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IMMOBILIZATION OF STARCH PHOSPHORYLASE FROM CABBAGE LEAVES: PRODUCTION OF GLUCOSE-1-PHOSPHATE

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Abstract - Starch phosphorylase has been isolated from cabbage (*Elephantopus scabar*) leaves 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 5.3 and pH 7.1 whereas in the direction of glucose-1-phosphate formation, it showed optimum pH at pH 7.0 with half pH-optima at pH 6.4 and 7.6. The optimum temperature for the enzyme activity has been found to be 37^{o}C with two half temperature optima at 34^{o}C and 40^{o}C. The partially purified enzyme has been immobilized using egg shell as solid support. The percentage retention of the enzyme on egg shell was nearly 56%. After immobilization, specific activity of the enzyme increased from 0. 0225 to 0.0452. 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.
Keywords: Starch phosphorylase; Immobilization; Glucose-1-phosphate; Egg shell; Cabbage.

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 the amount of 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 property of reusability. In spite of industrial 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 the 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 (Szulczynski, 1986) .

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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 played a role in the capacity of the leaf lumina 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 served 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 constituted 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 cabbage leaves 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.

Tissue

Fresh cabbage was purchased from the local vegetable market and stored in a refrigerator until use.

Enzyme Extraction and Partial Purification

Unless otherwise stated, the entire procedure was carried out at 0-4^{o}C in a cold room. Cabbage leaf tissue (10 g) was cut into small pieces and blended for 30 sec at low speed and 60 sec at high speed in a Waring blender with 90 ml of the isolation medium. The isolation medium (buffer A) was consisted of 0.01 M tris-HCl buffer, pH 7.5 containing 20 mM 2-mercaptoethanol and 0.05 M EDTA. The homogenate was filtered through two layers of a muslin cloth, made the volume 100 ml using buffer A 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 7.5 by the addition of 1% (v/v) ammonia. After storage for 3 h, it was centrifuged at 15000 x *g* for 20 min and the supernatant having most of the enzyme activity was brought to 60% saturation with powdered ammonium sulfate. After overnight storage, the suspension was centrifuged at 15000 x *g* for 20 min. The pellet was dissolved in buffer A, centrifuged and the supernatant was desalted using Sephadex-G-25 column chromatography. The desalted enzyme was used for study.

Enzyme Assay

The enzyme assay for the soluble and the immobilized enzymes 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 0.2 M sodium fluoride; 0.1 ml of 3% soluble starch and 0.5 ml of the enzyme preparation and water, and pre-incubated at 37^{o}C for 2 min. The reaction was started by addition of 0.1 ml of 0.05 M glucose-1-phosphate in the experimental tubes. After 30 min, the reaction was stopped by addition of 0.1 ml of 50% TCA and the tubes were put in an ice bath. Control tubes received glucose-1-phosphate after addition of TCA. The precipitate formed was removed by centrifugation in the cold condition. In the clear supernatant, inorganic phosphate formed was estimated using colorimetric method of Fiske and Subbarow (1925). The enzyme activity of the 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 the cold condition. The amount of the inorganic phosphate 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 experimental conditions.

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, and 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 in the experimental tubes. After 30 min, the reaction was stopped by keeping the tubes in a boiling water bath for 2 min, added NaH_2PO_4-Na_2HPO_4 in the control tubes and the precipitate formed was removed by centrifugation in the cold condition. In the clear supernatant, glucose-1-phosphate formed was estimated using 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 one h, the reaction was stopped by centrifugation at 3000 x *g* for 10 min in the cold condition. 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 experimental conditions.

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 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

Hen egg shell was prepared as described by Chatterjee et al (1990). Egg shells were broken into small pieces and kept in boiling water for 15 min. After decantation of water, washed several times with acetone and dried in an oven at 60°C. The pieces were then crushed to mesh 100. Two gm of prepared egg shell was added to 8 ml of the enzyme preparation (2.4 units; 108 mg protein) with stirring. The suspension was incubated for 4 h with gentle stirring. The pellet obtained after centrifugation under the cold condition was washed with buffer A until no protein was detected in the washings and then suspended.

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°C 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°C 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, 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^{o}C. The final product was tested using phosphoglucomutase and glucose-6-phosphate dehydrogenase coupled enzyme assay (Bergmeyer and Klotzsch, 1965).

RESULTS AND DISCUSSION

Enzyme Extraction and Partial Purification

30-60% ammonium sulfate fraction contained about 71 % of the enzyme activity compared to crude extract. After desalting using Sephadex G-25 chromatography, 63 % of the enzyme was recovered containing 3.04 units and 135 mg protein exhibiting specific activity of 0.0225 units/mg protein. The purification profile of the enzyme has been shown in Table 1.

