Trehalose, a disaccharide formed by the two glucose units linked in an α,α-1,1-glycosidic linkage is widely present in yeasts, fungi and plants. It has been receiving much attention due to its great importance in nature and industry. In nature, it plays a number of roles from being an energy source to a stress protectant, enhancing the resistance of cellular components against different abiotic stresses, such as high temperature, freezing, high osmotic pressure, dehydration, and high ethanol concentration. In industry, it can be used as a cryoprotectant for cells in medicine and microbiology, as an effective component in cosmetics, as a stabilizer for clinical reagents and bioproducts, and as a preservative for fresh foodstuffs.

Trehalose production has been reported through fermentation, enzymatic process from starch, sucrose and maltose, and extraction from transformed plants. So far, two pathways for trehalose biosynthesis have been reported in fungi. The first pathway is that it is synthesized in a two-step process in Candida albicans, Aspergillus niger, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Zygosaccharomyces rouxii, and others. First, trehalose-6-phosphate is formed from UDP-glucose and glucose-6-phosphate by trehalose-6-phosphate synthase (Tps1). Second, this compound is dephosphorylated into trehalose by trehalose-6-phosphatase (Tps2). Both tps1 and tps2 genes, encoding Tps1 and Tps2, respectively, have been cloned in A. niger, A. nidulans C. albicans, Hansenula polymorpha, Kluyveromyces lactis, S. cerevisiae, S. pombe, Yarrowia lipolytica, and Z. rouxii. The second pathway is that trehalose phosphorylase or trehalose synthase (TSase) in Agarius bisporus and Grifola frondosa catalyzes the reversible reaction of degradation and synthesis of trehalose from D-glucose and α-D-glucose-1-phosphate.

The starch is considered as best substrate for production of trehalose, due to its low cost and easy availability. In our previous study, we found that Saccharomyces fibuligera Sdu can use soluble starch as sole carbon and energy source and produce significant amounts of trehalose (a yield of 18.0%, w/w, cell dry wt basis within 48 h of fermentation). In another study, a mutant with low acid and neutral trehalase activities of S. fibuligera Sdu was found to accumulate more trehalose from soluble starch than its parent strain, when grown in YPS medium.

In this study, attempts have been made to purify and characterize trehalose-producing enzyme from mutant A11 of S. fibuligera Sdu, which has been...
reported to produce a large amount of trehalose from starch.

Materials and Methods

Yeast strain

A11: a mutant of S. fibuligera Sdu with low acid and neutral trehalase activities was used in the present study. Earlier, it was reported that this mutant produced higher amounts of trehalase from soluble starch in YPS medium than its parent strain. The mutant was maintained at 4°C on YPS medium containing 1.0% yeast extract, 2.0% polypeptone, 2.0% soluble starch, and 2.0% agar.

Preparation of cell-free extract

The cells of mutant A11 were cultivated in liquid YPS medium at 30°C by shaking for 9-10 h and collected by centrifugation at 7227 g and 4°C for 5 min and washed 3 times with ice-cold distilled water. The cell debris was removed by centrifugation at 16260 g and 4°C for 30 min and about 50 ml of the supernatant was concentrated to 3-4 ml by ultrafiltration (10 kDa cut-off) with a Labscale™ TFF system (Millipore, USA). The concentrated cell-free extract was used as the enzyme preparation.

Enzyme and protein assay

Tps1 activity in the enzyme preparation was measured using the assay described previously. Briefly, 0.4 ml of the reaction mixture containing 0.05 mM HEPES-KOH (pH 7.0), 5 mM UDP-glucose, 10 mM glucose-6-phosphate, 25 mM MgCl₂, 0.1 ml the enzyme preparation and a suitable amount of distilled water, was incubated at 37°C for 20 min. The reaction was terminated in 100°C water bath for 5 min. After cooling, the mixture was centrifuged at 2823 g for 5 min. The concentration of UDP formed in the supernatant was determined according to the decrease in OD₂₅₄ in 0.5 ml of the mixture containing 0.14 M HEPES-KOH (pH 7.6); 2 mM phosphoenolpyruvate, 0.3 mM NADH, 5 U lactic dehydrogenase and 5 U pyruvate kinase.

Protein concentration was measured by the method of Bradford using bovine serum albumin served as standard. One unit of enzyme activity was defined as the amount of enzyme that produced 1.0 μM of NAD⁺ per min at 37°C and pH 7.0.

