Chapter: IV

<table>
<thead>
<tr>
<th>Figure No</th>
<th>Figure captions</th>
<th>Page No</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>CM sepharose column, 10-80% fraction from 3 day old culture was chrometographed and developed against the linear gradient of 0 to 0.4 M NaCl, collecting 10 ml fractions, fractions were analyzed for phytase activity, acid phosphatase activity and A280</td>
<td>8</td>
</tr>
<tr>
<td>4.2</td>
<td>Sephadex G-200 column; the fraction containing phytase activity was chrometographed in gel filtration column using sodium acetate buffer and fractions were analyzed for phytase activity and A280</td>
<td>8</td>
</tr>
<tr>
<td>4.3</td>
<td>lane 1 molecular weight marker, lane 2 2.5%, lane 3 20%, lane 4 50%, lane 5 55%, lane 6 60%, lane 7 65%, lane 8 70%, lane 9, 77%, lane 10, 80% concentration of ammonium sulphate, when yeast culture grown on minimal medium containing sodium phytate</td>
<td>9</td>
</tr>
<tr>
<td>4.4</td>
<td>Native 7.5% gel stained with fast garnet B and CBB G250 stained gel of 10% SDS PAGE, revealed presence of multisubunit nature of yeast phytase, yeast phytase may contain 3</td>
<td>9</td>
</tr>
<tr>
<td>4.5</td>
<td>pH optimization of purified phytase in acetate buffer.</td>
<td>12</td>
</tr>
<tr>
<td>4.6</td>
<td>Temperature optimization of purified phytase</td>
<td>13</td>
</tr>
<tr>
<td>4.7</td>
<td>IEF-SDS PAGE analysis of phytase</td>
<td>15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table No</th>
<th>Table captions</th>
<th>Page No</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Purification table for phytase</td>
<td>7</td>
</tr>
<tr>
<td>4.2</td>
<td>Amino acid composition of yeast phytase</td>
<td>11</td>
</tr>
<tr>
<td>4.3</td>
<td>Substrate selectivity study of yeast phytase</td>
<td>14</td>
</tr>
</tbody>
</table>
Abstract

Present study is focused on purification of phytase from yeast *Saccharomyces boulardii*. The yeast strain produces intracellular and extracellular phytase. The phytase was purified by 3 step strategies. First step encompasses the ammonium sulphate precipitation of proteins, phytase precipitates at 80% saturation. The precipitates were dissolved and subjected to determination of total protein concentration and specific phytase activity was carried out. CM sepharose Cl 6b and Procion red He3B columns were used for purification of phytase. The purification results were confirmed by SDS and Native page analysis in the presence of dye fast garnet B. Furthermore, the optimum conditions for enzyme activity was evaluated during the studies, optimum temperature was optimized at 50°C, pH 4.5. The substrate specificity and amino acids composition were also studied.
4.1 Introduction

Protein purification is tightly regulated for normal functioning of a cell or organism. To understand protein structure and function in detail, they often need to be separated from other cellular components and isolated to homogeneity, it should retain all its native biological characteristics of structure and activity. To achieve this objective, one needs to take into account the physical and chemical property of proteins. General steps include (i) extraction (ii) precipitation and/or (iii) chromatography.

Inorganic phosphate is preferred extracellular phosphate source of yeasts. Upon its depletion, Phosphate is liberated from organic substrates by extra cellular phosphatases [Johnston and Carlson, 1992]. Phosphorous metabolism relies on several constitutive and phosphate-repressible acid and alkaline phosphatases in various yeasts using the pho regulon. Phytase and its regulation mechanism are best studied in the baker’s and brewer’s yeast, Saccharomyces cerevisiae. Two forms of acid phosphatases are known in the yeast S.cerevisiae. A constitutive form encoded by PHO 3 gene, and a repressible form encoded by three genes-PHO 5, PHO 10, and PHO 11. Acid phosphatases have also been studied in the other conventional yeast Schizosaccharomyces pombe; in several nonconventional yeast like Arxula adeninivorans, Yarrowia lipolytica, Klyveromyces lactis, Candida albicans, as well as in the methylotrophic yeast, Hansenula polymorpha and Pichia pastoris. Intracellular and extracellular phytase is reported in single yeast strain.