Table 1: Purification profile of starch phosphorylase from cabbage leaves.

Purification step	Enzyme activity (units)	Protein (mg)	Specific activity (units/mg)	%Recovery	Fold enrichment
Crude homogenate	4.86	648	0.0075	100	-
30-60% fraction	3.44	326	0.0105	70.88	1.4
Sephadex G-25 Chromatography	3.04	135	0.0225	62.6	3.0

Enzyme Immobilization

The partially purified enzyme was subjected to immobilization on hen egg shell. The specific activity of the immobilized enzyme was 0.0452 units/ mg protein whereas the soluble enzyme had 0.0225 units / mg protein, thereby immobilized enzyme showed about two fold purification. The percentage retention of starch phosphorylase upon immobilization was nearly 56 %. 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 phosphorylase from *Cuscuta reflexa* using egg shell as solid support. Therefore, in the present study, retention of the activity of starch phosphorylase after immobilization is more compared to earlier reports.

Optimum pH

In the direction of polysaccharide synthesis, the soluble enzyme showed optimum pH at pH 6.0 and half-maximal activity at pH 5.3 and 7.1 whereas immobilized enzyme showed optimum pH at 6.2 and half maximal activity at pH 5.6 and 7.2. The results are shown in the pH-activity graph (Fig. 1a).

In the direction of glucose-1-phosphate formation, the soluble enzyme showed optimum pH at pH 7.0 and half maximal activity at pH 6.4 and 7.6. After immobilization, the enzyme showed maximal activity at pH 7.2 with half maximal activity at 6.6 and 7.8 (Fig. 1b).

Weiber et al. (1975) showed lower optimum pH for activity of covalently coupled pectinesterase compared to soluble enzyme and concluded it due to the negative charge on glass beads and/ or altered conformation of the enzyme. Earlier, our laboratory showed alkaline shift in optimum pH of pectinesterase upon immobilization on egg shell and in gelatin (Nighojkar et al., 1995). The alkaline shift in the optimum pH was explained due to possible decrease in the internal matrix pH from the pH of the bulk phase or due to altered conformation of the enzyme protein upon immobilization. Chatterjee et al. (1990) showed lower pH optima upon immobilization of goat liver catalase on egg shell. Our earlier studies on immobilization of starch phosphorylase from waste water of potato starch factories found alkaline shift in the optimum pH upon immobilization on egg shell (Venkaiah and Kumar, 1995). Immobilization of Bengal gram starch phosphorylase on DEAE cellulose was found to show alkaline shift whereas immobilization on alginate beads showed lower optimum pH compared to the soluble enzyme (Upadhye and Kumar, 1996). Shifting in the optimum pH may be due to altered conformation of the enzyme upon immobilization.

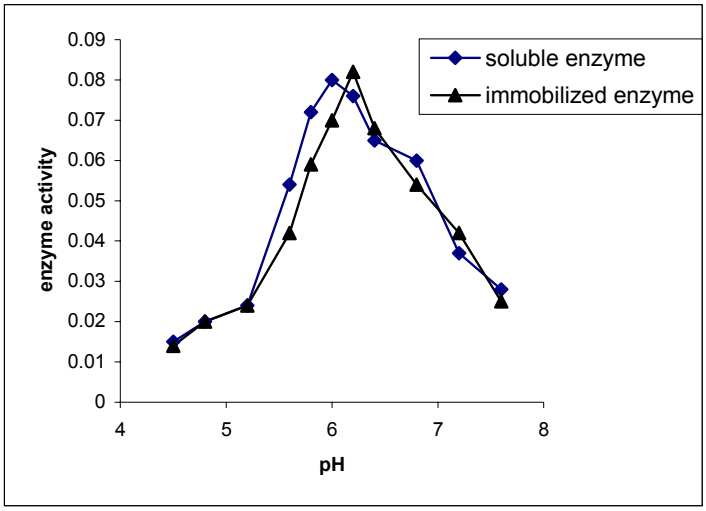

Figure 1a: pH- enzyme activity (in the direction of polysaccharide synthesis) relationship of starch phosphorylase

