>
</table>

$K_m$ and $V_{max}$ was 0.156mM and 220µm/min/mg using Lineweaver Burk plot. These values fall well within the range previously reported for microbial phytases. The enzyme was insensitive to PMSF, ethylmaleimide and iodoacetate (Table 2). The reducing agents like DTT had no effect on enzyme activity suggesting that –SH groups are not involved in the catalytic activity or this enzyme does not have free and accessible –SH groups. Acetone, glycerol inhibited phytase
activity slightly while other solvents stabilized it. Among the non-ionic detergents, Tween 20, Tween 80, Triton-X 100 stabilized the enzyme activity, while the anionic detergent (SDS), even at low concentration severely inhibited the activity phytase. The SSF phytase of *A. niger* NCIM 563 was inhibited by NBS suggesting the possible role of tryptophan in catalysis. The amino acid in active domain of phytase from *A. ficuum* is arginine, histidine, and tryptophan [24]. In the phytase of *B. amyloliquefaciens*, lysine, histidine, and tryptophan is related to active domain [11].

The chelating agent EDTA did not show any observable effect on the catalytic properties of phytase. None of the characterized phytases are known to require metal ion for the activity except that of *Bacillus spp* which is calcium dependent [12].

![Fig. 2 Substrate specificity studies of SSF phytase from A. niger NCIM 563](image)

1- Sodium phytate, 2- $p$-nitro-phenyl phosphate, 3- Sodium phenyl phosphate, 4- α-D-Glucose-1-phosphate, 5- D-Glucose-6-phosphate, 6- 1-Naphthyl phosphate, 7- 2-Naphthyl phosphate, 8- ATP, 9- ADP, 10- AMP, 11- β-NADP, 12- Sodium pyrophosphate, 13- Glycerol-1-phosphate, 14- Phenyl phosphate
Phytase has a broad substrate specificity and hydrolyzed most of the substrates used (Fig. 2). The MALDI-LC-MS/MS generated peptide sequences when compared and searched against the public database Swiss-Prot using the MS/MSIONS search tool on the MASCOT web site did not homology with the known microbial phytases.

3.4 Self-assembled phytase capsules in IL

Phytase enzyme was initially purified to homogeneity from the SSF broth of *A. niger*, as mentioned in section 3.1. In a typical reaction, phytase enzyme (20µg/mL) was added to IL [BMIM][BF4] and left at room temperature for 24h under shaking conditions. Fig. 3 shows the transmission electron microscopy (TEM) micrograph of phytase capsules obtained after self-assembly of phytase molecules in the IL [BMIM] [BF4]. The protein capsules are quasi-spherical in morphology with an apparently rough surface and an average diameter of 150-200nm. In a control experiment in water, no phytase self-assembly was observed. Controlled self assembly of Phy III enzyme in the IL ([BMIM] [BF4]), lead to the formation of phytase capsule.

![Fig 3](A & B) Lower and (C) higher magnification TEM images of self-assembled phytase (20µg/mL) capsules synthesized in ionic liquid [BMIM] [BF4].
3.5 Silica nanoparticles

Phytase capsules obtained via self-assembly in [BMIM] [BF4] have been further utilized as functional templates for the spontaneous growth of hollow silica nanocontainers. To obtain hollow silica nanoparticles, TEOS was added to IL [BMIM] [BF4] containing phytase capsules.

Fig. 4. (A) Lower and (B) higher magnification TEM images of solid silica nanoparticles synthesized in ionic liquid [BMIM] [PF6] using 20 µg/mL self-assembled phytase.

Fig. 4A is the TEM image of hollow silica nanoparticles synthesized in [BMIM] [BF4]. The hollow silica nanoparticles are 120-150 nm in diameter with a rough surface and quasi-spherical morphology. The higher magnification Fig. 4B TEM micrograph of one of the hollow silica nanoparticles indicates that these hollow silica nanoparticles are ~50 nm thick. The hollow silica nanoparticle synthesized by in situ self-assembly and templating of phytase capsule in [BMIM] [BF4] thus acts as an enzyme nanocontainer, in which phytase molecules are encapsulated during its synthesis while retaining the native activity of enzyme molecules for at least up to four cycles.
The self-assembly of phytase molecules in IL [BMIM][BF4] leading to the formation of enzyme capsules and their utilization as self-templating nanoreactors for the synthesis of hollow silica nanoparticles with controllable wall thickness is quite interesting and has not been previously demonstrated for SSF phytase. Phytase enzyme used in this study has been isolated from the fungus \textit{A. niger} and belongs to the histidine acid phosphatase (HAP) family of proteins. A common catalytically active site motif, RHGXRXP (Arg-His-Gly-X-Arg-X-Pro), is shared by all HAPs, wherein the catalytic histidine in this sequence is known to initiate a two-step reaction that results in the hydrolysis of substrate [23] Such catalytically active motifs that are present in phytase seem to be responsible for TEOS hydrolysis, leading to hollow silica nanoparticles. The hollow silica nanoparticle synthesized by in situ self-assembly and templating of phytase capsule in [BMIM] [BF4] thus acts as an enzyme nanocontainer, in which phytase molecules are encapsulated during its synthesis while retaining the native activity of enzyme molecules for at least up to four cycles.
Conclusions

The extracellular Phy III from *A. niger* NCIM 563 was purified to homogeneity. Characterization studies reveal some exclusive biochemical properties that bring out its potential for use as an animal feed additive. Among them is stability over broad pH range, high efficacy in hydrolyzing phytate P under emulated gastric conditions and phytate hydrolysis of heavy metal contaminated soils. We show that enzyme (phytase) molecules self-assembled in the presence of an ionic liquid, resulting in the formation of enzyme capsules. Phytase capsules synthesized using this approach was further used as templating nanoreactors for the synthesis of enzyme-containing hollow silica nanocontainers. In situ immobilized phytase enzyme in the silica nanocontainers, when subjected to enzyme-reusability application, establishes them as excellent reusable biocatalysts. This controlled templating of bionanomaterials in ILs of different properties will thus lead to applications in drug encapsulation and controlled release, biocatalysis, biosensing, and bioelectronics.
References


