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Some Properties of Inulinase from *Rhizoctonia solani*

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Abstract: The preparation and some biochemical properties of inulinase from *R. solani* which isolated from soil in Tekirdağ-Turkey, was carried out by dialysis and concentrated with silica gel-60 then, further proceeded with Sephadex G-150 and DEAE-Cellulose chromatographies. The specific activity of the enzyme was enhanced from 0.256-5.43 U mg⁻¹. The enzyme showed maximum activity at 35°C and pH 5.0, it was stable in the pH range of 5.0-6.5 and up to 40°C. The activity of enzyme was inhibited strongly by Hg²⁺ and Ag²⁺ and KCN and DTNB. The action mode of enzyme for inulin was determined as endohydrolitic-action by TLC.

Key words: Inulinase, *R. solani*, endo-action, properties, fructooligosaccharide

INTRODUCTION

Inulin is a fructose polymer which has been widely used for the production of high-fructose syrup by either enzymatic or acid hydrolysis processes^[1]. Inulin is hydrolysed in an acid medium at high temperature (80-100°C) due to bad solubility in water although fructose can be degraded easily under these conditions, resulting in undesirable side products such as difructose anhydrides^[2,3]. Enzymatic hydrolysis is usually achieved by microbial inulinases^[4-6]. However, it must be stable at high temperatures for any inulinase to be successful in industry because of the possibility of microbial contamination at room temperature^[7]. A thermally stable inulinase is required for any industrially acceptable preparation at room temperature because of the low solubility of inulin^[8]. In the present study, the partial characterisation and some properties of an inulinase obtained from *Rhizoctonia solani* isolated from Tekirdağ soil (Turkey) were investigated.

MATERIALS AND METHODS

Micro-organism: In this study, the fungus *R. solani* isolated from Tekirdağ-Turkey soil was used. It was maintained on potato dextrose agar slants.

Cultivation: The fungus was grown aerobically at 40°C for 24 h in a 500 mL shaking flask containing 100 mL of following Jerusalem artichoke powder 3.0%, NH₄NO₃ 0.23%, (NH₄)₂HPO₄ 0.37%, K₂HPO₄ 0.1%, MgSO₄ · 7 H₂O 0.005%, yeast extract 0.15% and water. The initial pH was

6.0^[9]. The medium was autoclaved at 115°C for 30 min. Micelles were removed by filtration using Whatman No. 1 filter paper. The culture filtrate was centrifuged at 5000 rpm for 10 min. The supernatants were used as the crude enzyme^[10,11].

Preparation of Jerusalem artichoke powder: Jerusalem artichokes were washed with cold water, sliced and then dried in Pasteur oven at 80°C. After milling the resultant fine powder was used directly as carbon source^[1].

Enzyme assay: 0.1 mL enzyme solution was mixed with 0.9 mL 0.1% inulin in acetate buffer (0.1 M, pH 5.0) and the mixture was incubated at 35°C for 15 min. Resulting reducing sugar was determined by the 3,5 dinitrosalicylic acid method^[12]. The amount of reducing sugar was estimated by comparison with a calibration curve that was made with fructose. One unit of inulinase activity was defined as one micromole of fructose produced per minute by the enzyme solution.

Enzyme preparation: The crude enzyme solution was dialysed against 0.1 M acetate buffer, pH 5.0 and then concentrated by embedding silica gel-60 powder which is powerful hygroscopic substance. The concentrated enzyme solution was applied to a DEAE-Cellulose column (2.5x25 cm) previously equilibrated with 0.1 M acetate buffer (pH 6.0). Active fractions were loaded onto a column of Sephadex G-150 (2.5x35 cm) equilibrated with 0.1 M acetate buffer pH 5.0. The fractions showing the enzyme activity were pooled. The active fractions obtained at this step were used as the enzyme solution.

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Protein was determined using bovine serum albumin as the standard^[13].

