PURIFICATION AND CHARACTERIZATION OF TWO INVERTASES FROM MUTANT STRAIN OF SACCHAROMYCES CEREVISIAE

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Abstract

In the present study, a mutant strain of Saccharomyces cerevisiae EMS-42 was used for the biosynthesis of invertase (E.C.3.2.1.26). Both types of invertases i.e., extracellular and intracellular invertase are present in S. cerevisiae. An extracellular invertase was purified to homogeneity by two step purification i.e., ammonium sulfate precipitation and DEAE-Sephadex A-50. The enzyme was present in the supernatant of 85% saturation being glycoprotein in nature. DEAE column chromatography eluted enzyme as single active fraction at 0.2 M NaCl. The enzyme was purified by 15 fold with recovery of 38%. The molecular mass of 110 kDa was determined after SDS-PAGE. The carbohydrate content was found to be 48%. The intracellular invertase contains both forms of glycosylated (large) and non-glycosylated (small). The similar above procedure was applied for glycosylated intracellular invertase (L-form) while three steps for non-glycosylated invertase (S-form). The L-form was purified by 19 fold with recovery of 32%. Like extracellular invertase, the molecular weight was (110 kDa) for L-form. Ammonium sulfate precipitation separated the enzyme (S-form) as insoluble fraction. The enzyme was eluted at 0.3 M NaCl using DEAE-Sephadex. A single band of molecular weight (55 kDa) was estimated after Sephadex G-50 with purification (16 fold) and recovery of 17%. Both types of invertases were isolated as monomeric protein. The optimum pH, temperature, MnCl₂ and the values of the Kₘ and Vₘₐₓ for non-glycosylated and glycosylated were found to be as 5, 50 and 60°C, (109 and 111%), (1.2 mM and 909 U/ml/min, 1.8 mM and 1429 U/ml/min), respectively.

Introduction

The yeast Saccharomyces cerevisiae is a rich source of both intracellular and extracellular invertase (β-D. fructofuranosidase, E.C. 3.2.1.26) catalyzes the reaction of detachment of the terminal nonreducing β-D. fructofuranoside residue in β-D. fructofuranosides (Amin et al., 2010). Its preferred substrate is saccharose but invertase is also able to catalyze the hydrolysis of raffinose, stachyose and inulin (Belcarz et al., 2002; Gore et al., 2009). There are many industrial uses of invertase in the production of invert syrup, non-crystallizing creams, jams, artificial honey, lactic acid and ethanol, confectionary, in digestive aid tablets, powder milk for infants and other infant foods etc. (Haq and Ali, 2007; Acosta et al., 2000; Phadtare et al., 2004; Safarik et al., 2009).

Invertase exists in two forms, glycosylated periplasmic protein and cytosolic non-glycosylated protein (Vitolo et al., 1995; Rashad et al., 2006). The secretion of enzyme located intracellularly which corresponds to repressed forms of invertase and extracellular one, containing nine or ten N-glycosidically linked oligosaccharides which corresponds to the de-repressed form of the enzyme (Hufläker & Robins, 1983) are regulated by catabolic repression. The high concentration of glucose in the culture medium completely repressed production of the enzyme, whereas the use of sucrose or raffinose as carbon source allowed derepression of invertase synthesis. It is known that both of these enzymes are synthesized on the matrix of the same structural gene and their protein moieties have a molecular mass of 60 kDa (Moreno et al., 1990).

Sonication is one of the most commonly employed methods for cell disruption (James et al., 1972). Ultrasound has been used to extract and release intracellular enzymes such as invertase from S. cerevisiae being secreted to the periplasmic space (Balasundaram & Pandit, 2001).

For the purification of proteins different precipitants such as ammonium sulphate, acetone, ethanol etc have been used as initial purifying agents (Pimpa 2004). The column chromatography has been a popular technique for isolation and quantifying the components from mixture of the compounds. For purification of invertase, mostly anion exchange chromatography and gel filtration techniques are used (Guimaraes et al., 2007; Uma et al., 2010).

