Extracellular Asparaginase from Candida utilis, Its Properties as Glycoprotein and Antitumor Activities

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Purified Candida asparaginase was proved to be homogeneous by gel filtration, ultracentrifugation and disc electrophoresis. The enzyme was found to have properties as glycoprotein containing mannose. The ratio of mannose to protein was 1 to 2 in purified enzyme. Specific activity was 5500 units per mg of protein. Isoelectric point was pH 4 to 4.5 and sedimentation coefficient was found to be about 8.2 S. Antitumor activity of Candida asparaginase was inferior to E. coli enzyme. It was thought as the reason why the Candida asparaginase had less affinity to L-asparagine and it was cleared faster from the blood than E. coli asparaginase.

In the preceding paper, the purification and the properties of extracellular asparaginase produced by Candida utilis were described. It is suggested that Candida asparaginase seems to be glycoprotein because of being always accompanied with carbohydrate in enzyme active fractions. The partially purified asparaginase described in the preceding paper was purified for the purpose of confirming this point. As the result, Candida asparaginase was proved to contain mannose and the ratio of mannose to protein was 1 to 2. The antitumor activity of Candida enzyme was inferior to E. coli asparaginase, especially had no effect against lymphoma 6C3HED solid tumor. Molecular weight, affinity to asparagine, clearance from the blood and others were discussed in relation to the antitumor activity.

MATERIALS AND METHODS

Partially purified asparaginase. Candida asparaginase (2420 unit/mg of protein) which was prepared by the method described in the preceding paper was used.

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E. coli asparaginase. E. coli asparaginase (EC-2) was prepared by the method of Schwartz and Campbell. The extracts of E. coli K12 cell were partially purified by ammonium sulphate precipitation, and column chromatographies with DEAE Sephadex A-50 and Sephadex G-200.

Assay of asparaginase activity, protein and carbohydrate. These assays were usually carried out according to the previous methods except asparaginase assay at low concentration of substrate. In a case of determination of Michaelis constant Km the reaction rate was not able to be measured because of low sensitivity of Nessler reaction for ammonia. So, 14C-uniform-labeled L-asparagine was used as substrate and L-aspartate produced in the enzyme reaction was determined by its radioactivity. The reaction was performed for 15 min at 37°C and stopped by heating it in a boiling water-bath for 2 min. Then, the supernatant was spotted on Whatman No. 1 paper and paper chromatography was carried out with water-saturated phenol. After the paper was dried, the radioactivity of aspartic acid spot was measured by using paper chromatogram scanner.

Purification by column chromatography

DEAE Sephadex A-50: DEAE Sephadex A-50 column was equilibrated with 0.01 M Tris-HCl buffer (pH 7.2) and enzyme solution was adsorbed on the column. After the column was washed thoroughly with the same buffer, step-wise elution with the buffer containing KCl was performed for the purpose of eliminating carbohydrates.

Sephacore 4B: Asparaginase fractions in DEAE Sephadex A-50 chromatography were collected and
fractionated by gel filtration in Sepharose 4B. In this procedure carbohydrate fractions were eluted earlier than the asparaginase fractions, so a few fractions near the peak of activity were separated from carbohydrate fractions.

**Disc-electrophoresis.** Disc-electrophoresis was performed in 7.5% and 3.75% polyacrylamide gels at pH 8.0 (7.5 gel). Enzyme solution (about 200 μg of protein) was layered and run at 2 mA per tube for 1.5 hr. Protein bands were stained with amido-black 10B. Carbohydrate bands were stained to red by the method of Keyser, in which periodate as oxidizing reagent and Schiff reagent were used.

**Paper chromatography of carbohydrate hydrolyzate.** Purified enzyme preparation was hydrolyzed with 1 N H2SO4 for 3 hr at 100°C and neutralized with Ba(OH)2 solution. Then supernatant of hydrolyzate was spotted on Toyo-Roshi No. 51 and developed three times by ascending method with ethylacetate–pyridine–water (2: 1: 2) mixture, and spots of carbohydrate were detected by aniline-phthalic acid reagent.

**RESULTS**

**Purification by chromatography**

**DEAE Sephadex A–50.** As shown in Fig. 1, stepwise elution was carried out with 0.01 M Tris-HCl buffer (pH 7.2) containing 0.15 M and 0.25 M KCl. In the first elution more than one half of the carbohydrates (perhaps glycoprotein) was eluted, but asparaginase activity was unable to be detected in these fractions. Fractions of asparaginase activity, protein and carbohydrate were eluted simultaneously by 0.25 M KCl buffer. Thereafter, neither protein nor carbohydrate was eluted at higher concentration of KCl.

**Gel filtration in Sepharose 4B.** It was impossible to separate carbohydrate rich fraction from asparaginase fraction by gel filtration in Sephadex G–200, but it became possible by the use of Sepharose 4B (Fig. 2). A few fractions near the peak of enzyme active fractions were collected and rechromatography in Sepharose 4B was carried out (Fig. 3). The peak of asparaginase activity, protein and carbohydrate
coincided perfectly with each other, so this preparation was proved to be homogeneous in gel filtration. The specific activity was 5500 unit per mg of protein.

**Homogeneity of the purified enzyme**

**UV absorption spectrum.** UV absorption spectrum of purified enzyme is shown in Fig. 4. This spectrum which has maximum absorption at about 278 nm and minimum at 250 nm is the typical spectrum of protein.