4.1.2 Present Study

Saccharomyces boulardii was further selected of purification of acid tolerant phytase. Microorganisms were selected on basis of two criteria, 1] suitability for solid-state fermentation; 2] Acid tolerance. Therefore selection of yeast strain is smart choice that partially fulfills both the criteria.

Phytase is a special class of acid phosphatase, since both phytase and nonspecific acid phosphatases hydrolyze synthetic phosphorylated substrates. Several reports are published for purification of phytase from various microorganisms; here in our study we are purifying phytase from yeast Saccharomyces. The objective of this study was to isolate and purify yeast phytase from major acid phosphatase in order to study its biochemical properties.
Part A: purification and characterization

4.2.1 Enzyme extraction

An overnight grown yeast culture with optical density 0.58 was centrifuged at 4000 RPM for 10 minutes at 20°C, the supernatant were collected in fresh vial and chilled for 20 min in ice cold beaker. Pellets were harvested and subjected to the sonication by probe sonication for 2 min, for lysis of cells in cell lysis buffer that contains PMSF. Cell debris was separated by 3-stage centrifugation in lysis buffer for 30 minutes. The exact volume of supernatant fraction was measured; same procedure was conducted for supernatant of cell lysates, marked as A and B respectively.

4.2.2 Ammonium sulphate precipitation of proteins

1000 ml of supernatant from A and B tubes were transferred into ice tray and incubated on magnetic stirrer for 60 minutes. Generally phytase can be precipitated up to 80% saturation. Solutions were centrifuged and pallets were harvested from various fractions of ammonium sulphate ranging from 0 to 80%. The pellets contain precipitated proteins was dissolved in sodium acetate buffer pH 4.5. Dissolved pellets were dialyzed against 100 mM sodium acetate pH 4.5 in semipermeable dialysis bag and the total protein content was estimated. The precipitates that formed during dialysis were removed by 8000g centrifugation for 10 minutes. This material did not contain any appreciable phytase or acid phosphatase activity.

4.2.3 Protein estimation

Total protein concentration was determined by Comassie blue G-250 dye binding using Bovine serum albumin as standard.

4.2.4 Column chromatography of proteins

The dialyzed ammonium sulphate fraction was loaded onto CM-sepharose CL- 6B cationic exchanger column purchased from sigma, equilibrated with 100mM sodium acetate, pH 4.5 at a flow rate of 50ml per hour, collecting 10 ml fractions. The column was extensively washed with loading buffer, than eluted with linear gradient from 0 to 0.4 M NaCl (400 ml) in 100 mM sodium acetate, pH 4.5. The fractions were subjected to phytase assay and positive fractions
were pooled and concentrated with membrane using an ultra-filtrator (Tarson), then dialyzed against 100 mM sodium acetate. The pooled fraction from previous steps was transferred to sephadex G-200 column equilibrated with 100 mM acetate buffer, at a flow rate of 100 ml per hour. The column eluent was collected and the fraction containing phytase activity was pooled.

### 4.2.5 Native and SDS PAGE Gel Electrophoresis

A 7.5 % SDS gel was prepared and partially purified protein along with *Saccharomyces cerevisiae* derived phytase obtained from Sigma-Aldrich, the band was observed by silver staining of the gel. In another study to determine the native molecular structure of protein, standard 7.5 % native gel electrophoresis was carried out without sodium dodecyl sulphate (SDS) at pH 4.5, following gel electrophoresis, gels were first equilibrated for 20 min at room temperature in 50 mM sodium acetate, prior to staining for enzyme activity. Enzyme activity was detected by immersing the gel in sodium phytate solution [pH 4.5] for half an hour coupled with Fast Garnet B was prepared in 0.6 sodium acetate pH 4.5 [Gibson and Ullah, 1988]. The gel was examined for in- gel activity of phytase immediately after the staining.