Materials and Methods

Microorganism: Saccharomyces cerevisiae IS-66 was isolated from different fruits and soil samples and identified by Wickerham (1951) and Lodder & Kreger-Van (1952). It was chemically mutated by ethyl methane sulfonate (EMS). One mutant EMS-42 was achieved after treating with EMS concentration of 100 μl/ml at 15 min exposure time and selected as hyperproducer for invertase. It was maintained on YPSA medium containing (g/l), Yeast extract 3, Peptone 6, Sucrose 15 and Agar 20.

2-deoxy D-glucose resistance: The potential mutant strains were cultured overnight on the YPS agar medium, harvested during the exponential phase of growth (1×10⁷ cells/ml), washed with sterilized distilled water and plated on the 2dg-YPR agar medium containing (mg/ml): yeast extract 3, peptone 5, raffinose 20, agar 20 and 2-deoxy-D-glucose (0.02-0.10). The concentration of 0.04 mg/ml was found optimal, as at this level EMS-42 gave consistent invertase production. Colonies exhibiting the most vigorous growth were tested for stability in invertase production by shake flask fermentation. The master culture was preserved in sterile 20 % (v/v) glycerol at -80°C.

Preparation of inoculum: Fifty millilitre of the YPS medium was transferred to the individual 250 ml Erlenmeyer flasks. Cell suspension was prepared from a
2-3 day old slant culture. One millilitre of the cell suspension was aseptically transferred into the flask and incubated at 30°C in a rotary shaking incubator (Model: 10X400.XX2.C, SANYO Gallenkamp, PLC, UK) at 200 rpm for 18 h.

**Invertase production in stirred fermentor:** Production of invertase was carried out in a laboratory scale stirred fermentor of 7.5 L capacity with working volume of 5 L (New Brunswick Scientific Bioflo 110, USA). The inoculum was transferred into YPS broth medium at level of 7.5%, v/v. The cultural conditions were maintained as agitation speed (240 rpm), aeration rate (1vvm), dissolved oxygen (10%) at 30°C for 24 h.

**Isolation of extracellular invertase:** The harvested cell free broth was centrifuged at 18,000×g for 30 min to get the supernatant crude extract having intracellular invertase. The ammonium sulfate was added at varying amounts (20-85%) to cell supernatant crude extract after sonication was spun at 12,000×g for 15 min at 4°C. The resultant precipitates and supernatants were dissolved in 0.5 M Tris-HCl buffer, pH 7.5 and dialyzed against same buffer.

**Ammonium sulfate precipitation:** The cell free broth containing enzyme activity were pooled, dialyzed and purified to homogeneity by following purification steps.

**Protein marker:** The molecular weight of the invertase was estimated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Hames (1990).

**Electrophoresis:** At each step of purification, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Hames (1990).

**Carbohydrate content:** The total carbohydrate content was detected by the phenol sulfuric acid method (Dubois et al., 1956) with mannose as standard.

**Characterization of purified invertase**

**Effect of pH and temperature on stability of invertase:** It was observed by taking hundreds microlitres of appropriately diluted enzyme solution incubated in 0.05 M citrate/0.05 M acetate buffer at different pH values ranging for 2-8 at 40°C for 15 min. At optimal pH, the invertase activity was tested at varying temperature values (20-80°C). For this, the reaction mixture was incubated for 15 min at different temperatures and residual activity in both parameters was determined under standard conditions (Akgol et al., 2001).

**Effect of additives on enzyme activity:** Different chemicals and metal ions such as NaCl, KCl, MnCl2, EDTA, BaCl2, MgCl2, CuSO4, HgCl2, CoCl2, FeSO4, CaCl2 and ZnSO4 preincubated with the purified enzyme at 1 mM at 30°C for 30 min. before determination of the enzyme activity. Blank was taken showing relative activity (100 %) before adding the metals.

**Determination of kinetic constant (Km):** The Km value of the invertase is determined using sucrose as a substrate and using Lineweaver-Burk plot (Lineweaver & Burk, 1934) by following conditions of sucrose (10-100 mM) in 0.05 M acetate buffer (pH 4.5), incubation time (15 min) and temperature (35°C). The amount of liberated reducing sugars was measured by Miller, (1959).
**Determination of maximum velocity (V\textsubscript{max}):** The maximum velocity (V\textsubscript{max}) of sucrose hydrolysis of invertase under same optimal conditions of kinetic constant was calculated.