To measure trehalose synthesis from α-D-glucose-1-phosphate and glucose, 0.1 ml of the reaction mixture containing 100 mM α-D-glucose-1-phosphate, 100 mM glucose, 100 mM HEPES buffer (pH 7.0) and 40 μl of the enzyme preparation was incubated at 35°C for 3 h and the reaction was stopped by boiling for 5 min. The inorganic phosphate liberated was determined by ammonium molybdate method.

Purification of Tps1

Purification of Tps1 was carried out using AKTA™ prime with Hitrap™ (Amersham Biosciences, Sweden).

Sepharose CL-4B gel filtration chromatography

The 3 ml of the enzyme preparation was loaded on a Sepharose CL-4B gel filtration column (16 x 100 cm) equilibrated and eluted with the buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM PMSF and 2 mM EDTA with a flow rate of 0.5 ml/min and 3 ml of fraction was collected in each tube. Tps1 activity and protein content in each tube were determined by using the methods as described above. The fractions showing the Tps1 activity were pooled.

DEAE-Sepharose Fast Flow anion exchange chromatography

The pooled fractions showing the Tps1 activities were applied to DEAE-Sepharose fast flow anion-exchange column (16 x 25 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.8). After washing the column (by eluting with 20 mM Tris-HCl buffer, pH 7.8), the proteins were eluted with a linear gradient of 0-1.0 M NaCl in the same buffer in a total volume of 250 ml with a flow rate of 1.0 ml/min and 2 ml of the elute was collected in each tube. Tps1 activity and protein content in each tube were determined by using the methods as described above. The fractions showing the Tps1 activity were concentrated to 100 μl by ultrafiltration (3 kDa cut-off).

Gel electrophoresis

The purity and molecular mass of Tps1 in the concentrated fractions showing the activity were analyzed in non-continuous denaturing SDS-PAGE with a 2-D electrophoresis system (Amersham Biosciences, Sweden) and by silver staining. The molecular mass standards used were β-galactosidase (116.2 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), α-lactalbumin (36 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), and soybean trypsin inhibitor (20.1 kDa).

Effect of the concentration of metal ions on the activity

The effects of the concentration of metal ions (Cu²⁺, Zn²⁺, Ca²⁺, Mg²⁺, Mn²⁺, Ni²⁺, Co²⁺, and Fe²⁺) were studied at 4°C. The reaction mixture contained 50 mM HEPES-KOH (pH 7.0), 5 mM UDP-glucose, 10 mM glucose, 25 mM MgCl₂, 0.1 ml the enzyme preparation, and 0-10 mM metal ions. The activity of Tps1 was determined as described above. The effects of metal ions on the activity of Tps1 were determined by using the methods as described above. The fractions showing the Tps1 activity were concentrated to 100 μl by ultrafiltration (3 kDa cut-off).

The effect of metal ions on the activity of Tps1 was studied at 4°C. The reaction mixture contained 50 mM HEPES-KOH (pH 7.0), 5 mM UDP-glucose, 10 mM glucose, 25 mM MgCl₂, 0.1 ml the enzyme preparation, and 0-10 mM metal ions. The activity of Tps1 was determined as described above. The effects of metal ions on the activity of Tps1 were determined by using the methods as described above. The fractions showing the Tps1 activity were concentrated to 100 μl by ultrafiltration (3 kDa cut-off).
The effect of pH on the enzyme activity was determined by incubating the purified enzyme between pH 4.0-9.0 using the standard assay conditions. The buffers used were 0.01 M acetate buffer (pH 4.0-5.0), 0.01 M phosphate buffer (pH 6.0-7.0) and 0.01 M Tris-HCl buffer (pH 7.5-9.0). The pH stability was tested by 30 min pre-incubation of the purified enzyme in appropriate buffers that had the same ionic concentrations at different pH values ranging from 4.0 to 9.0 at 4°C. The residual activities of Tpsl were measured immediately after this treatment with the standard method as mentioned above.

The optimal temperature for activity of the enzyme was determined at 0°C, 19°C, 28°C, 37°C, 42°C, 47°C and 52°C in the same buffer as described above. The temperature stability of the purified enzyme was determined by pre-incubating the enzyme at different temperatures ranging from 0°C to 52°C for 30 min, and measuring residual activity immediately, as described above. The pre-incubated sample at 4°C was used as reference to calculate the residual activity.

