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Full Length Research Paper

Immobilization of starch phosphorylase from seeds of Indian millet (*Pennisetum typhoides*) variety KB 560

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Starch phosphorylase has been isolated from the seeds of millet (*Pennisetum typhoides*) variety KB560 and partially purified using ammonium sulfate fractionation. The partially purified enzyme was desalted using Sephadex G-25 chromatography. In the direction of polysaccharide synthesis, the enzyme showed optimum activity at pH 6.0 with two half pH optima at pH 5.4 and pH 7.0 whereas in the direction of glucose-1-phosphate formation, it showed optimum pH at pH 7.6 with half pH optima at pH 6.7 and 7.9. The optimum temperature for the enzyme activity has been found to be 37°C with two half temperature optima at 34 and 40°C. The partially purified enzyme has been immobilized using brick dust as solid support. The percentage retention of the enzyme on brick dust was nearly 80%. After immobilization, specific activity of the enzyme increased from 0.816 to 2.89. Upon immobilization, there was a slight alkaline shift in the optimum pH when assayed in both the directions. The immobilized enzyme also displayed increased optimum temperature and thermo-stability and could be reused number of times. The increase in thermo-stability and reusability of the immobilized enzyme has been exploited for the production of glucose-1-phosphate, a cytostatic compound used in cardio-therapy. The glucose-1-phosphate produced has been purified with nearly 95% purity after adsorption chromatography on norite and ion exchange chromatography on DEAE cellulose.

Key words: Starch phosphorylase, immobilization, glucose-1-phosphate, brick dust, millet.

INTRODUCTION

Starch phosphorylase (EC 2.4.1.1.; α-1,4-glucan, orthophosphate, α-glucosyl transferase) catalyzing the reversible conversion of starch and inorganic phosphate into glucose-1-phosphate, plays an important role in starch metabolism in plants. It can be used for the production of glucose-1-phosphate, a cytostatic compound used in cardio-therapy (Weinhausel et al., 1994). Starch phosphorylase may also be used to estimate inorganic phosphate in serum under the pathological conditions as well as to detect amount of inorganic phosphate pollution in the environment. Immobilized enzymes are in great demand in industries due to their reusability. In spite of its great importance, starch phosphorylase has been immobilized from few sources only. Kumar and Sanwal (1981) for the first time immobilized starch phosphorylase from mature banana leaf on methylene bis acrylamide and also characterized immobilized enzyme. Potato phosphorylase was subsequently immobilized by coupling to an insoluble support through diazonium salts and covalent bonding between the enzyme and Eupergit C (Szulezynski, 1986). Zeeman et al. (2004) showed that plastidial α-glucan phosphorylase from *Arabidopsis* is not required for starch degradation since loss of its activity by T-DNA insertions resulted in no significant change in total accumulation of starch during the day or its remobilization at night. They also showed that it plays a role in the capacity of the leaf lamina to endure a transient water deficit. Chen et al. (2002) showed regulation of the catalytic behavior of starch phosphorylase from sweet potato roots by proteolysis. They showed the presence of 78 amino acids in the center of the enzyme protein that serves as a signal for rapid degradation. After nicking in the middle of the molecule, protein still retained tertiary and quaternary structure as well as full catalytic activity. Hsu et al. (2004) reported purification and characterization of a cytosolic starch phosphorylase from etiolated rice seedlings. Jorgensen et al. (2006) showed the presence of starch phosphorylase protein in potato tuber juice after starch isolation. They also showed that it con-

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stitutes a major protein (nearly 4% of total proteins in the juice). Michiyo et al. (2006) established a system to produce synthetic amylose from either sucrose or cellobiose using the combined action of α-glucan phosphorylase with sucrose phosphorylase or cellobiose phosphorylase.

Our laboratory is engaged in studying starch phosphorylase and its immobilization on various solid supports (Venkaiah and Kumar, 1994, 1995; Srivastava et al., 1996; Upadhye and Kumar, 1996). In the present study, isolation, partial purification and immobilization of starch phosphorylase from germinating millet seeds has been reported. The immobilized enzyme has been exploited for the production of glucose-1-phosphate.

