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EXTRACELLULAR INVERTASE FROM ASPERGILLUS ATHECIUS: ISOLATION AND IMMOBILIZATION

K.M. Madyastha*, A.R. Ganguli, V.G. Kubair, N. Kowser and D. Vidya

Department of Organic Chemistry, Bio-organic Section, and Chemical Engineering Indian Institute of Science, Bangalore-560 012, India.

SUMMARY

A fungal strain isolated from soil and identified as Aspergillus athecius, when grown on moistened wheat bran produced large amounts of extracellular invertase. Most of the invertase from the moldy bran was easily extracted by low ionic strength buffer (0.005 M, pH 5.7). The crude invertase immobilized on DEAE cellulose showed not only increased activity (~45%) but also greater thermal and storage stability than the free enzyme. The free and the bound enzymes showed a temperature optimum of 50-55°C and a pH optimum of 5.7 and 4.8 respectively. The K_m app. of the bound enzyme was lower than that of the free enzyme.

INTRODUCTION

The enzyme invertase has been studied in great detail and this is particularly true of invertase from yeast. Various methods such as prolonged autolysis, sonication and blending have been used to release invertase from variety of sources. Many studies have been reported on the preparation of immobilized invertase by adsorption on different supports (Maeda and Suzuki, 1973; Leemputtern and Horisberger, 1974; Boudrant and Cheftel, 1975; Imai et al. 1983 and Yamazaki et al. 1984).

We have isolated a fungal strain identified as Aspergillus athecius which was shown to produce large amounts of extracellular invertase when grown on moistened wheat bran. The present paper reports its isolation, immobilization and comparison of properties between the bound and the free invertase.

MATERIALS AND METHODS

Aspergillus athecius was maintained on potato-dextrose-agar slants. The slants were inoculated with spores and grown at 30°C for 4 days and then stored at 4°C.

Preparation of crude invertase. Erlenmeyer flasks (250 ml) containing sterile wheat bran (25 g) wetted with 50% of its weight with water were inoculated with 5 ml of spore suspension (~10^8 spores/ml), mixed well and incubated at 30°C for 72 h. Then to each flask 75 ml of citrate-phosphate buffer (0.005 M, pH 5.7) was added and allowed to soak for one hour. It was then filtered through cheese cloth and the filtrate centrifuged (10,000 g, 15 min). The clear supernate contained most of the invertase activity.

Immobilization. To washed DEAE cellulose (15 g, coarse), equilibrated in citrate-phosphate buffer (0.005 M, pH 5.7) at 25°C, crude invertase (~14 units/mg protein) was added while gently stirring until the supernate was devoid of any invertase activity. This suspension was stored overnight at 4°C, filtered and washed with citrate-phosphate buffer (0.005 M, pH 5.7) until the filtrate was free from absorbance at 280 nm. The immobilized enzyme was dried under vacuum and stored at 0-4°C.

Assay of invertase. Sucrose solution (2.5%) prepared in 0.005 M citrate-phosphate buffer (pH 5.7 for free enzyme, pH 4.8 for bound enzyme) was incubated with free or bound enzyme in a total volume of 10 ml at 50°C for 30 min. One ml of the reaction mixture was mixed with one ml of dinitrosalicylic acid reagent, heated in a boiling water bath for 5 min. and the colour developed was estimated at 540 nm (Miller, 1959). One unit was defined as the amount of enzyme releasing one µmole of reducing sugar in one min under the standard assay conditions.

pH optimum. The following buffers of 0.005 M were used: citrate-phosphate (pH 3.0-7.0), phosphate (pH 7.3-8.0) and borate (pH 8.0-10.0).

Thermal stability. It was determined by preincubating both the enzymes in their respective buffers at different temperatures for 30 min, rapidly cooled and then assayed for their residual activity.

Storage stability. Free and bound enzymes were stored for a maximum period of 10 days at 4°, 25° and 50°C and periodically assayed for their residual activity.

Stability of bound enzyme to continuous inversion of sucrose. A mixture of 200 mg of bound enzyme (~400 U) and 60 ml of sucrose solution (2.5%) prepared in 0.005 M citrate-phosphate buffer (pH 4.8) was incubated with shaking at 50°C for 30 min. It was then filtered and the amount of reducing sugar present in the filtrate was estimated. The residue (bound enzyme) was washed with distilled water and again suspended in 60 ml of buffered

sucrose solution for the next inversion. This process was repeated five times, each time the amount of reducing sugar present in the filtrate was measured.

