CHAPTER 2

STUDIES ON THE REGULATION OF PHYTASE

In bacteria, the synthesis of phosphatases is known to be influenced by the levels of inorganic phosphorus in the medium. Alkaline phosphatases are produced in measurable amounts only when inorganic phosphorus in the medium becomes limiting. On the other hand acid phosphatases are produced constitutively, being unaffected by the levels of inorganic phosphorus in the medium (Torriani, 1960).

The synthesis of total phosphatase is reported to be repressed by high inorganic phosphorus levels in A. awamori var. kawachii. However the type of phosphatase activity expressed depended on the levels of phosphorus in the medium. In a high phosphorus medium (10-68 mg/100 ml), activity was mostly directed towards β-glycerophosphate (GP) whereas in a low phosphorus medium (0.5-3.0 mg/100 ml) activity was directed towards glucose-6-phosphate (G-6-P), indicating that the specificity of the enzyme had changed. Moreover, the phosphatase produced in a low phosphate medium could be separated into four different fractions by ion exchange chromatography whereas that obtained in a high phosphorus medium was mostly eluted as a single fraction different from the previous four fractions. All fractions differed in their pH optima with GP and G-6-P as substrates. Although this study was not directed specifically towards phytase synthesis, it
was concluded that phytase was a general phosphatase since the use of different phosphate esters like phytin, ribonucleic acid, G-6-P and inorganic phosphate (P_i) in the growth medium resulted in the synthesis of phosphatases which were active against all the above organic substrates and GP, indicating that the enzymes of phosphorus metabolism were activated by non-specific phosphorus sources. Presumably the synthesis of phytase was controlled in a manner similar to that of any other phosphatase (Ohta et al., 1968).

In a similar study with *A. ficuum*, it has been shown that two types of phosphatases are synthesised. These differ in their pH optima. In a high phosphate medium; more of the pH 5.5 enzyme is produced while in a low phosphorus medium more of the pH 2.0 enzyme is produced. Both were active against calcium phytate as well as against some other phosphate esters. These enzymes were clearly different as evidenced by their different pH optima, substrate specificity, resistance to denaturation by heat, inhibition by P_i and the regulation of their synthesis by P_i (Shieh et al., 1969).

In an earlier study, Shieh and Ware (1968) reported that the inhibition of phytase synthesis by P_i is a general phenomenon since it was observed in all the species of molds and yeasts which produced phytase. However, the induction of phytase in *A. ficuum* was dependent on the total phosphorus in the medium since increasing ratios of carbon to phosphorus yielded higher phytase activity at low levels of phosphorus.
In a similar study with *A. ficuum*, Han and Gallagher (1987) showed that phytase production did not require the presence of a phytin/phytate source in the medium. They concluded that phytase synthesis was enhanced by low levels of $P_i$ in the medium. Levels were kept low by using phytic acid and insoluble calcium phytate which induced more synthesis than potassium dibasic phosphate. Nitrate salts favoured phytase synthesis while activity was negligible in media containing yeast extract or peptone. Phosphatase activity was favoured by ammonium salts. A similar effect has been reported by Ghareib (1990) in a study on phytase production by *A. carneus*. The substitution of ammonium sulphate with certain amino acids decreased enzyme yields significantly.

The type of phosphate source is important for the induction of phytase at low levels of $P_i$. In a study with *A. ficuum*, Gibson (1987) has shown that activity obtained in media containing Hylon starch was higher than that obtained in media containing $P_i$, corn starch, potato starch and glucose-6-phosphate. The reason stated for this is that the phosphoester linkage in Hylon starch is more resistant to hydrolytic cleavage than the ones in corn or potato starch, thus resulting in a low but steady supply of available phosphorus.

In general, the synthesis of fungal phytase is inhibited by high $P_i$ levels and is favoured when the concentration of $P_i$ is low. However, in *A. terreus*, it has been reported that the synthesis of phytase is not induced by $P_i$. Its synthesis requires the presence of a phytin source in the medium. The type of phytin
is also important. Rice bran induced maximum synthesis, while soybean did not induce any phytase synthesis. Barnyard grains, corn, french beans and wheat bran induced intermediate activity. The addition of starch and glucose had an inhibitory effect on enzyme synthesis. Among the different nitrogen containing salts, ammonium salts were the best for phytase synthesis, while in media containing nitrate salts, phytase activity was nil (Yamada et al., 1968).

