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Separation and Characterization of β-Glucanases from *Penicillium ochro-chloron*

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Two pools of glycogen exist in yeast; cytoplasmic glycogen serves as energy reservoir and cell wall bound glycogen is released on treatment with β-glucanase. Search for specific β-glucanase showed that endo(1→3)-β-D-glucanase and β-glucosidase are produced in high concentration in the cultural filtrate of *Penicillium ochro-chloron*. The activity of (1→3)-β-D-glucanase was inducible, when insoluble β-glucans from the yeast cell wall were added in the medium (1% w/v). (1→3)-β-D-glucanase and β-glucosidase were separated by DEAE-sepharose ion exchange chromatography. Both the activities were retained by the column. Stepwise elution was achieved by using salt gradient, which resolved the retained protein into five peaks. The first peak eluted at 0.05 M NaCl concentration showed (1→3)-β-D-glucanase activity whereas the β-glucosidase activity was eluted at 0.12 M NaCl concentration. The kinetic properties of (1→3)-β-D-glucanase showed that the enzyme was maximally active at temperature 60°C, pH 5 and at 55.5 μg/mL substrate concentration when laminann was used as a substrate. Hg^{2+} inhibits the enzyme activity to some extent while Co^{2+} enhances enzyme activity. The molecular weight of (1→3)-β-D-glucanase is in the range of around 45,000 daltons. β-Glucosidase shows maximum activity at temperature between 55–60°C, pH 5.5 and at substrate concentration 125 μg/mL when *p*-nitrophenyl β-D-glucopyranoside is a substrate. The molecular weight of β-glucosidase is in the range around 1,13,000 daltons.

Key Words: β-Glucanases, *Penicillium ochro-chloron*.

INTRODUCTION

The cell walls of yeast and many fungi are comprised of polysaccharides principally glucan and mannan. The cell wall hydrolysing enzymes were used for the hydrolysis of yeast cell walls and for the preparation of spheroplasts[1]. Enzymes of specific hydrolytic action are valuable tools for the structural analysis of yeast cell walls. β-Glucans are not static components but dynamic in nature. This is essential in the modification of cell wall in growth, budding, conjugation and other functions. As many as six different glucanases have been reported to be present to help in the modification[2,3]. Preliminary evidences suggest that different glucanases may be required at different stages of cell life cycle. The specific action of certain

(1→3)-β-D-glucanases has already been reported[4–6]. Two types of endo-(1→3)-β-D-glucanase were isolated from the culture medium of *Bacillus circulans* which lyse the yeast cell[7]. The enzymatic activity from a species of fungi *Imperfecti* on incubation with yeast glucan causes rapid reduction in turbidity has been shown[8, 9]. We searched for β-glucanase that dissolves the cell wall bound glycogen. *Penicillium ochro-chloron* culture secrete such enzymes.

EXPERIMENTAL

Laminarin, Gentiobiose, Cellobiose *para*-nitrophenyl-β-D-glycopyranoside (*p*-NPG), DEAE-sepharose glucose oxidase, horse radish peroxidase were purchased from Sigma Chemical Company, USA. The Biogel P-2, P-100 and P-200 were purchased from BioRad Lab, Cal., USA.

Microorganism and cultural conditions

The fungal culture of *Penicillium ochro-chloron* was obtained from Institute of Microbial Technology, Chandigarh, India. *Penicillium ochro-chloron* culture was maintained on agar slant containing potato dextrose medium. The cultural medium for the optimum growth of *Penicillium ochro-chloron* that secretes the required enzyme was prepared by adding 2% glucose in the potato extract medium at pH 6.5. The medium was dispensed in 1 L conical flask. Flasks were inoculated with a 72 h starter culture (10% v/v) grown in the same medium and then incubated at ±28°C for 15 days with occasional shaking. (1→3)-β-D-Glucanase and β-glucosidase were inducible and the activity in the cultural filtrate of *Penicillium ochro-chloron* was monitered over 20 days after incubation. Portions of the culture were sampled at the time shown in Fig. 1, centrifuged and assayed for (1→3)-β-D-glucanase and β-glucosidase activity.