### 4.2.6 Optical spectra of purified phytase

The purified phytase was dialyzed against 10 mM sodium acetate, pH 5.0 and than scanned from 500 to 230 nm against a buffer blank using Cystronic model-166.

### 4.2.7 Determination of molecular weight

Sephadex G-100 [Fraction range 4 000 – 100 000] column were manually prepared in sodium acetate buffer. Dextran blue dye was used to determine the fraction collection point, the slurry was carefully packed in glass columns equipped with stopcock. The care was taken to remove any air bubble from the column. As blue dextran eluted from the column, the 5 ml fractions were collected from the column. The sample were loaded with hemoglobin, myoglobin and gamma globulin and vitamin-B12 [Biored], internal molecular standards. Once the vitamin-B12 was eluted from the column, the sample collections were stopped and fractions were analyzed for phytase activity. The column was pre equilibrated in 100mM sodium acetate, at a flow rate of 15 ml per hour, 5 ml fraction. To determine the molecular weight of phytase the
graph of molecular weight of standard and eluted volume were plotted on semi-log graph and the molecular weigh of phytase was determined by comparing with standards.

4.2.8 Procion red He3B-Agarose column chromatography

Approximately 1.0 A280 unit of purified phytase was adjusted to a volume of 5 ml with sodium acetate, the protein was applied to a Procion Red He3B agarose column [Gibbs, 1988], pre-equilibrated in the same buffer. Upon loading the protein, the column was washed with buffer and developed by passing a linear salt gradient (0-500mM NaCl) at flow rate of 80ml per hour. The fractions were collected and their absorbance was measured.

4.2.9 Amino acid composition

A portion of the purified enzyme was reduced alkylated with vinyl chloride, and dissolved in spectroscopic grade trifluoro acetate prior to HPLC on a 5-Mn ultrapore C3 column. Protein was eluted with a gradient of 0.2 % TBA in methanol: propanol, 3:1, 10-70% at 2% increase per minute, at a flow rate of 0.5 cm per minute. The protein peak was used for amino acid analysis. Amino acid standard mixture was used to calculate the best fit one each of the amino acid residue.

Part B: Optimization of Enzyme activity

4.2.10 Effect of temperature on phytase activity

To determine the optimal incubation temperature for phytase activity, reaction mixture phytase [0.2 U/ml] was incubated at various temperature (25°C, 30°C, 37°C, 50°C, 75°C) s keeping other conditions at their optimum level.

4.2.11 Effect of media pH on phytase activity

To determine the effect of growth media pH on phytase production, the production media pH were varied from 2.0 to 6.0 with 1N HCl or 1N NaOH. The reaction was incubated at 50°C.

4.2.12 Time course for enzyme activity

Optimal time for phytase production at pH 4.5 and 55°C was studied by harvesting the production media at different days (0-7 days) and determining the phytase activity.
4.2.13 Effect of aeration on enzyme activity

To study the effect of agitation speeds, flasks with production medium was incubated at static, 120 and 200 rpm.

4.2.14 Effect of surfactants

A level of 0.25% v/v and w/v of Tween-80, SDS, and EDTA were separately added to phytase screen media. The medium without any surfactant served as control.

4.2.14 The effect of phosphate on phytase activity

To study the effect of phosphate on phytase production, the activity of this enzyme in nutrient broth, medium with tricalcium phosphate and medium with sodium phytate was assayed. The three medium was incubated under same condition as above. The phytase activity was measured.

4.2.15 Effect of metal ions on phytase activity

Effect of various divalent metal ions were evaluated at 0.1% concentration of metal ions
4.3 Results and Discussions

4.3.1 Chromatographic purification of phytase

A summary of the purification scheme is given in Table 4.1 page 10. The 30-60% ammonium sulfate fraction contained both acid phosphatase and phytase activities. These activities, however, were separable on the first strong cationic exchange column, CM-Sepharose CL-6B [Figure 4.1]. Under the conditions employed, the majority of phytase activity was not bound to the column, but the acid phosphatase activity was retained on the column and eluted at approximately 300 mM NaCl.