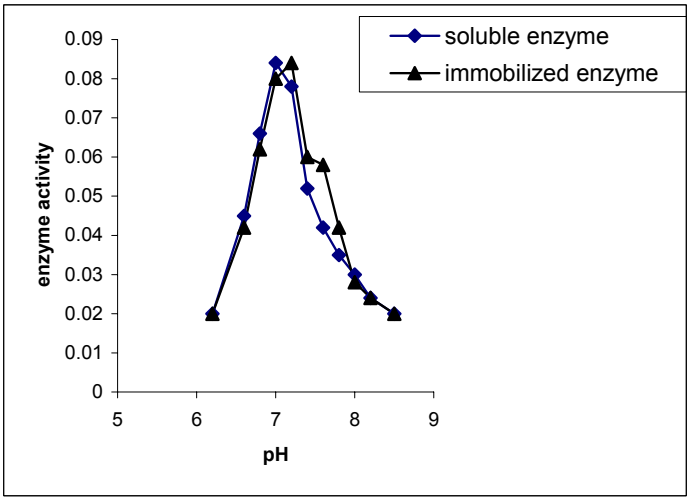

Figure 1b: pH- enzyme activity (in the direction of polysaccharide degradation) relationship of starch phosphorylase

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°C and 40°C whereas immobilized enzyme showed optimum temperature at 45°C and half maximal activity at 38°C and 50°C. The results are shown in Fig. 2a and 2b.

Consistent with the present results, Makkar and Sharma (1983) in case of *Lactobacillus bulgaricus* β-galactosidase, and our own laboratory in cases of potato starch phosphorylase (1995), *Cuscuta reflexa* starch phosphorylase (1996) also reported a considerable increase in the optimum temperature upon immobilization on egg shell. However, in our laboratory itself, in 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). Mostly upon immobilization, increase in the optimum temperature of the enzymes have been reported and has been explained due to more stability of the immobilized enzymes. The change in optimum temperature upon immobilization may be due to altered conformation.

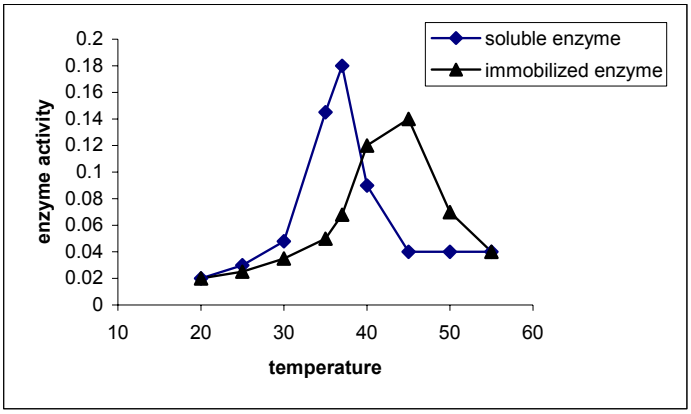

Figure 2a: Temperature- enzyme activity (in the direction of poly-saccharide synthesis) relation-ship of starch phosphorylase

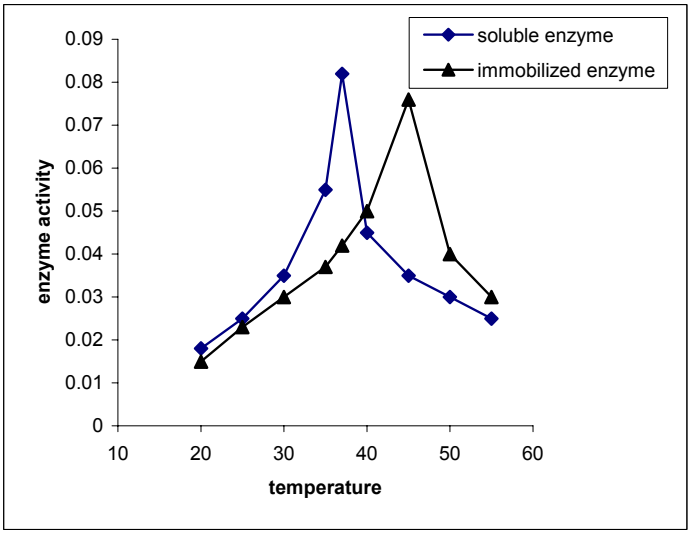

Figure 2b: Temperature- enzyme activity (in the direction of poly-saccharide degradation) relation-ship of starch phosphorylase

Thermal Stability

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 and *Cuscuta reflexa* starch phosphorylases (Venkaiah and Kumar, 1994; Srivastava et al., 1996).

Enzyme Reusability

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-phosphate.

Purification of the Product (Glucose-1-Phosphate)

The effluent collected from the immobilized enzyme column contained small amounts of starch

and inorganic phosphate as contaminants with glucose-1-phosphate. Nearly 40% contaminants were found removed by norite-celite column. After ion exchange column, glucose-1-phosphate was found to be nearly 95% pure as tested using phosphoglucomutase and glucose-6-phosphate coupled enzyme assay.

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