Enzyme properties: Enzyme preparation, is mentioned above, was used to investigate the effects of temperature, pH, K_m , V_{max} , various cations and chemicals and analysis of hydrolysis products.

Thin-layer chromatographic analysis: The hydrolysis products of inulin with *R. solani* inulinase was determined by Thin-Layer Chromatography (TLC) using 20 cm plates (Merck, TLC aluminium sheets 20x20 cm, silica gel-60 F₂₅₄). 1.0 mL enzyme was incubated with 1.0 mL of 1.0% inulin dissolved in 0.1 M acetate buffer (pH 5.0) after 15 min the reaction was stopped by boiling. Samples (3 μ L) were spotted by onto silica gel-60 aluminium sheets and 1% (w/v) inulin, fructose, glucose and sucrose standards (3 μ L) were also applied to the chromatograms. The chromatography was carried out using the solvent system, acetic acid: chloroform: water (35:30:5; v/v/v). Carbohydrates were detected by staining with aniline-diphenylamine reagent on the air dried plates. By using this procedure, fructose and fructooligomers were visualised as brown spots, glucose as a blue spot and sucrose as a dark green spot. The colours are clearly visible for up to 6 h^[14].

RESULTS

Enzyme preparation: Purification steps are shown in Table 1 for enzyme preparation. The purification of inulinase from *R. solani* yielded about 11.67%. The enzyme exhibit specific activity 5.43 U mg⁻¹ protein. The purification factor was 21.2 for inulinase of *R. solani* (Table 1).

Enzyme properties

Effect of pH on the activity and stability of inulinase: Effect of pH on activity was measured by incubation 0.1 mL of enzyme and 0.9 mL of buffers adjusted to pH of 4.0 to 8.0 containing inulin (0.1%). As the buffers Na-acetate pH: 3.0-5.0; phosphate pH: 6.0-7.0 and borate pH: 8.0 buffers. Stability of the enzyme at different pH values was studied by incubating the enzyme without substrate at different pH values ranging from 4.0 for 8.0 then estimating the residual activity. Optimum inulinase activity was found in the pH 5.0. The enzyme was stable between pH 5.0-5.6 as shown in Fig. 1.

Effect of temperature on the activity and stability of inulinase: The effect of temperature on the inulinase activity was determined as mentioned above for 10 min at pH 5.0 within temperature range of 30-80°C.

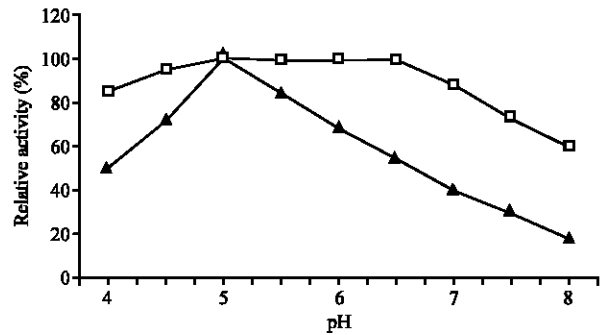


Fig. 1: Effect of pH on the enzyme activity. pH- Activity curve (▲): The reaction mixture containing 1 mL of 0.1% inulin and 0.1 mL of enzyme was incubated at 35°C for 10 min. pH-Stability curve (□): The enzyme solution was incubated at various pH for 20 min at 4°C and then residual enzyme activity was measured at pH 5.0 and 35°C

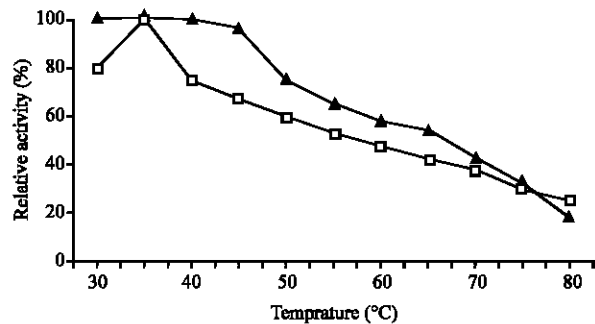


Fig. 2: Effect of temperature on the enzyme activity. Temperature activity curve (□): The enzyme reaction was carried out at pH 5.0. Temperature stability curve (▲): The enzyme was incubated at various temperature at pH 5.0 for 20 min and then residual enzyme activity was measured at pH 5.0 and 35°C