Determination of (1→3)-β-D-glucanase activity

Enzyme Assay: The reaction mixture containing 0.2 mL laminarin (1 mg/mL) and 0.7 mL citrate buffer (50 mm, pH 5.0) was placed in a water bath at 50°C. The enzymatic reaction was initiated with the addition of 0.1 mL of enzyme solution (dialysed cultural filtrate). The reaction mixture was incubated for 30 min with occasional stirring. Exactly after 30 min incubation, the reaction was teminated by immersing the tubes in a boiling water bath for 5 min.

The glucose released due to enzyme action was measured specifically using glucose oxidase peroxidase assay method[10].

Unit of (1→3)-β-D-glucanase activity

One unit of (1→3)-β-D-glucanase activity is defined as the amount of enzyme activity required to produce 1 μg of glucose per 30 min at pH 5.0 and at temperature 50°C.

Determination of β-glucosidase activity

Enzyme Assay: The reaction mixture containing 0.2 mL *p*-NPG (1 mg/mL)

and 1.7 mL citrate buffer (100 mM, pH 5.0) was placed in a water bath at 50°C. The enzymatic reaction was initiated with the addition of 0.1 mL of enzyme solution (dialyzed cultural filtrate). The reaction was terminated by addition of 1 mL of 1 M NaOH. The *p*-nitrophenol released due to enzyme action was measured at 420 nm.

Unit of β-glucosidase activity: One unit β-glucosidase activity is defined as the amount of enzyme activity required to produce 1 μg of *p*-nitrophenol per 30 min at pH 5.0 and at temperature 50°C.

Analytical Procedures

Estimation of glucose was done by glucose oxidase peroxidase method[10]. Proteins were determined by Lowry method[11] with Bovine serum albumin as standard. Total carbohydrate was determined by phenol sulphuric acid method[12].

Chromatography

Ion-exchanger chromatography: The dialyzed enzyme preparation was chromatographed using DEAE sepharose CL–4B ion exchanger chromatography. The column (17 × 1.5 cm) was equilibrated with 10 mM, potassium phosphate buffer, pH 7.0. The effluent fractions with (1→3)-β-D-glucanase and β-glucosidase activity were collected and used for further studies.

Gel filtration chromatography: The molecular weight of (1→3)-β-D-glucanase was determined by using Biogel P-100 gel filtration chromatography, carried out at +4°C. The column (69 × 1.2 cm) was equilibrated with 10 mM potassium phosphate buffer, pH 7.0. 1 mL of enzyme solution was loaded on to Biogel P-100 column. Elution was done using the same buffer with a flow rate 8 mL/h and 100 fractions of 1 mL each were collected and analyzed further. The protein standards used from molecular weight marker kit MW-GF-200 from Sigma Chemical Company USA were alcohol dehydrogenase MW = 1,50,000, albumin MW = 66,000, carbonic anhydrase MW = 29,000, cytochrome-C MW = 12,000. The void volume of the column was determined by pasing the blue dextran.

The molecular weight of β-glucosidase was determined by using Biogel P-200. All the experimental procedures used were similar as Biogel P-100 which is described earlier except the column dimensions (70 × 1.2 cm). The protein standards used were β-amylase MW = 2,00,000, alcohol dehydrogenase MW = 1,50,000, albumin MW = 66,000, carbonic anhydrase MW = 29,000, cytochrome-C MW = 12,000.