MATERIALS AND METHODS

Chemicals

Tris, glucose-1-phosphate, Sephadex –G-25, 2-mercaptoethanol, bovine serum albumin (fraction V) were purchased from Sigma-Aldrich, USA. All the other chemicals used were of high quality procured locally.

Seeds of millet

Millet variety KB 560 seeds were purchased from Seed Market, Indore. Seeds were washed with tap water thoroughly and subsequently with distilled water. Thereafter, these were surface sterilized with 0.1% mercuric chloride solution for 2 - 3 min. The surface sterilized seeds were kept for germination at 30°C in petri plates containing four folds of Whatman filter paper soaked with distilled water. No nutrient was provided except distilled water after every 24 h.

Germination profile

From the zero day (the day seeds were soaked), the enzyme activity was determined every day and up to seventh day and the germination profile was drawn.

Enzyme extraction and partial purification

All the procedure was carried out at 0 - 4°C in a cold room. The germinating seeds at various days were blended with 90 ml of isolation medium using a Waring blender for 30 s low speed (nearly 7000 rpm) and 60 s at high speed (nearly 15000 rpm). The isolation medium was consisted of 50 mM tris-HCl buffer, pH 7.6 containing 10 mM 2-mercaptoethanol, 20 mM EDTA and 1% (v/v) triton-X-100. The homogenate was filtered through two layers of a muslin cloth, made the volume 100 ml with the isolation medium and centrifuged at 15,000 x g for 30 min in a Sorvall RC 5B super speed cooling centrifuge. The supernatant containing the enzyme activity was taken as initial extract. To the initial extract, powdered ammonium sulfate was slowly added with constant stirring to get 0 - 30% saturation and the pH was maintained at 7.6 by the addition of dilute ammonia. After storage for 5 h, it was centrifuged at 15000 x *g* for 20 min and the supernatant having most of the activity was brought to 65% saturation with powdered ammonium sulfate. After overnight incubation, the suspension was centrifuged at 15000 x *g* for 20 min. The pellet was dissolved in buffer A (20 mM tris-HCl, pH 7.6 containing 5 mM 2-mercaptoethanol and 20 mM EDTA), centrifuged and the supernatant was desalted using Sephadex-G-25 column chromatography. The desalted enzyme was used for further study.

Enzyme assay

The enzyme assay for the soluble and the immobilized enzyme was carried out in the direction of polysaccharide synthesis as described by Kumar and Sanwal (1981) with some modifications. The enzyme assay system for the soluble enzyme was consisted of 0.2 ml of 0.2 M tris-maleate buffer, pH 6.0; 0.1 ml of 0.02M sodium fluoride; 0.1 ml of 3% soluble starch and 0.5 ml of the enzyme preparation and water (water was added with partially purified enzyme if amount of the enzyme preparation taken was less than 0.5 ml), pre-incubated at 37°C for 2 min. The reaction was started by the addition of 0.1 ml of 0.05 M glucose-1-phosphate. After 30 min, the reaction was stopped by the addition of 1 ml of 10% TCA and the tubes were put in an ice bath. The precipitate formed was removed by centrifugation in the cold. In the clear supernatant, inorganic phosphate formed was estimated using colorimetric method of Fiske and Subbarow (1925). The activity of immobilized enzyme was measured using a water bath shaker. The assay system was scaled up five times. The reaction was stopped by centrifugation at 10000 x *g* for 5 min in cold condition. The amount of the inorganic phos-phate formed in the supernatant was measured using the colorimetric method of Fiske and Subbarow (1925). One unit of the enzyme activity was taken as the amount of the enzyme required to liberate one micromole of inorganic phosphate in one min under the conditions of the enzyme assay.