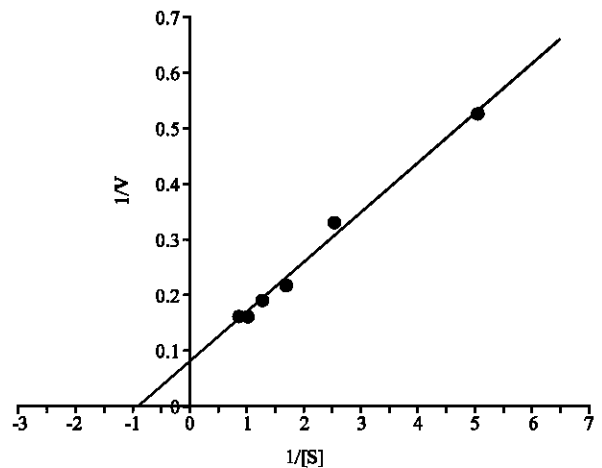


Fig. 3: Effect of substrate concentration (Lineweaver-Burk plot)

Table 1: Summary of purification of inulinase from *R. solani*

| Step | Total protein (mg) | Total activity (Units) | Specific activity (U mg ⁻¹) | Purification (fold) | Yield (%) |
|---------------------------------------|--------------------|------------------------|---|---------------------|-----------|
| Crude enzyme | 937.50 | 240.00 | 0.256 | 1 | 100.00 |
| Dialysis + concentrated by Silica gel | 206.80 | 124.50 | 0.602 | 2.35 | 51.87 |
| DEAE-Cellulose | 32.25 | 43.05 | 1.334 | 5.21 | 17.93 |
| Sephadex G-150 | 5.16 | 28.01 | 5.43 | 21.20 | 11.675 |

Table 2: The effects of some metal ions on the inulinase activity from *R. solani*

| Metal ions (1 mM) | Remaining activity (%) | Inhibition (%) | Metal ions (5 mM) | Remaining activity (%) | Inhibition (%) |
|-------------------|------------------------|----------------|-------------------|------------------------|----------------|
| None | 100.00 | - | None | 100.00 | - |
| Fe ²⁺ | 81.25 | 18.75 | Fe ²⁺ | 56.25 | 43.75 |
| Na ⁺ | 75.00 | 25.00 | Na ⁺ | 68.75 | 31.25 |
| Ca ²⁺ | 82.50 | 17.50 | Ca ²⁺ | 71.25 | 28.75 |
| Hg ²⁺ | 12.5 | 87.50 | Hg ²⁺ | 6.25 | 93.75 |
| Mn ²⁺ | 62.50 | 37.50 | Mn ²⁺ | 56.10 | 43.90 |
| Ag ²⁺ | 32.50 | 67.50 | Ag ²⁺ | 18.75 | 81.25 |
| Mg ²⁺ | 78.12 | 21.88 | Mg ²⁺ | 61.87 | 38.13 |
| Ba ²⁺ | 84.37 | 15.63 | Ba ²⁺ | 60.00 | 40.00 |
| Zn ²⁺ | 69.37 | 30.63 | Zn ²⁺ | 61.25 | 38.75 |
| Cu ²⁺ | 93.75 | 6.25 | Cu ²⁺ | 75.00 | 25.00 |

