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Immobilized Sucrose Phosphorylase from *Leuconostoc mesenteroides*

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ABSTRACT

Sucrose phosphorylase from *Leuconostoc mesenteroides* was immobilbilized by covalent linkage to several supports, and the specific activity recovery was 2–11%. The enzyme adsorbed onto DEAE-cellulose retained about 18% specific activity and was stable over eight months. The optimum pH (7.0) and temperature (30°C) did not change after immobilization. Also there was no improvement of thermal stability, and K_m for sucrose and phosphate was lower compared to the soluble enzyme.

Index Entries: Immobilization; immobilization sucrose phosphorylase; DEAE-cellulose adsorption; *Leuconostoc mesenteroides*.

INTRODUCTION

Sucrose phosphorylase (E.C.2.4.1.7) is an intracellular enzyme first found in *Leuconostoc mesenteroides* and strains of *Pseudomonas* (*1–3*). But, it was only with *Leuconostoc mesenteroides* that the optimization of sucrose phosphorylase production has been tried (*4–5*). The enzyme has potential biotechnological application, using either the phosphorolytic or the synthetic reaction direction. It can be used as an alternative way of fructose and glucose-1-phosphate production. Another possible use is as an analytical tool for sucrose assay, either in a soluble coupled enzyme-NAD+ assay (*6*) or after immobilization, in a enzyme based electrode. Until now,

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the enzyme electrode for sucrose assay is based on the invertase-glucose oxidase system with or without mutarotase (*7*). Such enzyme electrode needs care in the interpretation of results, since it will respond to both sucrose and glucose. The synthesis of sucrose from starch (*8,9*) might become an important industrial process in certain countries, as a result of the unstable market situation of sucrose. The increase of sucrose production from fructose and glucose-1-phosphate was achieved using a column with immobilized sucrose phosphorylase, connected by the outlet to a second column filled with activated charcoal. As the sucrose eluated from this second column was recirculated, the enzyme was continuously confronted with high substrate concentration (*9*). Another practical application of sucrose phosphorylase is the production of novel disaccharides, that may lead synthetic sugars with new and important functional properties. This approach is possible because sucrose phosphorylase has low specificity towards the glycosil receptor. A recent review about microbial sucrose phosphorylase discusses the enzyme production, immobilization, and importance of those applications (*10*). We wish to report the immobilization of sucrose phosphorylase from *Leuconostoc mesenteroides* by covalent linkage with several water insoluble supports, by adsorption onto DEAE-cellulose and the properties of DEAE-cellulose derivative, mainly the optimum temperature and thermal stability.

MATERIAL AND METHODS

Enzyme Source and Culture Conditions

Leuconostoc mesenteroides, isolated from sugar cane juice, was grown in a 10 L scale under anaerobic conditions at 28°C using a 10% inoculum in a liquid medium at pH 6.8–7.0. The medium composition was similar to the medium described by Doudoroff (*1*), except that triptone (10g/L) was replaced by peptone (DIFCO, 10g/L). Growth was monitored by spectrophotometry and the cells, harvested in late log phase, were washed in distilled water and stored at −15°C.

Enzyme Extraction and Partial Purification from *Leuconostoc mesenteroides* Cells

The frozen cells were ground with abrasive (quartz sand, 80 mesh) at the proportion of 1:4. The enzyme was extracted with 20m*M* Tris-HCl buffer, pH 7.0 (5mL/g of broken cells) after centrifugation at 7700*g* at 4°C for 10 min. The crude extract was partially purified after nucleic acid precipitation with 50m*M* (final concentration) $MnCl_2$ solution, followed by protein precipitation with solid ammonium sulfate (50–100% saturation) and DEAE-cellulose chromatography. This partially purified enzyme was used for all immobilization procedures.

Methods of Immobilization

Immobilization on nylon polyethyleimine beads proceeded as described by Ferreira (*11*). The beads (1 g), activated with glutaraldehyde (5%), were mixed with 6 mL of sucrose phophorylase solution containing 8.2 U/mg protein. Coupling proceeded at 4°C for 3 h with gently stirring.

Immobilization on polyacrylamide beads (1g) treated with glutaraldehyde (2.5%) was according to Carvalho et al. (*12*). A total of 5 mL of sucrose phosphorylase solution containing 6.5 U/mg protein was used for coupling. In both cases, the immobilizated derivative was filtered and sequentially washed with 0.5*M* NaCl (three times), followed by 0.1*M* sodium phosphate buffer pH 7.0.