The inhibitory effect of glucose has also been reported by (Nair et al., 1991) during a study on phytase production by A. ficuum in submerged batch process.

The physical state of the medium plays an important role in phytase synthesis. Thus, semisolid media containing phytin yield larger amounts of phytase as compared to liquid media. The repressing effect of \( P_1 \) is also less apparent in semisolid media than in liquid media (Han et al., 1987).

Sea water has been reported to have an enhancing effect on phytase synthesis. Extracellular phytase production by A. flavipes and intracellular phytase production by Macrophomina phaseolina were maximal when the culture medium was supplemented with 10% and 20% sea water respectively. A study of the effect of different metal ions showed that Mg++, Mn++, Ca++ and Fe+++ favoured enzyme synthesis by A. flavipes and Zn++, Co++, Mn++ and Mg++ had a stimulatory effect on enzyme production by M. phaseolina. Both Na+ and K+ had an inhibitory effect on phytase synthe-
sis by both organisms (Ghareib et al., 1986).

The effect of surfactants on the production of phosphatase and phytase by A. ficuum is different for the two enzymes. Both phosphatase and phytase production were increased by the addition of Triton X-100, Tween 80 and sodium oleate. However, the increase in phosphatase production was more than that for phytase when Triton X-100 was used. On the other hand, phytase activity was greatly enhanced as compared to phosphatase when sodium oleate was used. The underlying biochemical mechanism of this was not studied (Han et al., 1987).

Few bacteria are known to produce phytase. Three cultures were isolated by Cosgrove et al. (1970), out of which only one, a Pseudomonas species, was used in further studies (Irving and Cosgrove, 1971). Enzyme synthesis was induced both by inositol and phytate in this culture. The effect of inorganic phosphorus was not studied.

Wheat bran extract was used to induce enzyme synthesis in Bacillus subtilis by Powar and Jagannathan (1967). In a later study (1982) they reported that the enzyme was induced by yeast extract, wheat bran extract and sodium phytate in media containing either starch, glucose or maltose as carbon sources. Phytase was synthesised faster in a medium containing casein. Both inositol and glucose did not induce any enzyme synthesis. The effect of different metal ions and P_i was not studied.

Aerobacter aerogenes (now called K. aerogenes) was reported
to produce phytase in nutrient broth (Greaves et al., 1967). No studies were carried out to determine which component was responsible for phytase induction.

In a study with *Klebsiella* sp. no. PG-2, Shah and Parekh (1990) reported that phytase was induced only by phytate. No other carbon source, including inositol, induced enzyme synthesis.

**AIMS AND OBJECTIVES**

Preliminary work in the laboratory had shown that phytase activity could be induced in *K. aerogenes* by phytate, inositol and yeast extract. Glucose was found to be a repressor whereas acetate and citrate had no effect on enzyme synthesis (Tambe, 1989). However, these studies were carried out using media, the composition of which was not precisely defined. This was due to the inclusion of yeast extract, which also induced enzyme synthesis in the medium. Besides, the effect of inorganic phosphorus on enzyme synthesis was not studied. Keeping this in mind, it was thought of interest to study the regulation of phytase and compare it with that of other phosphatases in the same organism.

**MATERIALS AND METHODS**

**Culture**

*Klebsiella aerogenes* was used in the present study. It was grown in 20 ml aliquots of minimal medium containing 20 mM glycerol and 5 μM P$_i$ for inoculum preparation (for composition, see Appendix II). This was then inoculated into 100 ml aliquots of
the same medium containing different carbon sources and different phosphorus sources, at a strength of 2% v/v. The culture was incubated on a rotary shaker (120 rpm) at 30°C, for 12 h. The cells were then harvested by centrifugation, at 25000 x g for 20 min, in a refrigerated centrifuge. The cells were resuspended in 10 mM Tris-HCl buffer, pH 8 and washed. These were then finally resuspended in 10 ml of the same buffer.