**Results and Discussion**

An extracellular invertase from *S. cerevisiae* EMS-42 was purified through successive steps of ammonium sulfate (40-85%) precipitation and DEAE-Sephadex A-50 (Table 1). The key step involved a fractionation of insoluble and soluble forms of invertase apparently due to differences in carbohydrate content of the enzymes. The ammonium sulfate at the 85% saturation level separates the external (glycosylated) invertase as soluble fraction while giving insoluble in precipitated form. Gascon & Lampen (1968) separated the external invertase from the internal by ammonium sulfate precipitation method. Both precipitated and soluble fractions (85% ammonium sulfate supernatant) were dialyzed and run on SDS-PAGE as shown in Fig. 1. The one major peak as shown in Fig. 2 was eluted by using 0.2 M NaCl. When this peak was tested by electrophoresis, only one broader band (Fig. 3) was found with molecular mass of 110 kDa. Milintawisamai *et al.* (2007) found extracellular invertase from *C. humicola* as the result from 60-100% ammonium sulfate saturation followed by DEAE column chromatography and eluted extracellular protein fraction using 0.3-0.35 M NaCl with molecular mass of 110 kDa as a single active fraction. The purified extracellular was glycoprotein in nature with 48% carbohydrate content.

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Volume (ml)</th>
<th>Total activity (U)</th>
<th>Total Protein (mg/ml)</th>
<th>Specific activity (U/mg)</th>
<th>Fold Purification</th>
<th>Activity recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude broth</td>
<td>1000</td>
<td>53120</td>
<td>404</td>
<td>131</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Freeze dried ammonium sulfate supernatant (85%)</td>
<td>200</td>
<td>34016</td>
<td>210</td>
<td>162</td>
<td>1.2</td>
<td>64</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>8</td>
<td>20110</td>
<td>10.5</td>
<td>1915</td>
<td>15</td>
<td>38</td>
</tr>
</tbody>
</table>

The specific activity of the purified extracellular invertase was estimated to be 1915 U/mg, which is about 15 fold than that of the crude enzyme with final activity recovery of 38%. The optimum pH and temperature were found to be as 5 and 60°C, respectively (Figs. 7 & 8). Chavez *et al.* (1997) obtained invertase from *Candida utilis* with molecular weight of 150 kDa at pH of 5.5 temperature of 60-75°C. Table 3 depicts the data on the effect of additives such as NaCl, KCl, MnCl\textsubscript{2}, EDTA, BaCl\textsubscript{2}, MgCl\textsubscript{2}, CuSO\textsubscript{4}, HgCl\textsubscript{2}, CoCl\textsubscript{2}, CuCl\textsubscript{2}, FeSO\textsubscript{4}, CaCl\textsubscript{2} and ZnSO\textsubscript{4} at the concentration of 1 mM on glycosylated invertase. Of the all, MgCl\textsubscript{2}, MnCl\textsubscript{2} and CoCl\textsubscript{2} was found to be as slightly stimulatory with relative activity from 102-111% while remaining caused reduction in relative activity. The addition of HgCl\textsubscript{2}, CuSO\textsubscript{4} and CuCl\textsubscript{2} completely inhibited the enzyme. By using Lineweaver-burk plot, the K\textsubscript{m} and V\textsubscript{max} values were observed to be as 1.8 mM and 1429 U/ml/min, respectively (Fig. 9). Hernalsteens & Maugeri (2008) gave the K\textsubscript{m} (13.4 g/l) and V\textsubscript{max} (21 μmol/ml/min) for sucrose by invertase in *Candida* sp.
The intracellular invertase from mutant strain was purified successively through three steps i.e., ammonium sulfate (20-85%) saturation, DEAE-Sephadex A-50 and Sephadex G-50 (Table 2). The procedure of ammonium sulfate was also used for the separation of two forms of intracellular invertase, one in small amount (S-form) being non-glycosylated was recovered from (20-85%) saturation in precipitated form. On the other hand, the second form in large amount (L-form) was found from supernatant of 85% ammonium sulfate saturation being glycoprotein in nature. It means that extracellular invertase isolated from the cell free broth was secretory periplasmic enzyme. Carlson et al., (1983) and Batista et al., (2004) reported that two forms of invertase (secreted and non-secreted) are exist in S. cerevisiae. It can be extracted from the cells in the same glycosylated form as shown in Fig. 1 (lane 8, arrow indicated) & Fig. 4 (lanes 2-3). In both cases the bands were broad having same molecular weight of 110 kDa. The further purification of L-form invertase was obtained in the same manner as extracellular invertase. For the purification of S-form invertase, the collected precipitates were dialyzed and then loaded on DEAE-Sephadex column. After anion-exchange, out of four protein peaks one peak was eluted at 0.3 M NaCl showing enzyme activity as shown in Fig. 5. Kern et al., (1992) found intracellular invertase in yeast at NaCl (0.15-0.3 M) by using Q-Sepharose and found it of 115 kDa. Similar finding by Trimble & Maley (1977) who obtained carbohydrate free invertase composed of two 60 kDa subunits in S. cerevisiae. After pooling active fractions, the concentrated protein was further purified on Sephadex G-50 column. As that result only one protein peak showing invertase activity was obtained (Fig. 6). After SDS-PAGE only one protein band was found having approximately molecular weight of 55 kDa as shown in (Fig. 4, lane 5). After Phenol-Sulfuric test the L-invertase was found to be glycoprotein and S-invertase as carbohydrate-free protein. Both glycosylated and non-glycosylated invertase proteins were found to be monomeric being single bands. In contrary it was reported as dimer, tetramers, hexamers, octamer, (Trimble & Maley 1977; Chu et al., 1983; Rodriguez et al., 1995).