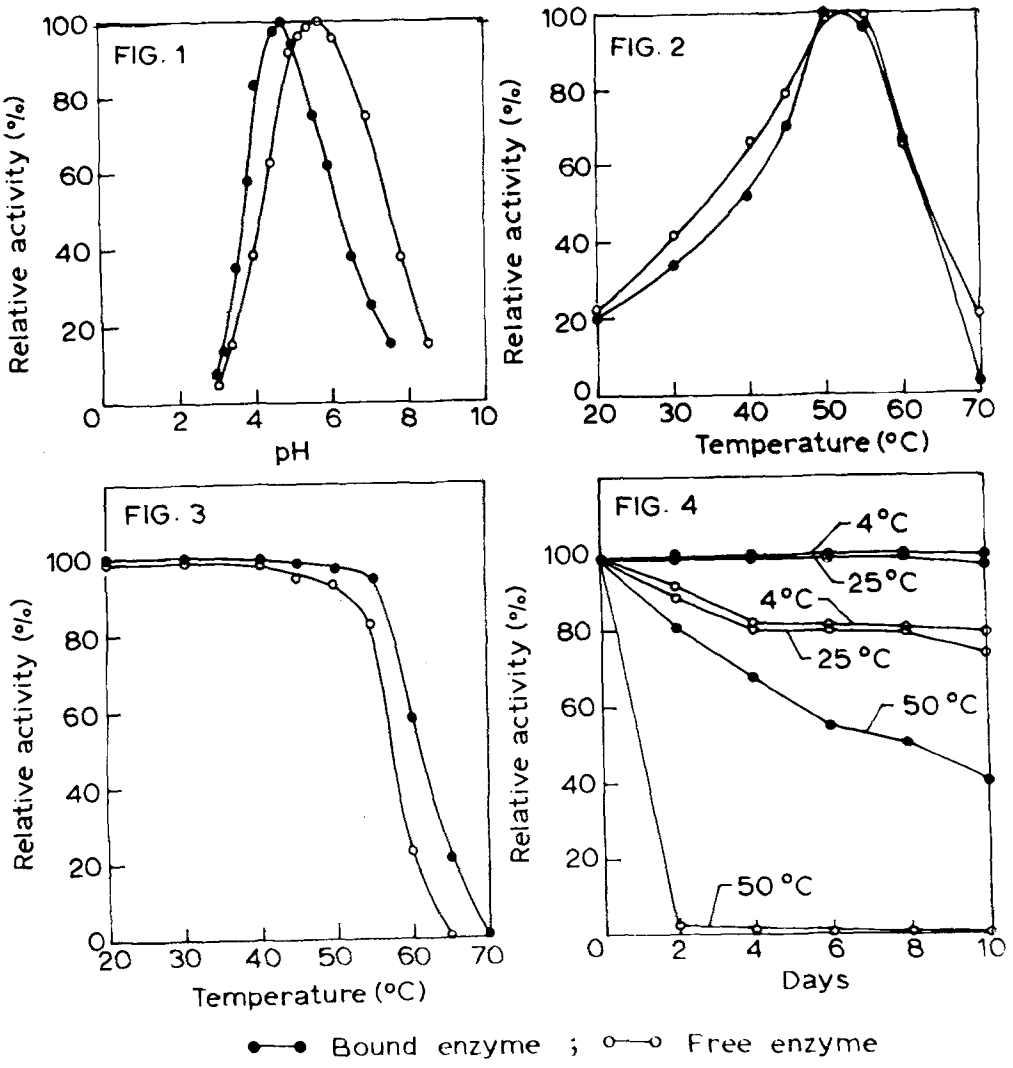
Kinetics. K_m for free enzyme and K_m app. for the bound enzyme were determined in a sucrose concentration range of 1.4×10^{-2} M - 1.4×10^{-1} M in citrate-phosphate buffer following standard assay conditions.