The enzyme assay was also carried out in the direction of glucose-1-phosphate synthesis as described by Kumar and Sanwal (1981). The enzyme assay system for the soluble enzyme consisted of 0.2 ml of tris- maleate buffer (0.2 M, pH 7.0), 0.1 ml of 5% freshly prepared soluble starch, 0.1 ml of 0.2 M sodium fluoride and 0.5 ml of the enzyme preparation and water (water was added with partially purified enzyme if amount of the enzyme preparation taken was less than 0.5 ml)., pre-incubated at 37°C for 2 min. The reaction was started by the addition of 0.1 ml of 0.5 M NaH_2PO_4-Na_2HPO_4, pH 7.0. After 30 min, the reaction was stopped by keeping the tubes in a boiling water bath for 2 min and the precipitate formed was removed by centrifugation in the cold. In the clear supernatant, glucose-1-phosphate formed was estimated using the phosphoglucomutase and glucose-6-phosphate coupled enzyme assay method (Bergmeyer and Klotzsch, 1965). The activity of immobilized enzyme was measured using a water bath incubator shaker. The assay system was scaled up five times. After 1 h, the reaction was stopped by centrifugation at 3000 x *g* for 10 min in the cold. The amount of glucose-1-phosphate formed was estimated in the supernatant as described above with the soluble enzyme. One unit of the enzyme was taken as the amount of the enzyme required to synthesize one micromole of glucose-1-phosphate in one min under the conditions of the enzyme assay.

Protein estimation

Protein was estimated using the method of Lowry et al. (1951) as modified by Khanna et al. (1969) using bovine serum albumin as a standard. In the case of immobilized enzyme, protein was estimated by subtracting the amount of protein present in the concentrated pooled washings of the immobilized enzyme from the amount of the protein taken for immobilization.

Enzyme immobilization

Our Laboratory is involved in enzyme immobilization on various solid supports including brick dust. In the present study, brick dust

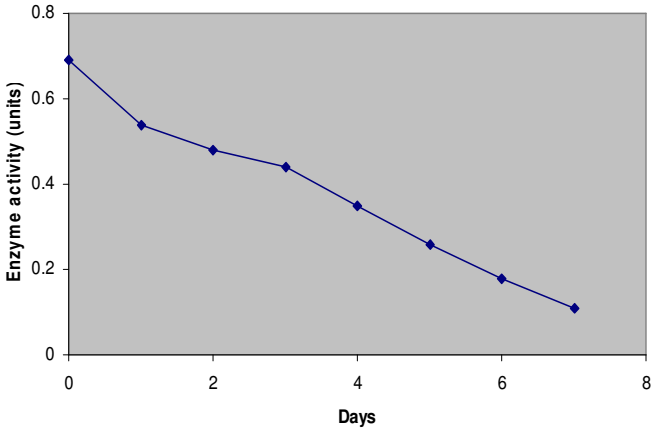

Figure 1. Enzyme activity profile of starch prosphorylase in germinating millet seeds.

of 1 to 1.5 mm mesh size was washed under running tap water. Thereafter, it was treated with 0.5 N hydrochloric acid for 30 min and subsequently washed with distilled water to remove hydrochloric acid. This treatment of hydrochloric acid was repeated once. After that, washed brick dust was equilibrated with 0.5 M tris-maleate buffer, pH 6.0 (buffer B). To 8 ml of desalted enzyme preparation (58 mg protein), 5 gm of equilibrated wet brick dust was added with slow but constant stirring. Thereafter, glutaraldehyde was added to the suspension to a final concentration of 0.9% and the suspension was incubated at 0 to 4°C for 4 h with frequent but gentle stirring. After 4 h, decantation was done and brick dust was washed with buffer B till no protein was detected in the washings. Finally, brick dust was suspended in 8 ml of buffer B.

Optimum pH

In the direction of polysaccharide synthesis, enzyme assay was carried out in the range varying from pH 4.5 to pH 8 using tris-maleate buffer to find out the optimum pH at which the enzyme shows maximum activity. Whereas, in the direction of glucose-1-phosphate formation, enzyme assay was carried out in the range varying from pH 6.2 to pH 8.5.

Optimum temperature

In both polysaccharide synthesis and glucose-1-phosphate formation directions, enzyme assay was carried out in the range of 20 to 55°C to find out the optimum temperature.