Table 2. Purification steps of intracellular invertase (S and L forms).

<table>
<thead>
<tr>
<th>Forms of intracellular invertase</th>
<th>Purification steps</th>
<th>Volume (ml)</th>
<th>Total activity (U)</th>
<th>Total protein (mg/ml)</th>
<th>Specific activity (U/mg)</th>
<th>Fold purification</th>
<th>Activity recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-form invertase</td>
<td>Crude extract</td>
<td>500</td>
<td>29700</td>
<td>287</td>
<td>103</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Ammonium sulfate (20-80%)</td>
<td>100</td>
<td>10346</td>
<td>95</td>
<td>109</td>
<td>1.1</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>DEAE-Sephadex</td>
<td>28</td>
<td>7300</td>
<td>28</td>
<td>260</td>
<td>2.5</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Sephadex G-50</td>
<td>1.5</td>
<td>5011</td>
<td>03</td>
<td>1670</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Freeze dried ammonium sulfate supernatant (85%)</td>
<td>100</td>
<td>14221</td>
<td>72</td>
<td>98</td>
<td>1.9</td>
<td>46</td>
</tr>
<tr>
<td>L-form invertase</td>
<td>DEAE-Sephadex</td>
<td>2.5</td>
<td>9820</td>
<td>05</td>
<td>1964</td>
<td>19</td>
<td>32</td>
</tr>
</tbody>
</table>

Fig. 3. The SDS-PAGE of purified extracellular invertase.
* Lane 1 Protein marker. Lanes 2-3, purified extracellular invertase