![Absorbance Graph](image)

**Figure 4.1** CM sepharose column, 10-80% fraction from 3 day old culture was chrometographed and developed against the linear gradient of 0 to 0.4 M NaCl, collecting 10 ml fractions, fractions were analyzed for phytase activity, acid phosphatase activity and A280.

A gel filtration step (Sephadex G-200) was employed as the second step in the purification scheme. The fractions containing phytase activity eluted as a shoulder of the first major peak [Figure 4.2].
Figure 4.2 Sephadex G-200 column; the fraction containing phytase activity was chromatographed in gel filtration column using sodium acetate buffer and fractions were analyzed for phytase activity and A280.

The 20-80% [Figure 4.3] ammonium sulphate contained both phytase an acid phosphatase activity Figure4.2. Acid phosphatase activity gradually decreases at increasing concentration of ammonium sulphate. These activities however separable, with strong cationic exchanger CM-sepharose, column was under the given condition majority of acid phosphatase enzyme retained by the matrix while unbound phytase was pooled out from the fractions. The acid-phosphatase enzyme was eluted with 200mM NaCl concentration shows very low phytase activity peak.

Figure: 4.3 (starting from right to left) lane 1molecular weight marker, lane 2 5%, lane 3, 20%, lane 4 50%, lane 5 55%, lane 6, 60%, lane 7 65%, lane 8 70%, lane 9, 77%, lane 10, 80% concentration of ammonium sulphate, when yeast culture grown on minimal medium containing sodium phytate.
4.3.2 Analysis of purity and homogeneity

The concentrated fraction from Sephadex G-200 column was examined by 7.5% SDS PAGE. 3 bands were observed, from them two bands were observed in proximity was of 62KD to 70 KD respectively, while 3 band which was observed was smaller than just of 40KD, when gels were stained for detection of carbohydrate with PAS [periodic acid Schiff reagent] no detectable bands were observed. 7.5% native gel electrophoresis shown one bands indicating the presence of enzyme were observed, the same bands were eluted from PAGE gel and subjected to SDS PAGE analysis, while three bands in SDS containing gel was observed [Figure 4.4], which partially confirmed that purified phytase may have three subunits, the molecular weight is compared with available literature which indicates the subunit may be Phy A and Phy B and Phy C subunits like Aspergillus niger phytase.
Table 4.1 Purification table for phytase

<table>
<thead>
<tr>
<th>Steps</th>
<th>Volume</th>
<th>Total enzyme (nkat)</th>
<th>Total protein (mg)</th>
<th>Total activity (U/ml)</th>
<th>Specific activity (nkat/mg)</th>
<th>Fold purification</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysate</td>
<td>972</td>
<td>48,348</td>
<td>49847</td>
<td>48451284</td>
<td>0.9</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation 10-80%</td>
<td>925</td>
<td>20280</td>
<td>19825</td>
<td>18338125</td>
<td>1.0</td>
<td>0.3</td>
<td>37</td>
</tr>
<tr>
<td>CM-Sephrose column</td>
<td>412</td>
<td>38432</td>
<td>32856</td>
<td>13536672</td>
<td>1.1</td>
<td>1.6</td>
<td>73</td>
</tr>
<tr>
<td>Sephadex-G100</td>
<td>380</td>
<td>32045</td>
<td>18923</td>
<td>7190740</td>
<td>1.9</td>
<td>0.5</td>
<td>53</td>
</tr>
</tbody>
</table>
Lane 1
Proteins loaded in 2.5 µg concentration

Lane 1, 25 µg, lane 2, 10 µg proteins were loaded

Phy B Phy A

Phy C

Figure 4.4: a. Native 7.5% gel stained with fast garnet B. b. CBB G250- stained gel of with SDS, shows the multi-subunit nature of yeast phytase, yeast phytase may contain 3 subunit Phy A, Phy B, Phy C [starting from top 72, 62 and 40 KD respectively].
4.3.3 Interaction of Phytase with Procion Red HE3B-Agarose affinity column

When purified fraction of yeast phytase was applied to a Procion Red HE3B-agarose column, the protein was totally adsorbed and was only eluted in a salt gradient. The interaction of the protein with the ligand was moderate, since a salt concentration of 250-300 mM was needed for complete desorption of the phytase.