Unless mentioned otherwise, conditions for inoculum preparation, growth, harvest and resuspension were kept the same in all studies.

**Phosphatase assay:**

The conditions for phosphatase assay were as given in table 3.1.

**Protein assay:**

Protein was estimated by the method of Lowry et al. (1951), using bovine serum albumin (BSA) as standard.

Since the synthesis of phosphatases is sensitive to the levels of inorganic phosphorus, studies were carried out to determine the following:

1) Concentrations of inorganic phosphorus which are limiting and non-limiting for growth.

It was necessary to determine the minimum level (limiting) of \( P_i \) which would support growth and the maximum level (non-limiting) of \( P_i \) beyond which no significant increase in growth would be observed.
Conditions for phosphatase assay

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pNPP / GP, equivalent to 50 µmols Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>100 µmols</td>
</tr>
<tr>
<td>Cells</td>
<td>equivalent to 500 µg protein</td>
</tr>
<tr>
<td>Total volume</td>
<td>2 ml</td>
</tr>
<tr>
<td>Temperature of incubation</td>
<td>45°C</td>
</tr>
<tr>
<td>Time of incubation</td>
<td>20 min</td>
</tr>
<tr>
<td>Start of reaction</td>
<td>Addition of cells</td>
</tr>
<tr>
<td>Termination of reaction</td>
<td>Addition of 2 ml of 10% TCA</td>
</tr>
<tr>
<td>Treatment of blank</td>
<td>2 ml 10% TCA added to the assay mixture before adding cells</td>
</tr>
<tr>
<td>Parameter measured</td>
<td>P_1 by the method of Fiske and Subba Row (1925)</td>
</tr>
<tr>
<td>Enzyme units</td>
<td>µmol of P_1 liberated per min under assay conditions</td>
</tr>
</tbody>
</table>

These were determined by growing the culture in minimal medium with 20 mM glycerol (60 mM carbon) and different concentrations of inorganic phosphorus. The inoculum was prepared as stated earlier and was inoculated in 50 ml aliquots of the medium in 250 ml flasks containing different concentrations of phospho-
rus. The absorbance of the cultures was read in a colorimeter at 660 nm (red filter), at 6h, 9h and 12 h after inoculation.

2) pH profile of phosphatases at limiting and non-limiting concentrations of phosphorus.

A study was carried out to determine the optimum pH for phosphatase activity. 100 ml aliquots of minimal medium containing 20 mM glycerol and either 5 μM or 50 mM phosphorus were inoculated. These were incubated as before, for 12 h. The cells were harvested and resuspended as stated before and assayed for phosphatase activity at different pH using the buffers given in Table 3.2.

<table>
<thead>
<tr>
<th>pH</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2 to 3.6</td>
<td>Glycine-HCl</td>
</tr>
<tr>
<td>3.6 to 6.0</td>
<td>Acetate</td>
</tr>
<tr>
<td>6.2 to 8.0</td>
<td>Imidazole-HCl</td>
</tr>
<tr>
<td>8.0 to 9.5</td>
<td>Tris-HCl</td>
</tr>
<tr>
<td>9.0 to 10.5</td>
<td>Glycine-NaOH</td>
</tr>
</tbody>
</table>
3) Effect of various carbon sources on the production of phosphatases at high and low levels of P₁

Studies were done to check whether the profile of phosphatase activity changes with the carbon source. The inoculum was prepared as before and inoculated into 100 ml aliquots of minimal medium containing either 5 μM or 50 mM phosphorus and either glycerol, glucose or inositol as the sole sources of carbon. All were taken at a concentration of 60 mM carbon in the medium. The cultures were incubated as before and harvested similarly. The cells were resuspended and assayed for phosphatase activity, at pH 4.6 and 10.5, which were found to be the optimum pH. The assay conditions were maintained as before. The substrates used for assay were glucose-6-phosphate (G6P), β-glycerophosphate (GP), phytate (Phy) and p-nitrophenyl phosphate (pNPP). The buffers used were acetate for pH 4.5 and glycine-NaOH for pH 10.5.

4) Effect of various carbon sources on the production of phosphatase activity in the presence of an organic phosphorus source.