RESULTS AND DISCUSSION

Production of the (1→3)-β-D-glucanase and β-glucosidase

Penicillium ochro-chloron produce (1→3)-β-D-glucanase and β-glucosidase; both the enzymes are extracellular. A certain amount of (1→3)-β-D-glucanase and β-glucosidase is produced constitutively; however. a little increase in the activity of (1→3)-β-D-glucanase was detected when yeast β-glucan was added to the medium. Though the activity of (1→3)-β-D-glucanase increases by addition

of yeast β-glucan, this substrate is not commercially available in large scale quantities for the production of enzyme. So, dextrose medium was used throughout. The incubation period required for maximum production of these enzymes is 15 days. After that up to 20 days a slow increase in the activity of both the enzymes is observed as shown in Fig. 1.

When excess glucose was added to the culture medium, it was observed that the growth rate is fast and the duration required for the maximum production of enzyme is more; however, in glucose limitations slow growth is observed. Hence glucose limitations and slow growth are paralleled by the appearance of extracellular (1→3)-β-D-glucanase and β-glucosidase. The (1→3)-β-D-glucanase and β-glucosidase might have been used in morphological changes in *Penicillium ochro-chloron*. When glucose is depleted from the medium, (1→3)-β-D-glucanase and β-glucosidase are seen to play a role in the morphological event by hydrolysing reserve (1→3)-β-D-glucan and (1→6)-β-D-glucan and supplying the glucose needed for maintenance.

Enzyme purification

A cultural filtrate of *Penicillium ochro-chloron* was removed after completion of incubation period as described in experimental section. The cells were separated from the culture by centrifugation and the supernatant fluid was extensively dialyzed against potassium phosphate buffer (10 mM, pH 7.0) at +4°C until the entire glucose from the cultural filtrate was removed and used as the source of crude enzyme. The fractionation of enzyme proteins was achieved by using DEAE sepharose ion exchange column chromatography, equilibrated with potassium phosphate buffer (10 mM, pH 7.0). The dialyzed cultural filtrate containing (1→3)-β-D-glucanase and β-glucosidase activities was loaded on to a column of DEAE sepharose with a flow rate of 30 mL/h and the column was thoroughly washed with the same buffer. The stepwise elution was achieved by using salt gradient 0.05 M, 0.1 M, 0.12 M, 0.15 M NaCl. Elution profile is shown in Fig. 2. The first peak which was eluted at 0.05 M NaCl showed (1→3)-β-D-glucanase activity and it specifically acts on laminarin, as well as on yeast β-glucan, but does not act on gentiobiose, cellobiose, *p*-NPG, while β-glucosidase activity was eluted at 0.12 M NaCl concentration and showed activity on gentiobiose, cellobiose, laminarin, yeast β-glucan and *p*-NPG. The activity of these enzymes varies from substrate to substrate. The purification obtained for (1→3)-β-D-glucanase was 55.72-fold and for β-glucosidase 30-fold.

Kinetic properties of (1→3)-β-D-glucanase

Kinetic properties were studied under standard conditions.

Effect of temperature on enzyme activity: As a result of the investigations it was observed that the activity increases up to 60°C and then decreases with further rise in temperature. The enzyme activity is completely lost at 80°C. It is concluded that 60°C is the temperature of maximum activity signifies that the enzyme is suitable for industrial use.