Effect of different metal ions on Tpsl activity

To examine the effect of different metal ions on Tpsl activity, the enzyme assay was performed in the reaction mixture as described above, with various metal ions at a final concentration of 25 mM. The metal ions used were CuSO₄, SnCl₂, MgSO₄, FeCl₃, CaCl₂, KCl, MnCl₂, HgCl₂ and CoCl₂. The activity assayed in the absence of metal ions served as control.

Effect of protein inhibitors on Tpsl activity

The effect of protease inhibitors (EDTA, PMSF and iodoacetic acid at a final concentration of 1.0 and 5.0 mM, respectively) on Tpsl activity was measured in the reaction mixture as described in ‘Materials and Methods’. The purified enzyme was pre-incubated with the respective inhibitors for 10 min at 4°C, followed by the standard enzyme assay as described above. The activity assayed in the absence of the protein inhibitors served as control.

Results

Determination of pathway for trehalose synthesis in mutant A11

The biosynthesis of trehalose in yeasts occurs predominantly via the trehalose synthase complex and this pathway has been studied extensively. An alternative pathway for trehalose synthesis and degradation is provided by trehalose phosphorylase in *Grifola frondosa* and *Agaricus bisporus*\(^{10,17}\). In order to know the biosynthesis of trehalose in mutant A11, in the present study, Tpsl and trehalose phosphorylase activities were determined in the cell extract. Although Tpsl activity was detected in the cell extract, no trehalose phosphorylase activity was found (data not shown).

Purification of Tpsl

Tpsl was purified from the concentrated cell extract, prepared from the cells cultivated for 9-10 h at 30°C. Elution profile of Sepharose CL-4B gel filtration chromatography (Fig. 1A) shows that peak 2 with Tpsl activity from 150 to 240 min displayed a single peak. Therefore, the fractions were collected and the pooled fractions were applied to DEAE-Sepharose fast-flow anion-exchange column. The elution profile (Fig. 1B) shows that peak 4 with Tpsl activity displayed a single peak, when NaCl concentration reached 0.69 M. Therefore, the fractions were collected and concentrated by ultrafiltration. The results in Table 1 show that the enzyme was purified to homogeneity, with a 30-fold increase in the specific activity and a yield of about 61%, compared to the concentrated cell-free extract.

Gel electrophoresis

SDS-PAGE was used to determine protein purity and molecular mass of the finally concentrated elute as described by Laemmli\(^{21}\). The results in Fig. 2 show only a single protein band from the finally concentrated elute with a relative molecular mass of 66 kDa. Thus, it may be concluded that Tpsl from mutant A11 was composed of monomer\(^{27}\).

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**Table 1: Summary of purification procedure of trehalose-6-phosphate synthase**

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total protein (mg)</th>
<th>Total activity (U/min)</th>
<th>Specific activity (U/min.mg)</th>
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<th>Yield (%)</th>
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<td>Cell extract</td>
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<td>2.4</td>
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<td>4.8</td>
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<td>3.2</td>
<td>66</td>
</tr>
<tr>
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<td>0.44</td>
<td>31.7</td>
<td>72</td>
<td>30</td>
<td>61</td>
</tr>
</tbody>
</table>

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**The biosynthesis of trehalose in yeasts occurs predominantly via the trehalose synthase complex and this pathway has been studied extensively.**

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**Grifola frondosa** and *Agaricus bisporus*\(^{10,17}\). In order to know the biosynthesis of trehalose in mutant A11, in the present study, Tpsl and trehalose phosphorylase activities were determined in the cell extract. Although Tpsl activity was detected in the cell extract, no trehalose phosphorylase activity was found (data not shown).
indicated by the separation of the enzyme from the standard protein markers 116 kD, 66.2 kD, 45 kD, and 35 kD.

**Effect of pH and temperature on Tps1 activity**

Tps1 activity was measured at various pHs in buffers with the same ionic concentrations. The maximum activity was observed at pH 6.6. The activity profile of the enzyme was stable just around the optimal pH, but the enzyme activity decreased significantly at pH values lower or higher than 6.6 (Fig. 3). These results suggested that the enzyme was very sensitive to change of pH. Tps1 activity, measured as a function of temperature from 0 to 52°C showed the highest activity at 37°C (Fig. 4). The enzyme was stable up to 19°C, but inactivated rapidly at temperature above this and was completely inactivated at 52°C (results not shown).