The effect of different carbon sources in the presence of an organic phosphorus source, on the profile of phosphatase activity was studied. The inoculum was prepared as before and inoculated into 100 ml aliquots of minimal medium containing either glycerol, inositol or glucose at 60 mM carbon and pNPP at 30 mM concentration. The medium was buffered at pH 7.2 using 50 mM Tris-HCl. Conditions for incubation were maintained as before. The cells were harvested as before and assayed for phosphatase activity.
5) Effect of insoluble phosphates as sources of phosphorus on the production of phosphatases.

The inoculum was prepared as before and inoculated into 100 ml aliquots of minimal medium containing insoluble hydroxyapatite and calcium phytate at a concentration of 50 mM and either glycerol, inositol or glucose at a concentration of 60 mM carbon. The cells were harvested as before and assayed similarly.

6) Effect of supplementation of insoluble organic phosphorus source, with soluble phosphorus, on the production of phosphatases.

100 ml aliquots of minimal medium containing calcium phytate were supplemented with soluble P₁ at a concentration of 1 mM and the culture was grown as before and assayed for phosphatase activity.

7) Effect of combined sources of phosphorus and carbon on the production of phosphatases.

The culture was grown as before in minimal medium containing 5 μM phosphorus and 20 mM glycerol. It was then inoculated into 100 ml aliquots of minimal media containing either glycerophosphate, glucose-6-phosphate or phytate at a concentration of 60 mM carbon. Glucose was also used in combination with phytate and glycerophosphate, keeping the carbon concentration at 60 mM. The cultures were incubated as before and assayed for phosphatase activity.
RESULTS AND DISCUSSION

1) Concentrations of inorganic phosphorus which are limiting and non-limiting for growth

Of the various concentrations of P ranging from 5 μM to 100 mM, which were tried out, it was found that a concentration of 5 μM was limiting while 50 mM was the minimum concentration that was non-limiting (figure 3.1). At higher concentrations no increase in growth was obtained, indicating that at these concentrations some other nutrient or experimental condition could be limiting. Hence it was decided that a concentration of 5 μM could be used for growing the culture under phosphorus limiting conditions while a concentration of 50 mM could be used for non-limiting conditions.

2) pH profile of phosphatases at limiting and non-limiting concentrations of phosphorus

Two peaks of enzyme activity were obtained with both the substrates, one at a pH of 4.6 and the other at 10.5 (fig. 3.2). This is in agreement with the reported values for Escherichia coli (Torriani, 1960). Hence it was decided to use these pH in assays of phosphatases in further studies.

3) Effect of carbon sources on the production of phosphatases at high and low concentrations of inorganic phosphorus

It can be seen from the accompanying figures (3.3-3.6) that acid phosphatase is a constitutive enzyme while alkaline phospha-
Fig. 3.1: Effect of inorganic phosphorus concentration on growth

O.D. (680 nm)

Phosphorus concentration (mM)

0 20 40 60 80 100 120

6 h 9 h 12 h
Fig. 3.2: pH optima of phosphatase activity in *Klebsiella aerogenes*

1) Only data for pNPP has been shown
2) Activity relative to the maximum obtained has been shown
Fig. 3.3: Acid phosphatase activity in *K. aerogenes* grown on different carbon sources at 5 μM inorganic phosphorus.

All carbon sources were used at a concentration of 60 mM C.
Fig. 3.4: Acid phosphatase activity in *K. aerogenes* grown on different carbon sources at 50 mM inorganic phosphorus.

**Specific activity**

- **Glycerol**
- **Glucose**
- **Inositol**

**Assay substrates**
- pNPP
- G6P
- GP
- Phy

All carbon sources were used at a concentration of 60 mM C.
Fig. 3.5: Alkaline phosphatase activity in *K. aerogenes* grown on various carbon sources at 5 μM inorganic phosphorus.

All carbon sources were used at a concentration of 60 mM C.
Fig. 3.6: Alkaline phosphatase activity in *K. aerogenes* grown on various carbon sources at 50 mM inorganic phosphorus.