Immobilization by the CM-cellulose azide method (*13*) proceeded using 22 mL the soluble enzyme (3.6 U/mg protein) adjusted to pH 8.0 with sodium borate and mixed with 0.5g of the activated support. Washings for removal of proteins not covalently linked were carried out according to Crook et al. (*14*). All these immobilized derivatives were stored until used as suspensions in 0.1*M* sodium phosphate buffer pH 7.0 at 4°C.

Immobilization by adsorption was carried out using DEAE-cellulose 303C (Nutritional Biochemical Corporation) after successive treatments with 0.5*M* HCl and 0.5 NaOH solutions. A sample of 4 mL of the soluble enzyme (1.12 U/mg protein) was mixed with 1 g of pretreated DEAE-cellulose. The mixture was maintained with gentle stirring at 4°C for 1 h, followed by filtration and washings with deionized water at 4°C. The adsorbed enzyme was stored until used at 4°C in suspension in deionized water.

Enzyme Assay

The reaction mixture contained 100 m*M* sucrose in 50m*M* sodium phosphate buffer pH 7.0, a final vol of 1 mL for the soluble enzyme (0.5 mL), and 5.0 mL for the immobilized enzyme (0.5g). Incubation was performed in a shaking bath at 40°C. Sucrose phosphorylase activity was determined by the Fiske and Subbarow method (*15*). One unit of sucrose phosphorylase activity was defined as the amount of enzyme that causes an initial uptake of 1 μmol of Pi after 20 min at pH 7.0 and at 40°C. This definition of activity was similar to Doudoroff (*1*).

Protein determination was carried out by a spectrophotometric method (*16*). Immobilized protein was determined from the difference between the amount of protein added and recovered after immobilization and washings.

Experiments for determination of optimal pH, optimal temperature, and thermal stability were made in duplicate with the soluble and the immobilized derivative that was most stable to reuse.

Optimal pH was determined by incubation of the enzyme, at 40°C, in sucrose (100 m*M*) and phosphate (50 m*M*), prepared at pHs 6.0–9.0. 50

Table 1
Sucrose Phosphorylase Immobilized Derivatives

Support	Linkage	Coupling mg protein/ g support	Specific activity recovery (%)
Nylon-PEI glutaraldehyde	covalent	1.7	5
Polyacrylamide glutaraldehyde	covalent	66.0	11
CM-cellulose azide	covalent	165.0	2
DEAE-cellulose	adsorption	74.0	18

PEI = polyethyleimine

m*M* sodium phosphoate buffer (pH 6.0–7.5), and 50 m*M* Tris-HCl buffer (pH 7.5–9.0) were used.

Optimal temperature was investigated using the standard assay conditions at the temperatures ranging from 20–60°C. The heat stability was determined by the preincubation of the soluble or DEAE-cellulose enzyme, in 20 m*M* Tris-HCl buffer, at optimal pH 7.0, at the temperature range of 30–60°C for 30 min. After rapid cooling, the residual activities were assayed as described for the enzyme assay.

K_m values were determined at 40°C, for sucrose (0.1–1.5 m*M*) prepared in 50 m*M* phosphate buffer at pH 7.0, and for phosphate (0.1–1.0 m*M*) in presence of 100 m*M* sucrose, prepared in 20 m*M* Tris-HCl buffer, pH 7.0. The calculations were performed by the Hofstee method (*17*).

RESULTS AND DISCUSSION

Leuconostoc mesenteroides sucrose phosphorylase, immobilized by covalent linkage to nylon polyethyleimine, polyacrylamide beads, and CM-cellulose azide, retained respectively 5, 11, and 2% of the specific activity (Table 1). The polyacrylamide bound enzyme was the less stable derivative, losing all activity after 24 h of discontinuous use. Although the DEAE-cellulose sucrose phosphorylase retained only 18% of the specific activity, it was the most stable. During the first 6 d of utilization at 40°C, the adsorbed enzyme lost 40% of maximum activity, and 20% of the total adsorbed proteins. Afterward, the preparation remained stable to batch discontinuous use at pH 7.0 and 40°C for about eight months.