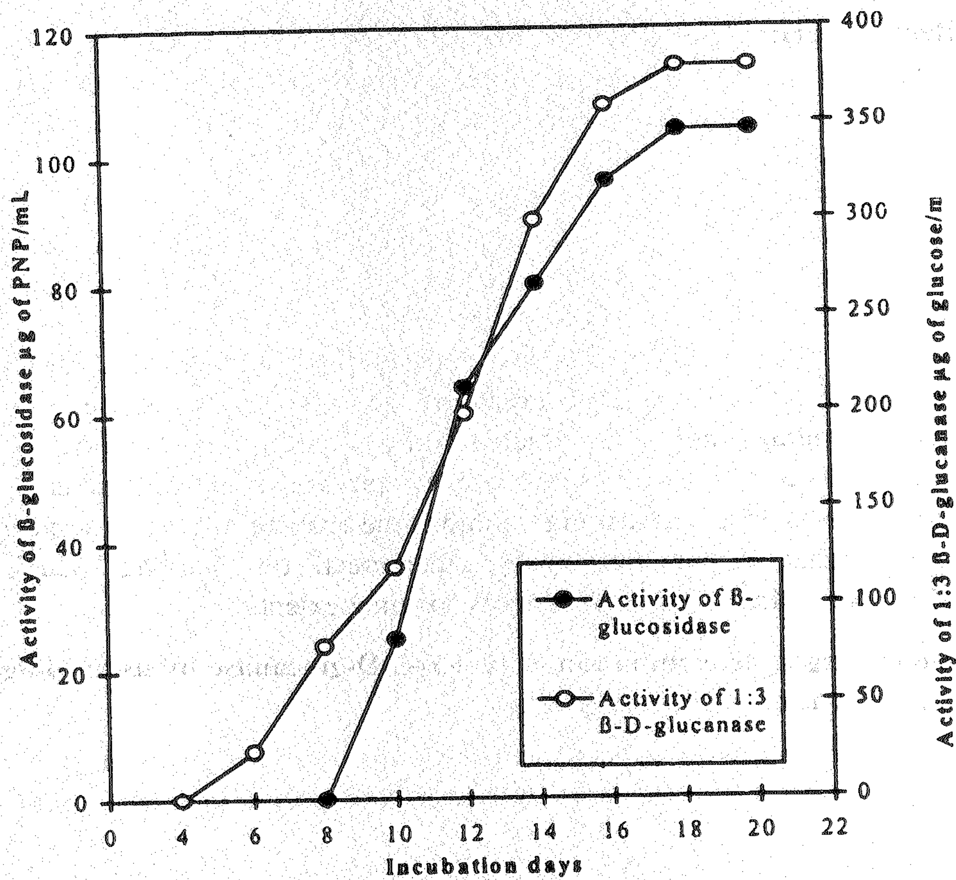

Fig. 1. Effect of incubation days on activity of enzymes

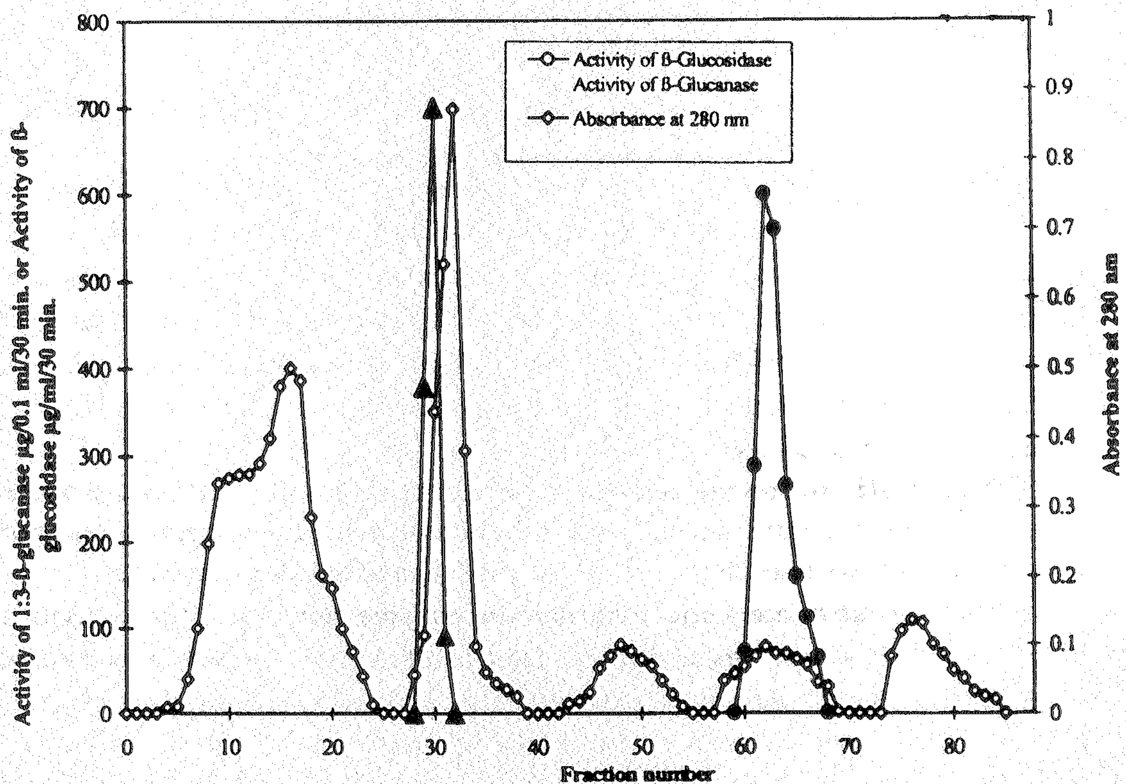

Fig. 2. Elution profile of (1→3)-β-D-glucanase and β-glucosidase

Effect of pH: The effect of pH on (1→3)-β-D-glucanase activity was investigated at various pH ranges. The observations indicate that the activity increases progressively above pH value 2.5, reaches maximum at pH 5 and declines thereafter.

Effect of substrate concentration: The activity of enzyme (1→3)-β-D-glucanase was also studied as a function of laminarin concentration in a given medium. Initially, the increase in laminarin concentration results in a rapid increase in the activity up to 200 μg laminarin concentration and in the range of 200–1000 μg of laminarin it almost remains constant. The K_m (Michaelis constant) value is 55.5 μg/mL of laminarin for (1→3)-β-D-glucanase.

Effect of metal ions: The effect of various metal ions at a final 10 mM concentration was studied on the activity of enzyme under the standard assay conditions. It was observed that Hg^{2+} inhibits the enzyme activity to some extent while Co^{2+} enhances enzyme activity; other metal ions like Mg^{2+}, Cu^{2+}, Ag^{+}, Ca^{2+}, Zn^{2+}, Na^{+}, Mn^{2+} inhibit the activity to some extent.

Molecular weight determination of (1→3)-β-D-glucanase by using Biogel-P-100 gel filtration chromatography