Thermal stability

Thermal stability studies were carried out by incubating the enzyme in capped tubes in a water bath at different temperatures between 30 and 55°C. Every 30 min, suitable aliquots were withdrawn from the tubes and stored on ice before carrying the enzyme assay.

Enzyme reusability

Each day, starch and sodium phosphate buffer were passed through the immobilized enzyme column at 30°C for 5 h followed by washing with 5 times the bed volume of 0.02 M tris-HCl buffer, pH 7.5 and incubation under the cold condition. A single fraction was collected on each run and glucose-1-phosphate in the effluent was determined by the coupled enzyme assay method (Bergmeyer and Klotzsch, 1965).

Glucose-1-phosphate (product) purification

The effluent collected from the immobilized enzyme column was passed through a norite-celite (2:1) column (1.5 x 10 cm), previously equilibrated with distilled water, and introduced as a glucose-1-phosphate trap with the column. Glucose-1-phosphate was eluted from the column at a flow rate of 10 ml/h using water as the eluent. The effluent was further purified by ion-exchange chromatography using a DEAE cellulose column (1.5 x 10 cm) previously equilibrated with distilled water. Glucose-1-phosphate was eluted from the column using 0.02 M potassium acetate buffer, pH 7.0 at a flow rate of 10 -12 ml/h. The product thus obtained was precipitated with two volumes of 95% ethanol and dried in a vacuum oven at 60°C. The final product was tested using phospho-glucomutase and glucose-6-phosphate dehydrogenase coupled enzyme assay (Bergmeyer and Klotzsch, 1965).

RESULTS AND DISCUSSION

Germination profile

Starch phosphorylase activity was determined during germination of millet seeds starting from zero day up to 7 days. It was found that activity of the enzyme decreased with germination time, maximum being at the zero day. Therefore, zero day germinated seeds were used for extraction and partial purification of the enzyme (Figure 1).

Enzyme Extraction and partial purification

Ammonium sulfate fraction (30 - 65%) contained about 72 % of the enzyme activity compared to crude extract. After desalting by using Sephadex G-25 chromatography, almost 100% of the enzyme was recovered containing 47 units and 58 mg protein exhibiting specific activity of 0.81 units/mg protein. The purification profile of the enzyme has been shown in Table 1.

Enzyme Immobilization

The partially purified enzyme was then subjected to immobilization on brick dust. The specific activity of the immobilized enzyme was 2.89 units/mg protein whereas the soluble enzyme had 0.81 units/mg protein, thereby immobilized enzyme showed about 3.5 fold purification. The percentage retention of starch phosphorylase upon immobilization was nearly 80% (Table 2). Earlier, Venkaiah and Kumar (1994) showed retention of 46% activity of starch phosphorylase from sorghum leaves using egg shell as solid support. Srivastava et al. (1996) reported retention of about 50% activity of starch phos-

Table 1. Purification profile of starch phosphorylase from millet seeds.

Purification step	Enzyme activity (units)	Protein (mg)	Specific activity (units/mg)	Recovery (%)	Enrichment (fold)
Crude homogenate	66	254	0.260	100	-
30 - 65% ammonium sulfate fraction	48	59	0.814	72.7	3.13
Sephadex G-25 Chromatography	47	58	0.810	71.2	3.11

Table 2. Immobilization of starch phosphorylase on brick dust.

Support	Soluble enzyme			Immobilized enzyme			Retention of enzyme activity (%)	Purification (fold)
	Total Activity (units)	Total Protein (mg)	Sp. act. (units/mg protein)	Total activity (units)	Total protein (mg)	Sp. Act. (units/mg protein)		
Brick dust	47.6	58.3	0.81	38.1	13.2	2.89	80	3.54