4.3.4 Molecular weight determination of native enzyme

To assess the molecular mass of native yeast phytase, enzymes was obtained from the Procion Red HE3B-agarose affinity column (step 4, fractions 20-40) and precipitated with ammonium sulfate (0-80% saturation). The precipitated protein was suspended in 1.5 ml of 10 mM sodium acetate, pH 4.5, and 2 mM mercaptoethanol and applied to Sephadex G-100 gel permeation column. Fractions (5.25 ml) were collected and assayed for phytase. The molecular weight was estimated around 70000 KD by comparing with the standards.

4.3.5 Amino Acid Composition

The peak of protein obtained from HPLC was used for amino acid analysis (Table 4.2). Soybean phytase has fairly high concentrations of glutamic and aspartic acid residues, contributing to its apparent pI of 5.5. The amino acid composition based on a total of 652 residues gave a protein molecular weight of 72,048.

4.3.6 Optical Properties

The purified protein against 10 mM sodium acetate, pH 4.0, and its UV and visible spectra were recorded. The protein showed a typical absorption peak at 280 nm. No detectable absorbance in the visible region was observed, indicating that the enzyme is not an iron or manganese-containing metaloprotein.
Table 4.2: Amino acid composition of yeast phytase

<table>
<thead>
<tr>
<th>Residue</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>39</td>
</tr>
<tr>
<td>Asp</td>
<td>78</td>
</tr>
<tr>
<td>Glu</td>
<td>49</td>
</tr>
<tr>
<td>Phe</td>
<td>29</td>
</tr>
<tr>
<td>Gly</td>
<td>52</td>
</tr>
<tr>
<td>His</td>
<td>9</td>
</tr>
<tr>
<td>Ile</td>
<td>43</td>
</tr>
<tr>
<td>Lys</td>
<td>43</td>
</tr>
<tr>
<td>Leu</td>
<td>52</td>
</tr>
<tr>
<td>Met</td>
<td>62</td>
</tr>
<tr>
<td>Pro</td>
<td>29</td>
</tr>
<tr>
<td>Arg</td>
<td>33</td>
</tr>
<tr>
<td>Ser</td>
<td>48</td>
</tr>
<tr>
<td>Thr</td>
<td>16</td>
</tr>
<tr>
<td>Cys</td>
<td>22</td>
</tr>
<tr>
<td>Val</td>
<td>23</td>
</tr>
<tr>
<td>Tyr</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>652</td>
</tr>
<tr>
<td>Mr</td>
<td>72,048 KD</td>
</tr>
</tbody>
</table>
Part B: Enzyme activity and optimization studies

4.3.7 pH optima

Enzyme assays were performed from pH 2 to 7 using a variety of buffers (Fig. 4.5). The pH optimum was determined to be 4.5–4.8. Activity fell rapidly at pH 6.0 in acetate, and imidazole buffers, while no detectable activity could be observed in malate buffers after pH 5.6. The result indicate that optimum pH of purified phytase is 4.5, irrespective to the type of buffer.

![Figure 4.5 pH optimization of purified phytase in acetate buffer.](image)

4.3.8 Temperature optima

The temperature profile of purified phytase was conducted from 25 to 75°C using a 4 hr incubation period under standard assay conditions at each temperature. The temperature optimum was found to be 50°C under these conditions, and activity fell off rapidly at 60°C [Figure 4.6]. The literature survey of various phytase with their source is supporting this result. Lily pollen, *Aspergillus*, *Bacillus* all other purified phytase have their temperature optima in the range of 50-55 °C [Granier, 2006].
4.3.9 Substrate selectivity