All carbon sources were used at a concentration of 60 mM C.
Taste is a phosphate repressible enzyme as evidenced by the activities obtained with cultures grown in media containing 5 μM and 50 mM inorganic phosphate. The activities towards GP and G-6-P were low. A comparison of the specific activity towards pNPP as substrate shows that the level of activity in the two media does not differ considerably. On the other hand phytase activity is detected with inositol in the low phosphate medium and with all three carbon sources in the high phosphate medium. This can be explained by the fact that limiting concentrations of inorganic phosphorus can induce phytase. Thus in the low phosphate medium the amount of total activity (if induced) was too low to be detected on account of the poor growth in this medium. On the other hand activity was detectable in the high phosphate medium because of better growth. Since the cultures were harvested in the late log phase of growth it is presumed that the enzyme is induced by the limiting concentration of inorganic phosphorus in the medium at that stage. When glucose was used as the carbon source, it was observed that the levels of acid phosphatase activity were low in both high and low phosphate media.

A comparison of the alkaline phosphatase activities under these conditions reveals that although the total activity is nearly the same in both, the specific activity changes significantly, being high in the low phosphate medium and low in the high phosphate medium. With glucose as carbon source, the specific activity of the enzyme does not vary under the two conditions suggesting that glucose has an effect overriding that of inorganic phosphorus.
No alkaline phytase activity was detected under any condition.

4) Effect of carbon source on the production of phosphatases in the presence of an organic phosphorus source

With an organic phosphorus source (pNPP) no alkaline phosphatase activity was detected suggesting that this substrate is primarily degraded by acid phosphatase. Besides, the rapid hydrolysis of this organic phosphate in the growth medium would lead to levels of $P_i$, inhibitory to alkaline phosphatase synthesis.

The activity of acid phosphatases in cells grown on glucose, was low. On the other hand significant activity was obtained with glycerol and inositol as carbon sources (fig.3.7). As stated earlier, acid phosphatase activity was low in the presence of glucose, and hence this would also explain the relatively poor growth obtained when glucose was combined with an organic phosphorus source.

5) Effect of insoluble phosphorus sources on the production of phosphatases

No growth was observed in any of the flasks indicating that both insoluble phosphates i.e., hydroxyapatite and calcium phytate, could not be utilised. This reflected the inability of the culture to solubilise these phosphates.
Fig. 3.7: Acid phosphatase activity in *K. aerogenes* grown on different carbon sources and 30 mM pNPP.

All carbon sources were used at a concentration of 60 mM C.
6) Effect of supplementation of insoluble phosphorus source, with soluble phosphorus, on the production of phosphatases

The addition of low amounts of soluble inorganic phosphate to calcium phytate stimulates growth. Acid phosphatase activities against all the four substrates, were produced to the same extent with all carbon sources (fig. 3.8). Phytase activity was the same in all cases suggesting that a poorly soluble source of phosphorus is a good inducer of phytase activity. Activity obtained with inositol was not different from that obtained with glycerol and glucose suggesting that the role of inositol is secondary to that of the availability of phosphorus.

7) Effect of combined sources of phosphorus and carbon on production of phosphatases

With combined sources of phosphorus and carbon, it was found that alkaline phosphatase activity was very low (fig. 3.9). In fact it was not detectable at all when phytate was used. On the other hand acid phosphatase activity, with all the substrates, was obtained to the same extent in all media (fig. 3.10).

The amount of phytase activity was higher in the case of phytate medium, pointing to the inductive effect of phytate. However, in the presence of glucose, this activity was brought down to the levels prevalent in the other media.

The influence of inorganic phosphorus on the synthesis of acid and alkaline phosphatases by *K. aerogenes* was similar to that reported with *E. coli* (Torriani, 1960) and *A. ficuum* (Ohta
Fig. 3.8: Acid phosphatase activity in *K. aerogenes* grown on different carbon sources, 1 mM Pi and 50 mM Ca-phytate.

All carbon sources were used at a concentration of 60 mM C.
Fig. 3.9: Acid phosphatase activity in *K. aerogenes* grown on combined sources of carbon and phosphorus.