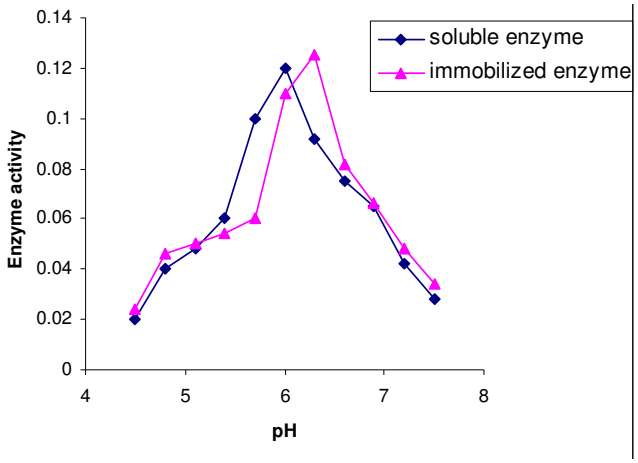

Figure 2A. pH-enzyme activity (in the direction of polysaccharide synthesis) relationship of starch phosphorylase.

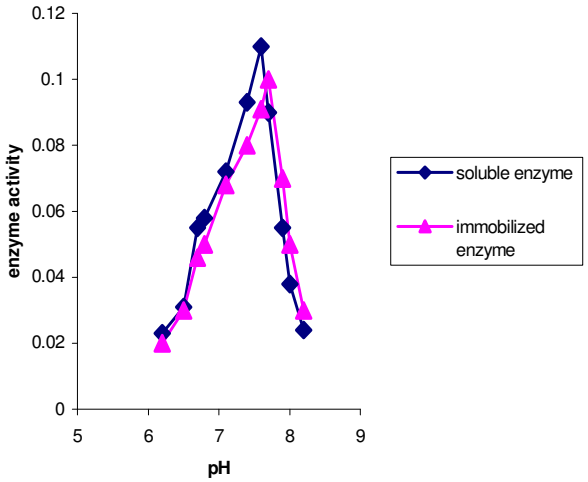

Figure 2B. pH –enzyme activity (in the direction of polysaccharide degradation) relationship of starch phosphorylase.

phorylase from *Cuscuta reflexa* using egg shell as solid support. Upadhye and Kumar (1996) reported 79 and 36% retention of starch phosphorylase activity after immobilization on DEAE- cellulose and alginate beads, respectively. Therefore, in the present study, retention of activity of starch phosphorylase after immobilization is more compared to earlier reports.

Optimum pH

In the direction of polysaccharide synthesis, enzyme assay was carried out in the range varying from pH 4.5 to pH 8 to find out the optimum pH at which the enzyme shows maximum activity. The soluble enzyme showed optimum pH at pH 6.0 and half-maximal activity at pH 5.4 and 7.0 whereas immobilized enzyme showed optimum pH at 6.2 and half maximal activity at pH 5.7 and 7.0 (Figure 2A).

In the direction of glucose-1-phosphate formation, enzyme assay was carried out in the range varying from pH 6.2 to pH 8.5. The soluble enzyme showed optimum pH at pH 7.6 and half maximal activity at pH 6.7 and 7.9. After immobilization, the enzyme showed maximal activity at pH 7.7 with half maximal activity at 6.8 and 8.0 (Figure 2B).

Optimum temperature

Enzyme assay was carried out in the range of 20 to 55°C to find out the optimum temperature. In both the directions (in the direction of polysaccharide synthesis and glucose-1-phosphate formation), the soluble enzyme showed optimum temperature at 37°C and the half-maximal activity at 34 and 40°C whereas immobilized

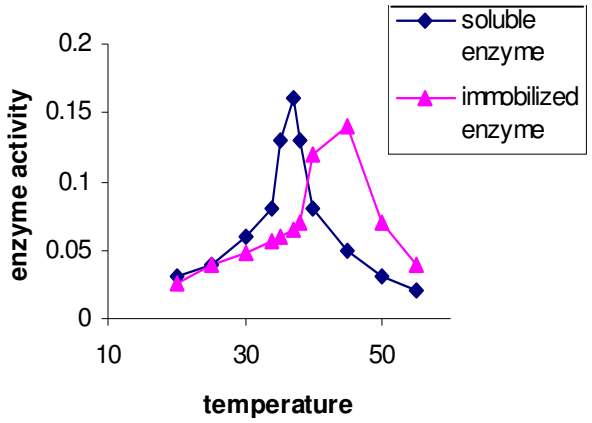

Figure 3A. Temperature-enzyme activity (in the direction of poly-saccharide synthesis) relation-ship of starch phos-phorylase.