The pooled fractions of the peak showing activity obtained on DEAE sepharose column were loaded on Biogel P-100. During molecular sieving on Biogel P-100 the enzyme was eluted at 29th fraction while other standard proteins like alcohol dehydrogenase, albumin, carbonic anhydrase and cytochrome-C eluted out at 19th, 23th, 35th and 42nd fractions respectively. It was inferred from the elution pattern of enzyme (1→3)-β-D-glucanase on Biogel P-100 that the molecular weight of the enzyme is in the range of around 45,000 daltons.

Kinetic properties of β-glucosidase

Kinetic properties of β-glucosidase were studied under standard assay conditions.

Effect of temperature on enzyme activity: The effect of temperature on the activity of β-glucosidase was that activity increases up to 55°C and remains stable up to 60°C, thus, the temperature optima for the enzyme β-glucosidase is between 55–60°C and decreases rapidly with further rise in temperature. The enzyme activity is completely lost at 90°C. The observation that 55–60°C is the temperature of maximum activity indicates thermostability of enzyme which is suitable for industrial use.

Effect of pH on enzyme activity: The effect of pH on β-glucosidase activity was indicated in the pH range 2.5–8.0. The activity increases progressively above pH 2.5 and shows maximum activity at pH 5.5, and declines thereafter.

Effect of substrate concentration on enzyme activity: The activity of enzyme β-glucosidase was also investigated as a function of *p*-NPG concentration in a given medium. Initially increase in laminarin concentration results in a rapid increase in activity upto 400 μg *p*-NPG concentration and in the range of 400–1000 μg of *p*-NPG it remains almost constant. The K_m (Michaelis constant) value is 125 μg/mL of *p*-NPG for β-glucosidase.