Fig. 4. The SDS-PAGE after ammonium sulfate and chromatography for intracellular invertase.
* Lane 1, protein marker
Lanes 2-3, intracellular crude extract
Lane 4, 60% ammonium sulfate precipitate
Lane 5, purified non-glycosylated intracellular S-invertase
The specific activity of the purified intracellular S-invertase and L-invertase were estimated to be as 1670 U/mg, 1964 U/mg and fold purification of 16, 19 with recovery of 17% and 32%, respectively as shown in Table 2. The optimum pH (5) and temperature (50°C) of non-glycosylated invertase was found to be observed (Figs. 7 & 8). The effect of chemicals and metal ions such as NaCl, KCl, MnCl₂, EDTA, BaCl₂, MgCl₂, CuSO₄, HgCl₂, CoCl₂, CuCl₂, FeSO₄, CaCl₂ and ZnSO₄ on non-glycosylated invertase was also investigated (Table 3). The findings after adding all additives was almost similar to glycosylated invertase but with slight decrease of relative activity. In contrast with glycosylated, the nonglycosylated internal form of invertase is extremely sensitive to proteolysis (Williams et al., 1985). From Lineweaver-burk plot, the $K_m$ and $V_{max}$ values for intracellular non-glycosylated were found to be as 1.2 mM and 909 U/ml/min, respectively (Fig. 10). Similar findings by Belcarz et al., (2002) were obtained $K_m$ values against sucrose for S (slow) and F (fast) forms of invertase in Candida utilis as 2 and 1.5 mM, respectively.

**Table 3. Effect of additives on stability of purified glycosylated and non-glycosylated invertase.**

<table>
<thead>
<tr>
<th>Additives (1 mM)</th>
<th>Non-glycosylated (%)</th>
<th>Glycosylated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NaCl</td>
<td>93 ± 0.2</td>
<td>96 ± 0.5</td>
</tr>
<tr>
<td>KCl</td>
<td>96 ± 1.0</td>
<td>96 ± 1.1</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>109 ± 0.04</td>
<td>111 ± 0.09</td>
</tr>
<tr>
<td>EDTA</td>
<td>102 ± 1.5</td>
<td>103 ± 2.5</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>97 ± 0.3</td>
<td>98 ± 1.3</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>105 ± 1.4</td>
<td>107 ± 0.9</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>17 ± 1.5</td>
<td>17 ± 1.0</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>2.9 ± 2.0</td>
<td>3.0 ± 1.9</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>102 ± 1.0</td>
<td>104 ± 0.8</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>86 ± 2.2</td>
<td>89 ± 1.3</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>78 ± 1.8</td>
<td>80 ± 1.6</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>19 ± 1.2</td>
<td>21 ± 0.8</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>88 ± 2.4</td>
<td>90 ± 2.5</td>
</tr>
</tbody>
</table>

*Fig. 7. Effect of pH on stability of purified glycosylated and non-glycosylated invertase.*

*Hundreds microlitres of each enzyme solution was incubated in 0.05 M citrate/0.05 M acetate buffer at pH values ranging for 2-8 at 40°C for 15 min. The residual activity was determined under standard conditions.*

*Fig. 8. Effect of temperature on stability of purified glycosylated and non-glycosylated invertase.*

*The enzyme activity was measured in the temperature range of 20-80°C. The reaction mixture (pH 5.0) was incubated for 15 min and residual activity was determined under standard conditions.*
Proteins were noticed after SDS-PAGE. The intracellular non-glycosylated (55 kD) as monomeric glycosylated invertase having 48% carbohydrate content was selected because it contained both types of invertases. It was previously mutagenized chemically by EMS for the production of a byproduct of continuous ethanol fermentation. Production, separation and purification of yeast invertase as a byproduct of continuous ethanol fermentation. Production and partial characterization of an invertase from Candida utilis: Comparison with natural and recombinant yeast invertases. J. Biotechnol., 53(1): 67-74.

The intercept on the y-axis corresponding to 1/V max = 0.0011, Slope = 0.0013.

\[
y = 0.0013x + 0.0011 \\
R^2 = 0.7333
\]

The intercept on the y-axis corresponding to 1/V max = 0.0007, Slope = 0.0013.

\[
y = 0.0013x + 0.0007 \\
R^2 = 0.7294
\]

The mean difference is significant at p<0.05. Y bars indicate the standard deviation (±sd) among the three parallel replicates.

**Conclusion**

*Saccharomyces cerevisiae* was selected because it contained both types of invertases. It was previously mutagenized chemically by EMS for the production of extracellular invertase as major secretory enzyme. A 15 fold purification of extracellular invertase with recovery of 38% was achieved. The molecular weight (110 kD) of glycosylated invertase having 48% carbohydrate content and intracellular non-glycosylated (55 kD) as monomeric proteins were noticed after SDS-PAGE.

**References**


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