Table 3: The effects of other chemicals on the inulinase activity from *R. solani*

| Chemicals (1 mM) | Remaining activity (%) | Inhibition (%) | Chemicals (5 mM) | Remaining activity (%) | Inhibition (%) |
|-------------------|------------------------|----------------|-------------------|------------------------|----------------|
| None | 100.0 | - | None | 100.0 | - |
| KCN | 50.0 | 50.0 | KCN | - | 100.0 |
| Iodosobenzoate | 83.5 | 16.5 | Iodosobenzoate | 70.0 | 30.0 |
| β-Mercaptoethanol | 65.0 | 35.0 | β-Mercaptoethanol | 46.5 | 53.5 |
| pCMB | 60.0 | 40.0 | pCMB | 46.0 | 54.0 |
| DTNB | 30.0 | 70.0 | DTNB | - | 100.0 |

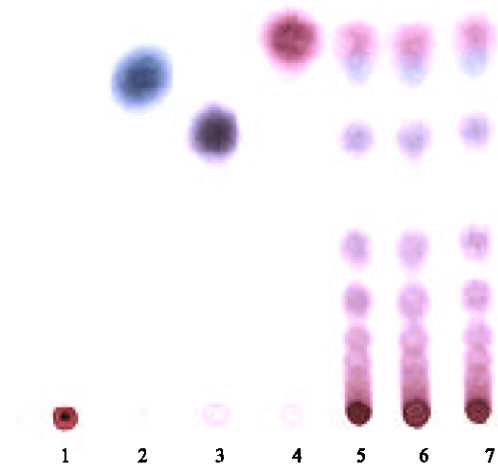


Fig. 4: Determination of hydrolysis product of inulinase from *R. solani* (TLC) 1: Inulin, 2: Glucose, 3: Sucrose, 4: Fructose (as standards), 5: 15 min hydrolysis, 6: 30 min hydrolysis, 7: 60 min hydrolysis

Thermostability was determined by incubation of the enzyme without substrate at temperature ranging from 30-80°C for 20 min. After treatment the residual inulinase activities were assayed at optimum temperature. The optimum temperature of inulinase was 35°C. The enzyme was stable for 20 min. at temperature ranging from 30-40°C (Fig. 2).

The effect of metal ions: Metal salts prepared at two different concentrations (1 and 5 mM) were incubated with the enzyme samples at 35°C and for 20 min to investigate the effect of metal ions on the enzyme activity. At the end of this period, enzyme activity was measured by adding substrate solution to the reaction medium. The highest inhibition was observed at 5 mM Hg²⁺ and Ag²⁺ treatment (Table 2).

Effects of other chemicals: Iodosobenzoate, KCN, β-mercaptoethanol, p-CMB and DTNB were prepared at two different concentrations (1 and 5 mM). Substrate solutions were added to reaction mixture after it was incubated with enzyme sample at 35°C for 20 min. Then enzyme activities were measured and compared with the values of enzyme activity which untreated by these compounds. It was obtained that DTNB and KCN (5 mM) inhibited the enzyme activity completely and p-CMP, β-mercaptoethanol caused the significant inhibition (Table 3).

Effect of substrat concentration: Figure 3 summarises the effects of different concentrations of inuline on enzyme activity of *R. solani*. When calculated by Lineweaver-Burk plot, K_m and V_{max} values for inulin under assay conditions were 1.3 mM and 13.88 mol mL⁻¹ min⁻¹, respectively.

The analysis of hydrolysis products: Thin layer chromatography analysis of the hydrolysis products of

inulin observed that major products are fructooligosaccharides which suggested that the enzyme showed endo-type hydrolysis activity. In case of exoinulinase, the products of inulin hydrolysis are only fructose (Fig. 4).

DISCUSSION

R. solani was isolated from soil in Tekirdağ-Turkey. This fungus was selected for the present study after screening several fungal strains for their capacity to synthesis extra cellular inulinase after growing on Jerusalem artichoke based growth medium. When it was grown on a medium 3.0% Jerusalem artichoke, for 24 h at 40°C, fungus produced 280 units of an extra cellular inulinase. The inulinase was prepared by dialysis, concentrated in silica gel 60 and chromatography on sephadex G-150 and DEAE cellulose.