Effect of metal ions: The effect of various metal ions at a final 10 mM concentration was studied on the activity of enzyme under the standard assay conditions. It was observed that ions like Mg^{2+}, Ca^{2+}, Na^{2+} exhibit no significant effect on enzyme activity. It was also found that Co^{2+}, Cu^{2+}, Hg^{2+}, Zn^{2+} inhibit enzyme activity to some extent.

Molecular weight determination of β-glucosidase by using Biogel P-200 gel filtration chromatography

The pooled fractions of the peak showing activities obtained on DEAE sepharose column were loaded on Biogel P-200. During the molecular sieving on Biogel P-200 the enzyme was eluted at 28th fraction. Standard proteins like β-amylase, alcohol dehydrogenase, albumin, carbonic anhydrase and cytochrome-C were eluted out at 22th, 26th, 32nd, 42nd and 45th fractions respectively. It was inferred from the elution pattern of enzyme β-glucosidase on Biogel P-200 that the molecular weight of the enzyme is in the range of around 1,13,000 daltons.

Depletion of glucose from the medium and slow growth are indications of the appearance of extracellular (1→3)-β-D-glucanase and β-glucosidase in the *Penicillium ochro-chloron*. This behaviour is typical of a number of glycosidases produced by bacteria and fungi which are subjected to catabolite repression and whose synthesis is stimulated under conditions that favour less growth. The hypothesis that postulates a participation of lytic enzymes in growth and extension of the cell wall is well known. According to this hypothesis, β-glucanases will collaborate in the extension of the cell wall by acting on the structural glucan and could be one of the determinants of its shape. This could be quite feasible since the cell wall of penicillia has been shown to contain (1→3)-β-D-glucan and (1→6)-β-D-glucan. Moreover, (1→3)-β-D-glucanase and β-glucosidase when partially purified from *Penicillium ochro-chloron* act on the isolated cell walls of this organism and release glucose similar to the role of β-glucanases from *Penicillium italicum*[13].

Fractionation of enzyme β-D-glucanase and β-glucosidase was done by using DEAE sepharose ion exchanger column chromatography. Both the activities were retained by the column. The stepwise elution was achieved by using salt gradient, which resolved the retained protein into four peaks. The first peak which was eluted at 0.05 M NaCl concentration showed (1→3)-β-D-glucanase activity while the β-glucosidase activity was eluted at 0.12 M NaCl concentration.

The (1→3)-β-D-glucanase was maximally active at pH 5, temperature 60°C and at 55.5 μg/mL substrate concentration. Study regarding the effect of metal iions on the enzyme activity showed that Hg^{2+} inhibits enzyme activity to some extent while Co^{2+} enhances enzyme activity. The molecular weight of (1→3)-β-D-glucanase determined by using Biogel P-100 is in the range of around 45,000 daltons.

Our study of the kinetic properties of β-glucosidase shows maximum activity at temperature between 55–60°C, at pH 5.5 and at substrate concentration 125 μg/mL. The molecular weight of β-glucosidase determined by using Biogel P-200 is in the range aound 1,13,000 daltons.

In conclusion, it was shown that (1→3)-β-D-glucanase and β-glucosidase are constitutively present in *Penicillium ochro-chloron* and the purified preparation of (1→3)-β-D-glucanase is used for the purpose of lysis of cell wall as well as for the solubilisation of glycogen fraction from the insoluble mass. The non-specific β-glucosidase separated from cultural filtrate of *Penicillium ochro-chloron* shows more activity on cellobiose, also useful for biomass conversion.

These β-glucanases have turned out to be very useful tools in protoplast presentations of *Saccharomyces* sp. and to establish the presence of the covalently linked yeast glycogen. The specific enzymic hydrolysis of cell wall β-glucans of yeast leach about 80% of cellular glycogen without cell lysis[14]. The two pools of glycogen serve differnt physiological functions. The cytoplasmic glycogen serves as an energy reservoir and the cell wall glycogen contributes to the process of flocculation.

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