**Discussion**

The results obtained in this study suggested that the mutant Tps1 protein in mutant A11 was not inhibited by trehalose-6-phosphate synthase Tps1 activity was stable at pH 6.6, but inactivated at pH values lower or higher than 6.6. The activity profile of the enzyme was stable at pH 6.6, but inactivated rapidly at temperature above 19°C.
inactivated at 28°C, indicating that Tps1 became very unstable at temperature higher than 19°C.

Effect of different cations on Tps1 activity

Fig. 5 shows that Ca$^{2+}$, K$^+$ and Mg$^{2+}$ had an activating effect on the enzyme, with K$^+$ showing the highest (188%) activation. However, Mn$^{2+}$, Cu$^{2+}$, Fe$^{3+}$, Hg$^{2+}$ and Co$^{2+}$ inhibited the enzyme activity, with Hg$^{2+}$ showing the highest inhibition (3.76%). To study, the optimal concentrations for activation of the enzyme, the different concentrations of Ca$^{2+}$, K$^+$ and Mg$^{2+}$ were added to the reaction mixture as described above, followed by the standard enzyme assays. The optimal concentrations of Ca$^{2+}$, K$^+$ and Mg$^{2+}$ were found to be 10 mM, 35 mM and 35 mM, respectively (results not shown). The inhibition by Hg$^{2+}$ ions indicated the possible role of thiol-containing amino acids in the enzyme function.

Effect of protease inhibitors on activity of Tps1

The effect of protease inhibitors of the enzyme is shown in Table 2. The chelating agent EDTA strongly inhibited the enzyme activity, indicating that the purified enzyme was a metallo-enzyme. This result was identical to that of Fig. 5. Iodoacetic acid and PMSF at 1 and 5 mM also strongly inhibited the enzyme activity, suggesting that Cys residues and Ser residues were essential for the enzyme activity. However, the enzyme could be protected by DTT (data not shown).

Discussion

The results showed that trehalose-6-phosphate is formed from UDP-glucose and glucose-6-phosphate by trehalose-6-phosphate synthase (Tps1), suggesting that the trehalose synthesis pathway in the mutant A11 was similar to that in S. cerevisiae. Stress treatments, such as heat and ethanol shock caused increase in trehalose-6-phosphate, Tps1 and mRNA encoding Tps1 and trehalose content in S. cerevisiae cells, due to the presence of a heat shock element in the promoter of tps1 gene. However, our previous results showed that heat shock did not result in increase in trehalose content in cells of S. fibuligera. Also, under the normal growth conditions, the mutant A11 accumulates larger amounts of trehalose from starch than S. cerevisiae. This implies that regulation of tps1 gene in S. fibuligera appears to be different from that in S. cerevisiae. But, the relationship between the synthesis of trehalose and stress response in this mutant can be explained, only after the gene encoding Tps1 is cloned.

Although Tps1 obtained from different sources catalyze the same reaction, it has shown variation in the structure from different organisms. Tps1 from mutant A11 is composed of monomer (Fig. 3). In contrast, in S. cerevisiae, Tps1 (56 kDa) is a subunit of the trehalose-6-phosphate synthase-complex, having a high molecular mass (600-800 kDa) and
contains three other proteins Tps2, Tsl1 and Tps3. In E. coli, Tps1 is not part of a complex and is independent of the phosphatase activity. In Selaginella lepidophylla, trehalose-6-phosphate synthase complex is a multi-protein complex (440 kDa) with three subunits of 50, 67, and 115 kDa (Tps1)14. In Z. rouxii, full-length Zr tps1 cDNA is composed of 1476 nucleotides, encoding a protein of 492 amino acids with a molecular mass of 56 kDa16. Sequence analysis of H. polymorpha tps1 revealed an open-reading-frame of 1,428 bp coding for a putative protein of 476 amino acids (54.4 kDa)22. Tps1 from this mutant was stable only at low temperature and in the narrow range of pH (Fig. 3), suggesting that the intracellular enzyme was very sensitive to the change of temperature and pH. The results in Fig. 5 demonstrated that Ca2+, K+ and Mg2+ had an activating effect on the enzyme. These ions were also found to be the activators of Tps1 in Selaginella lepidophylla14, and the maximal effects were observed at 1 mM, 50 mM and 5 mM, respectively. Tps1 from some other organisms is activated by K+ at concentrations up to 0.4 M14. However, Ca2+ does not affect E. coli Tps1.

In conclusion, the results of the present study demonstrated that Tps1 of the mutant A11 was significantly different from that in other fungi. However, to understand its gene structure and regulation, the attempts are being made to clone tps1 gene from the mutant.

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References

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