In order to determine the substrate selectivity of phytase, several synthetic substrates were utilized for Km and Vmax determinations. The results are summarized in Table 4.3. Of all the compounds tested, phytate gave the lowest Km value, while p-nitrophenyl phosphate, pyrophosphate, and ATP also gave low Km values. The compounds, 2-glycerophosphate, bis(p-nitrophenyl) phosphate, and phenyl phosphate exhibited higher Km values. The theoretical Vmax values as calculated from the double reciprocal plot are also shown in Table 4.3. Of all the compounds tested, bis(p-nitrophenyl) phosphate, ATP, phenyl phosphate, and p-nitrophenyl phosphate gave a higher Vmax as opposed to 2-glycerophosphate, inorganic pyrophosphate, and phytate.
Table 4.3. Substrate selectivity study of yeast phytase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km</th>
<th>Vmax (pKat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytate</td>
<td>52</td>
<td>149</td>
</tr>
<tr>
<td>p-nitrophenyl phosphate</td>
<td>32</td>
<td>212</td>
</tr>
<tr>
<td>2-Glycerophosphate</td>
<td>300</td>
<td>260</td>
</tr>
<tr>
<td>Bis-Peranitrophenyl phosphate</td>
<td>250</td>
<td>1000</td>
</tr>
<tr>
<td>Phenyl phosphate</td>
<td>128</td>
<td>625</td>
</tr>
<tr>
<td>ATP</td>
<td>40</td>
<td>512</td>
</tr>
<tr>
<td>Pottasium dihydrogen phosphate</td>
<td>23</td>
<td>452</td>
</tr>
<tr>
<td>Rock phosphate</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

4.3.10 Effect of phosphate and other metal ions

The yeast phytase activity was strongly inhibited by 0.1M phosphate concentrations, however the activity of yeast phytase was not inhibited by any other metal ions in over study, no significant change in presence and absence of metal ion was observed.

4.3.11 Production time, effect of surfactant and aeretion agitation on phytase activity

The phytase activity was observed after 24-h of incubation and this observation was continue up to 96-h of incubation, the peak activity was observed at 72-h (0.82 U/ml). Our purified phytase has not showed any activity loss in presence of surfactent studied. Likewise the activities was not affected by aeration and agitation throughout the study. The result indicates that the purified phytase is stable under the presence of metal ions, surfactant and shaking conditions under study, the purified phytase has proven robust under the conditions given. However, the studies contradict the results obtained by the others [Gibbion and Ullah, 1988]. This robustness of purified phytase can make this phytase as ideal choice. This study was used as base for designing the coating stratagies for purified phytase.
4.3.12 $K_{\text{cat}}$ determination

The number of times each enzyme site converts substrate $[^{32}\text{P}]\text{Phytate}$ to product $[^{32}\text{P}]\text{Inorganic phosphate}$ per unit time. The turnover number ($K_{\text{cat}}$) of purified yeast phytase was estimated to be 0.6 for the substrate phytate assuming a molecular weight of 72 KD.

4.3.13 IEF-SDS PAGE analysis of phytase

Preparative SDS-IEF [Figure 4.7], resulted in three distinct bands with pI values of between pH 4.8 and 5.0. The comparison with phytases from other sources [seeds: Maiti and Biswas, 1979; Gibson and Ullah, 1988; pollen: Baldi et al., 1988; and vegetative tissues: Laboure et al., 1993] revealed high similarities between to plant phytase compared to purified bacterial phytase.

![Figure 4.7 IEF-SDS PAGE analysis of phytase [pH 4.7]](image)

The study on phytase activity, comparison with standard in Native PAGE, IEF-SDS PAGE confirms that the purification strategies are appropriate for purification of intracellular as well as extracellular phytases, the study of phytase localization in yeast cell was also performed by cell fractionation followed by sucrose density gradient centrifugation. The enzyme activity of
each fractions were measured and concluded that the phytase was mainly associated with membranes, as maximum activity was observed in membrane fractions.
4.4 Conclusions

*Saccharomyces boulardii* derived phytase is robust in nature. The enzyme gave optimum pH activity at 4.5, temperature 55° C and optimum buffer is sodium acetate. The yeast-derived phytase does not show any significant effect of surfactant and divalent metal ions on enzyme activity. The purification strategy, which employ over here can successfully separate phytase from other acidphosphatase. The 4-step purification strategy is easy to optimize on large scale and for bulk purification of phytase on industrial scale. The nature of yeast-derived phytase is less similar with fungal phytase.