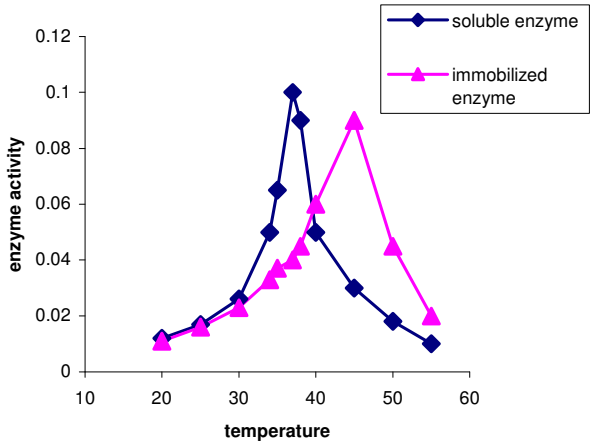

Figure 3B. Temperature-enzyme activity (in the direction of poly-saccharide degradation) relationship of starch phosphorylase.

enzyme showed optimum temperature at 45°C and half maximal activity at 38 and 50°C. The results are shown in Figures 3A and 3B.

Consistent with the present results, Makkar and Sharma (1983) in the case of *Lactobacillus bulgaricus* β-galactosidase, and our own laboratory in cases of potato starch phosphorylase (Venkaiah and Kumar, 1995), *Cuscuta reflexa* starch phosphorylase (Srivastava et al., 1996) and cabbage leaves starch phosphorylase (Garg and Kumar, 2007) also reported a considerable increase in the optimum temperature upon immobilization on egg shell. However, in our laboratory itself, in the case of Bengal gram starch phosphorylase, decrease in the optimum temperature was noted upon immobilization both on DEAE cellulose and alginate beads (Upadhye and Kumar, 1996).

Thermal stability

Thermal stability studies were carried out by incubating the enzyme in capped tubes in a water bath at different temperatures between 30 and 55°C. Every 30 min, suitable aliquots were withdrawn from the tubes and stored on ice before enzyme assay. The soluble enzyme had a half life of 5 h at 30°C and 30 min at 50°C. Immobilized enzyme had a longer half life of 9 h at 30°C and 1 h at 50°C. The increased thermal stability of the immobilized enzyme is very useful for the continuous production of glucose-1-phosphate from starch. Our present results are consistent with our earlier results on immobilization of sorghum leaves, *C. reflexa* and cabbage starch phospho-rylases (Venkaiah and Kumar, 1995; Srivastava et al., 1996; Garg and Kumar, 2007).

Enzyme reusability

Each day, starch and sodium phosphate buffer were passed through the immobilized enzyme column at 30°C for 5 h followed by washing with 5 times the bed volume of 0.02 M tris-HCl buffer, pH 7.5 and incubation under cold conditions. A single fraction was collected on each run and glucose-1-phosphate in the effluent was deter-mined by the coupled enzyme assay method (Bergmeyer and Klotzsch, 1965). The 20 batches could be carried out with almost same efficiency and thereafter efficiency decreased. The property of reuse up to so many times may be exploited in the production of glucose-1-phos-phate.

The effluent collected from the immobilized enzyme column contained small amounts of starch and inorganic phosphate as contaminants with glucose-1-phosphate. The contaminants were found to be removed by adsorption chromatography through a norite-celite (2:1) column (1.5 x 10 cm), previously equilibrated with distilled water, and introduced as a glucose-1-phosphate trap with the column. Glucose-1-phosphate was eluted from the column at a flow rate of 10 ml/h using water as the eluent. The effluent was further purified by using ion-exchange chromatography using a DEAE cellulose column previously equilibrated with distilled water. Glucose-1-phosphate was eluted from the column using 0.02 M potassium acetate buffer, pH 7.0. The product thus obtained was precipitated with two volumes of 95% ethanol and dried in a vacuum oven at 60°C. The final product was tested using coupled enzyme assay and found to be nearly 95% pure.

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