Inulinase from *R. solani* showed maximum activity at pH 5.0, the enzyme was stable in the pH range 5.0-6.5 and the similar pH values were reported for the fungal and bacterial inulinases. The data can be compared with pH values of 4.75, 5.2, 6.0, 6.1 and 7.0 for inulinases from *K. marxianus* Var. *bulgaricus*, *P. trzebinski*, *P. rugulosum*, *B. stearrowthermophilus* and *B. subtilis* 430 A, respectively^[15-18]. Moreover, *Chrysosporium pannorum* inulinase was reported to have pH stability in the range of 4.5-7.0^[19]. Maximum activity of the inulinase from *A. awamori* was determined at pH 4.5 and more than 80% of the maximum activity retained in the interval pH from 3.0 to 5.0^[3].

The maximum enzyme activity of *R. solani* was determined at 35°C, the enzyme was stable up to 40°C at pH 5.0. Similarly the optimum reaction temperature of the *Arthrobacter globiformis* 564-1's inulinase was 40°C, the enzyme was stable up to 40°C at the pH 6.5^[20]. It has been reported the inulinase of *Chrysosporium pannorum* was stable up to 45°C, but lost 15% of its activity at 50°C and lost its activity at 60°C completely with 30 min. and the optimum temperature was around 50°C^[19]. The inulinases from *A. niger*^[21] and *A. oryzae*^[7] display a temperature optimum of 50°C, while the most fungal inulinases have optimum between 30-55°C^[22-25].

The enzyme reaction was performed in the reaction mixture containing 1 and 5 mM of each metal ions at pH 5.0 and 35°C. As shown in Table 1, both Hg²⁺ and Ag²⁺ (5 mM) inhibited the enzyme activity strongly. This observation was also reported by other researchers^[26,27] and it is suggested that some -SH groups are essential for the activity of inulinase produced by molds. Mn²⁺, Fe²⁺, Na⁺, Ca²⁺ and Cu²⁺ (5 mM) caused decreasing in the inulinase activities by 43.9, 43.75, 31.25, 28.75 and 25%,

respectively. The other cations (Ba²⁺, Zn²⁺, Mg²⁺) inhibited the activity by approximately 40%. The activity of purified enzyme from *K. marxianus* was investigated in the presence of several cations. Manganese had not any effect on the activity, Ca²⁺ inhibited the enzyme by approximately 27%, Ba²⁺, Zn²⁺ and Na⁺ inhibited 50%, while ferric chloride completely inhibited the enzyme^[17].

The enzyme K_m value and V_{max} for inulin was calculated approximately as 1.3 mM and 13.88 mol mL⁻¹ min⁻¹, respectively. The K_m of *R. solani* from the present study could be compared with K_m values of 1.2x10⁻² mM from *C. acetobutylicum* ABKn8^[22], 0.21 mM from *P. purpurogenum*^[18], 0.42 mM from *P. trzebinski*^[28], 0.20 mM from *Penicillium* sp. Tn-88^[29]. K_m values of some *Aspergillus* species were found as follows; 0.12 mM for *A. versicolor*, 0.3 mM for *A. oryzae* and 0.003 mM for *A. awamori*^[3,7,8].

As shown in Fig. 4 inulinase from *R. solani* hydrolysed inulin by endo-action. Fructooligosaccharides, glucose and fructose was identified as products of inulinase action. The results indicated that inulinase by *R. solani* is endo-inulinase. The endoinulinases are potentially useful for large scale production of fructooligosaccharide from inulin containing agricultural crops. Because they hydrolyse fructans which type inulin by endo-action mode by produced a series of fructooligosaccharides. Therefore, they are important for the production of fructooligosaccharides which is known as soluble diet fibrils and increased the population of bifidobacteria is localized in human intestine^[29-31].

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