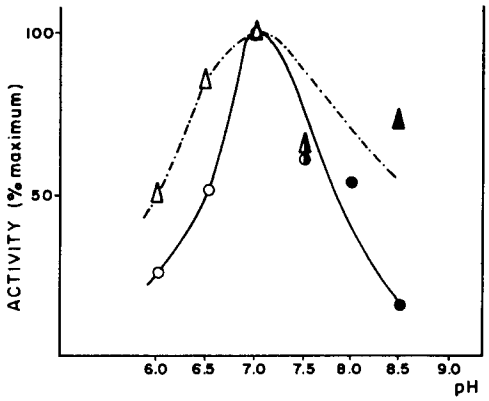

Fig. 1. pH profile. Soluble (○, ●) and DEAE-cellulose enzyme (△, ▲) were assayed under standard conditions except for the pH of the reaction mixture. Sodium phosphate buffer, 50 m*M* (pH 6.0–7.0) and 50 m*M* Tris-HCl buffer (pH 7.5–9.0) was used.

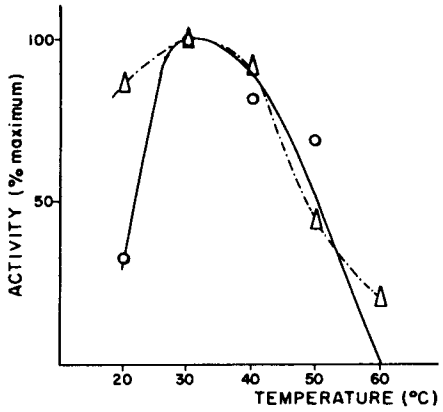

Fig. 2. Temperature profile. Soluble (○) and DEAE-cellulose (△) enzyme were assayed under standard conditions with a temperature range from 20° to 60°C.

This operational stability stands well as compared with that reported for the enzyme entrapped in tri-acetate fibers that was used continuously (up to 5 d) and repeatedly at 37°C and pH 6.0 without loss of activity (*18*). Also, the enzyme stability compared well with the enzyme immobilized on porous ceramic beads, whose half-life varied from 35 d at 30°C to 5 d at 40°C (*19*). When the properties of the soluble and DEAE-cellulose enzyme are compared, it is shown that the optimum pH 7.0 (Fig. 1) and the optimal temperature value of 30°C (Fig. 2) did not change after the immo-

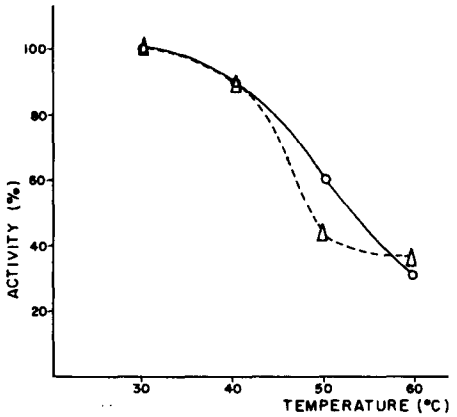

Fig. 3. Heat stability of soluble and DEAE-cellulose enzyme. Soluble (○) and DEAE-cellulose (△) enzyme were preincubated in 20 m*M* Tris-HCl buffer pH 7.0 at the temperature range of 30–60°C for 30 min. After rapid cooling, the residual activities were assayed at 40°C under the assay conditions.

bilization. There was no improvement of the thermal stability (Fig. 3). Nevertheless, the soluble enzyme stability is already higher than that reported for the enzyme from another strain of *Leuconostoc mesenteroides* that lost all activity after treatment at 60°C for 1 min (*2*).

K_m for sucrose was calculated as 0.163 ± 0.01 m*M* for the soluble enzyme and 0.082 ± 0.03 m*M* for DEAE-cellulose enzyme. K_m for phosphate was 0.427 ± 0.095 m*M* for the soluble enzyme, and 0.172 ± 0.079 m*M* for DEAE-cellulose enzyme. Similarly, a decrease of K_m value for sucrose has been reported for invertase adsorbed to DEAE-cellulose and assayed in sucrose prepared in phosphate buffer pH 7.0 (*20*). On the other hand, an increase of K_m values was found for DEAE-cellulose glucose isomerase when the microenvironment density charge was increased (*21*).

Considering the stability of the DEAE-cellulose sucrose phosphorylase, it might find application in a sucrose enzymatic assay. But, as the optimal temperature (30°C) and the thermal stability did not increase after the immobilization, this preparation is not suitable as an alternative way for frutose production, as was the glucose isomerase adsorbed to DEAE-dellulose (*21*). In our laboratory, research is under way with a fungal sucrose phosphorylase, despite being a soluble enzyme, that is already more thermostable.

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