All combined sources were taken at a concentration of 60 mM C.
Fig. 3.10: Alkaline phosphatase activity in *K. aerogenes* grown on combined sources of carbon and phosphorus.

All combined sources were taken at a concentration of 60 mM C.
Acid phosphatases are produced constitutively whereas alkaline phosphatases are repressed at high concentrations of inorganic phosphorus. However, alkaline phosphatase activity was not detectable in cultures grown with either an organic phosphorus source (pNPP) or an insoluble phosphorus source (calcium phytate). Two reasons can be cited for this observation:

1) The hydrolysis of pNPP, which is a good substrate for acid phosphatases, could possibly lead to levels of inorganic phosphorus inimical to the synthesis of alkaline phosphatase.

2) Usually, insoluble phosphates need to be solubilised by organic acids, leading to conditions which are unsuitable for the activity of alkaline phosphatase. Expectedly, under such conditions the synthesis of alkaline phosphatases would be minimal/absent and that of acid phosphatases would be high, as is evident in the present case.

The production of phytase, which is an acid phosphatase, is influenced by low levels of inorganic phosphorus. Irrespective of the carbon source, the enzyme was induced at low levels of inorganic phosphorus, which may be initially present or may result from the exhaustion of soluble phosphorus in the medium. Low levels of phosphorus are also obtained by the inclusion of calcium phytate in the medium. However, it is necessary to add small amounts of soluble phosphorus (1 mM) to the medium to initiate growth. This culture seems to be incapable of solubilising insol-
uble phosphates when these are present as sole sources of phosphorus. However, supplementation with low amounts of soluble phosphorus enables the organism to grow and produce significant amounts of phytase. In fact, the levels of activity are the same in all the cases unlike when free inorganic phosphorus is used. In the latter case, the inductive effect of inositol can be seen as evidenced by the higher activity obtained as compared to that obtained with the other two carbon sources viz., glucose and glycerol.

Glucose has a repressive effect on phytase synthesis. This is evident from the low activities obtained when glucose was added to phytate and GP containing media. Under these conditions, phytate seems to be the best inducer (in fact, maximum specific activity is seen in this case), while GP does induce activity to some extent. However, the addition of glucose in both the cases brought down the production of phytase. The high inductive effect of phytate can be put down to three reasons:

1. phytate is a poorly hydrolysable source of phosphorus,
2. thereby leading to
3. low levels of inorganic phosphorus and
4. the release of inositol which is also an inducer.

On the other hand, GP does not induce significant amounts of activity. From these studies, it seems that phytase is a distinct acid phosphatase since only its synthesis is affected by the addition of glucose. Other phosphatases are unaffected.

Thus, it can be concluded that phytase is a distinct phos-
phatase. However, like other acid phosphatases, it is induced by low levels of inorganic phosphorus. An insoluble source of phosphorus is a good inducer for the production of acid phosphatases since its presence can lead to constant low levels of inorganic phosphorus in the medium. Inositol is a good inducer of phytase. However, its inductive effect is minimal when calcium phytate is present in the medium. Phytate is the best inducer for the synthesis of phytase.

CONCLUSIONS

It may be concluded that when glycerol and inositol are used as carbon sources,
1) acid phosphatases are constitutive
2) alkaline phosphatases are repressed by phosphate
3) alkaline phosphatases are not synthesised in media containing poorly utilisable sources of phosphorus such as insoluble calcium phytate. They are also not synthesised in media containing organic phosphorus.
4) a low availability of phosphorus and phosphate starvation are good inducing conditions for phytase synthesis. These conditions are met by low concentrations of $P_i$, insoluble calcium phytate and organic phosphorus source (combined with and independent of carbon source).
5) Phytase is induced by inositol and phytate.
6) Glucose represses the induction of phytase by phytate.

The effect of glucose is dissimilar to that of the other two carbon sources viz., glycerol and inositol. Acid phosphatase
activities are low in glucose media while the inhibition of alkaline phosphatase synthesis by phosphate is removed, when glucose was used as the carbon source.

Phytase is a distinct phosphatase as evidenced by the absence of activity in cells grown with glucose and pNPP, although these cells had the ability to hydrolyse pNPP at pH 4.5.

REFERENCES


