Extraction of Extracellular L-Asparaginase from *Candida utilis*

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L-Asparaginase was extracted from *Candida utilis* cells using various reducing agents, 2-mercaptoethanol, dithiothreitol, or cysteine. The extraction of the enzyme depended upon the kind and concentration of reducing agents, temperature, time of incubation, and pH of buffer used. The enzyme was typically extracted by incubating the cells at 50°C for 4 h in an extraction solution containing 20 mM 2-mercaptoethanol in 20 mM potassium phosphate buffer (pH 7.0). The enzyme can be extracted from either cell precipitate or cell culture broth. The yeast cells were viable after extraction of L-asparaginase.

L-Asparaginase (L-asparagine aminohydrolase EC 3.5.1.1) catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonium ion. The enzyme has been extensively investigated since it was found that the enzyme from certain microorganisms has antitumor activity. The enzyme has been isolated and characterized from *Escherichia coli*, *Serratia marcescens*, *Saccharomyces cerevisiae*, *Bacillus coagulans*, *Aspergillus nidulans*, *Streptomycyes griseus*, *Proteus vulgaris*, and *Achromobacteraceae*. Since asparaginases from bacterial sources have been of significant clinical utility in the treatment of acute lymphoblastic leukemia, it is possible that yeast counterparts might be useful in this respect. The occurrence of two forms of asparaginase in *Saccharomyces cerevisiae* has been demonstrated. One of these enzymes, asparaginase I, is synthetized constitutively and is located within the cell. The other enzyme, asparaginase II, is a mannan glycoprotein in the cell wall and functions in the hydrolysis of extracellular asparagine.

Intracellular enzymes have the disadvantage that it is necessary to disrupt cells and separate the disrupted cells to recover the enzymes. Both these operations might lead to extensive loss of enzymes. Therefore, it would be promising if we could handle an extracellular enzyme. In this study we have extracted L-asparaginase from cell precipitates or cell culture broth of *Candida utilis* by treating with 2-mercaptoethanol, dithiothreitol, or cysteine as a reducing agent.

The microorganism used in this research was *Candida utilis* NRRL Y-900, which was obtained from the Northern Regional Research Center in Peoria, Illinois (U.S.A.). The standard medium contained (per liter): 20 g of d-glucose, 2 g of yeast nitrogen base (without amino acids and ammonium sulfate), and 2 g of peptone. Cells were cultivated for 18 h at 30°C with a shaking incubator at 120 rpm from a 0.5% inoculum. The cells were collected by centrifugation.

The L-asparaginase activity was measured colorimetrically as previously described by Dunlop et al. with some modification. After cultivation, yeast samples (0.2 ml) were collected by centrifugation, and washed with distilled water. The precipitate was resuspended in an assay mixture composed of 10 mM L-asparagine and 100 mM hydroxylamine in 75 mM sodium borate buffer at pH 10.5. The total assay volume was 1.0 ml and the enzyme reaction was done at 55°C for 30 min. After reaction, the reaction mixture was centrifuged, and 0.5 ml of clear supernatant was assayed for L-aspartic acid β-hydroxylamine by formation of a colored complex with FeCl₃. The enzyme assay of the soluble fraction was done in a assay mixture containing 10 mM L-asparagine and 100 mM hydroxylamine in 75 mM sodium borate buffer (pH 10.5). The enzyme reaction was started by addition of 0.1 ml of enzyme solution to the assay mixture. The total assay volume was 1.0 ml and the enzyme reaction was done at 55°C for 30 min. After reaction, FeCl₃ solution was added to form L-aspartic acid β-hydroxylamine. One unit of enzyme activity is defined as the amount of enzyme to produce one nmol of β-hydroxylamine synthesized per min per ml of cell culture.

Various reducing agents have been tested for their abilities to extract L-asparaginase from cell precipitates of *Candida utilis*. The cell precipitate was suspended in 20 mM potassium phosphate buffer (pH 7.0) containing one of the reducing agents tested, and incubated at 50°C for 2 h. Then they were centrifuged, and the supernatant was used as a enzyme preparation. Among reducing agents tested, 2-mercaptoethanol, dithiothreitol, and cysteine effectively extracted the enzyme from cell precipitate, but ascorbic acid and glutathione did not (Table). The extraction of the enzyme from the cells depended on the concentration of each reducing agent used. The maximal extraction of the enzyme was achieved by adding 20 mM 2-mercaptoethanol. The reducing agent had no effect on the enzyme activity. Asparaginase I of *Saccharomyces cerevisiae* is most active in the hydrolysis of L-asparagine, but it can hardly use D-asparagine. However, asparaginase II of the yeast was equally active with D- or L-asparagine. The enzyme from *Candida utilis* can uses both L-asparagine and D-asparagine as a substrate. No L-asparaginase activity was synthesized in either cell precipitate and culture broth when L-asparaginase was used as a sole source of nitrogen, and no enzyme activity was extracted by ultrasonification treatment of cells. Therefore, it seems likely that *Candida utilis* cells have L-asparaginase in cell wall, and the properties of the enzyme coincide well with those of asparaginase II of *Saccharomyces cerevisiae*. *Candida utilis* cells treated with 20 mM 2-mercaptoethanol were viable, and the survival rate was found to be 25%. Therefore, extracted cells can be used as an inoculum for subsequent cultivation. It has also been reported

Table: Effects of Kinds of Reducing Agents on Extraction of L-Asparaginase from Cell Culture Precipitate of *Candida utilis*

<table>
<thead>
<tr>
<th>Reducing agent (20 mM)</th>
<th>Enzyme activity (unit/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.06</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>0.80</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>0.78</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>0.77</td>
</tr>
<tr>
<td>Glutathione (reduced)</td>
<td>0.12</td>
</tr>
<tr>
<td>D-Ascorbic acid</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Incubation was done for 2 h at 50°C. The buffer used was 20 mM potassium phosphate buffer, pH 7.0.

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that exo-inulase was extracted from *Kluyveromyces fragilis* by treating it with cysteine.\(^{14}\) The exo-inulase of the strain is external to the cell membrane in the cell wall (periplasmic space) and the yeast survived after extraction of exo-inulase.

The maximal extraction of the enzyme by 2-mercaptoethanol was done by adjusting the pH of culture broth to 7.0 and incubating it at 50°C. The enzyme was fairly stable at 50°C but rapidly inactivated above 50°C. It would be convenient if we could extract the enzyme from cell culture broth directly by omitting preparative centrifugation and resuspension steps. Thus, the culture broth was adjusted to pH 7.0, and mixed with 20 mM (final concentration) 2-mercaptoethanol. The suspension was then incubated at 50°C for a given time, and sample were withdrawn periodically and centrifuged. The supernatant and precipitate were assayed for enzyme activity. As shown in Fig., the enzyme activity in cell precipitate decreased as the enzyme activity in the soluble fraction increased. The recovery of the enzyme activity after 2-mercaptoethanol treatment was 82.0%. The results indicated that the direct extraction of the enzyme from cell culture broth is possible. Again, the cells were viable after extraction, and may be usable further for other purposes.

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References