Protein estimation. Protein was estimated by the method of Lowry et al. (1951). The amount of protein adsorbed on DEAE cellulose was determined from the difference in the amount of protein added for immobilization and the protein remaining in the supernate after adsorption and in the washings.

RESULTS AND DISCUSSIONS

Large amounts of invertase was produced extracellularly when this organism was grown on moistened wheat bran than in a liquid medium. Most of the invertase from the moldy bran can be easily extracted by gently shaking it with low ionic strength citrate-phosphate buffer (0.005 M, pH 5.7). The crude extract contained about 50 units of activity per ml (sp. activity, 14.17 µmoles/min/mg). The crude enzyme readily gets adsorbed on DEAE cellulose equilibrated in citrate-phosphate buffer (0.005 M, pH 5.7). One gram of DEAE cellulose was found to bind $\sim$ 1600 units of the crude enzyme. The catalytic activity increased upon immobilization (sp. activity, 20.5 µmoles/min/mg bound protein). With a partially purified enzyme (enzyme eluted from DEAE cellulose using 0.25 M citrate-phosphate buffer, pH 5.7) having a specific activity of 220 units/mg protein, an astonishingly large amounts of the enzyme activity (33,000 units) could be bound on one gram of DEAE cellulose. It was observed that immobilized invertase had a narrow pH optimum as compared to the bound enzyme (Fig. 1) and the pH optimum shifted by 0.9 units to the acidic side. It was noticed that free and bound enzymes had an optimum pH 5.7 and 4.8 respectively (Fig. 1). The shift in the pH optimum for the bound enzyme was also observed by other investigators (Maeda and Suzuki, 1973). The activity of the bound invertase drops drastically at pH values above 6.0. Similar observations have been made

Figure 1-4 : pH optima (Fig. 1), Temperature optima (Fig. 2), Thermal stability (Fig. 3) and Storage stability (Fig. 4) of free and bound invertase. Experiments were carried out as described under "Methods".



in the case of invertase bound to polyvinyl alcohol (Imai et al. 1983). The free and bound enzymes showed the highest activity in citrate-phosphate buffer. However, other buffers such as phthalate, succinate, citrate (0.005 M, pH 5.7 for free enzyme, 4.8 for bound enzyme) tested did not have any adverse effects on the enzyme activity.

Both free and bound enzymes showed a temperature optimum of 50-55°C (Fig. 2). Similar results have been reported in other cases (Gregor and Rauf, 1975; Walters, 1976). Reports are also available regarding the elevation of temperature optimum for the bound enzyme as compared to that of the free enzyme (Ikeda and Fukui, 1973) as well as immobilized enzymes showing lower temperature optimum (Kawai and Eguchi, 1975).

Immobilized enzyme showed an increase in thermal stability when compared to that of free enzyme (Fig. 3). Upto 50°C, both free and bound enzymes were comparatively stable, whereas at 60°C, the residual activities for the bound and free enzymes after a 30 min. preincubation period were 56 and 20%, respectively. The free invertase lost 21% of its initial activity upon storage at 4°C for 10 days, whereas in the same period, the bound enzyme lost barely 2% of its initial activity (Fig. 4). When stored at 50°C, the free enzyme had lost almost all its activity in two days whereas the bound enzyme retained ~ 80% of its activity during this period (Fig. 4). The half life of free and bound enzymes at 50°C were found to be two hours and eight days, respectively. However, at 25°C, the free and the bound enzyme retained 76 and 97% of their original activities respectively after 10 days of storage. Earlier it has been reported that yeast invertase immobilized on DEAE cellulose when stored for 12 days below 30°C had lost about half of its activity, whereas the free enzyme retained 75% of its activity in the same period (Suzuki et al. 1966). It was noticed that the bound

enzyme after being used continuously for five times under a batch system, did not loose any appreciable activity indicating the stability to continuous inversion.

There are reports on the changes in the K_m values upon immobilization of enzymes (Chibata, 1978). In the present investigation it was found that K_m for the free enzyme was 25.5 mM whereas K_m app. of bound enzyme was 19.0 mM. The decrease in the K_m value of the bound invertase makes it attractive for the commercial application of the immobilized enzyme. Besides, properties such as enhanced catalytic activity, increased thermal and storage stability upon immobilization are advantageous for the industrial application of this enzyme.

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