![UV Absorption Spectrum](image)

**FIG. 4. UV Absorption Spectrum of the Purified Enzyme.**

**Ultracentrifugation.** Ultracentrifuge analysis of purified enzyme was carried out in a Hitachi ultracentrifuge type UCA-1. A sharp single boundary was observed in sedimentation pattern as shown in Fig. 5.

**Disc electrophoresis.** The protein band migrated very slowly in 7.5% gel. It is thought that this acrylamide gel acted as molecular shieve on the enzyme molecule. So in 3.75% gel the protein band migrated faster, but the band was broad and not clear. Only one main band was found in both of 7.5% and 3.75% gel (Fig. 6).

**Properties as glycoprotein**

Purified preparation of *Candida* asparaginase was proved to be homogeneous as mentioned above. And as the elution peak of asparaginase activities coincided with that of protein and carbohydrate in column chromatographies, it was suggested that this asparaginase was probably glycoprotein. The experiments described below were undertaken in order to make this point clearer.

**Disc electrophoresis.** Determination of asparaginase activities and staining of protein and carbohydrate were compared in three gels on which electrophoresis was performed at the same time. Asparaginase activity was assayed by the method as follows. After electrophoresis, the gel was sliced in 5 mm length...
and extracted in the buffer solution for 3 hr, then activity of extracted solution was determined by the usual method. Consequently, each band of asparaginase activity, protein and carbohydrate was located at the same position (Fig. 7).

**Identification of carbohydrate.** As shown in paper chromatogram of Fig. 8, detected spot of sugar was only one spot which indicated the same value of $R_f$ with authentic D-mannose. D-Glucose and D-galactose were not detected, but a spot radiating fluorescence by UV ray was found at the same position with authentic D-glucosamine, which also radiated fluorescence. In view of the above facts it seemed to be concluded that carbohydrate moiety of Candida asparaginase was mannan consisted of mannose and glucosamine-like substance.

**Carbohydrate content.** As explained above, it was found that Candida asparaginase contained mannan moiety. So carbohydrate content of purified enzyme was determined by phenol-sulfuric acid method. As the result, mannose was found to be 1.82 mg in 3.4 mg of enzyme preparation (as protein). Therefore, the ratio of mannose to protein was 1 to 2 in purified enzyme.

**Sedimentation coefficient.** Sedimentation velocity was determined from the schlieren patterns of the enzyme (Fig. 5). The pattern was photographed at 10, 20, 30, 40, 50 min and a plot of the distance of the peak from the center of rotation vs. time gave a straight line. The sedimentation coefficient was calculated from the slope, and found to be 8.2S.

**Isoelectric point.** The isoelectric point was estimated by the method of Lampson. After DEAE Sephadex A-50 was equilibrated with 0.2 M acetate buffer (pH 6.0), Candida asparaginase was adsorbed. Then linear stepwise elution with a series of the same buffer ranging from pH 6.0 to 3.5 at 0.5 intervals was carried out. The isoelectric point of this enzyme was...
assumed to be pH 4 to 4.5 according to elution pattern shown in Fig. 9.

**Molecular weight.** The determination of molecular weight was carried out by the Andrews' method of gel filtration. The gel filtration by Sepharose 4B was used for this enzyme. Because in Sephadex G-200, the enzyme was eluted near the void volume on account of high molecular weight. Plots of elution volume ($V_e$) or $V_e/V_s$ from Sepharose 4B column against logarithum of molecular weight for marker proteins did not indicate linearity. But $V_e$ of apoferitin and Candida asparaginase showed the same value when gel filtrations of the two proteins were performed separately or simultaneously. From these result molecular weight of Candida asparaginase was presumed to be about 480,000 in appearance.

**Michaelis constant $K_m$.** The reaction mixture was composed of 0.5 ml of L-asparagine ($2.5 \times 10^{-6}$ M to $5.05 \times 10^{-4}$ M) containing 0.27 µCi of $^{14}$C-L-asparagine, 0.25 ml of 0.05 M Tris-HCl buffer (pH 7.4), 0.25 ml of asparaginase solution (0.56 unit/ml). Lineweaver-Burk plot of concentration of substrate and rate of reaction is shown in Fig. 10. A $K_m$ value of $7.7 \times 10^{-5}$ M was found.

**Antitumor activity**

1) About $10^6$ cells of lymphosarcoma 6C-3HED (ascites form) were inoculated into the peritoneal cavities of C3H mice. Candida asparaginases were injected intraperitoneally the first, third and fifth days after the implantation. The result showed that the survival time of tumor bearing mice was prolonged as dose of the enzyme increased. (Fig. 11) Each mouse in administration of 3 doses of 200 unit and 400 unit survived more than 30 days, but all of one group (five mice) survived by 3 doses of 250 unit of *E. coli* L-asparaginase in a control. So Candida asparaginase is less in antitumor effect than *E. coli* asparaginase.

2) About $10^6$ cells of lymphoma D-28 which was isolated by Yamamoto were inoculated into the peritoneal cavities of DDD mice. (Lymphoma cells were taken from the spleens of mice inoculated intraperitoneally 10 days previously.) The weight of spleens increased to about one gram 10 days after the inoculation of tumor cells. At this time, the administration of asparaginase was performed intraperitoneally or intravenously. If tested asparaginases have antitumor effects, spleens regress to normal weight (0.12 to 0.15 g) after 24 to 48 hr. The result which Candida and *E. coli* asparaginases were tested to lymphoma D-28 is shown in Table I. *Candida* asparaginase was not effective in intraperitoneal injection, but effective in intravenous injection. However, *E. coli* asparaginase was highly effective intraperitoneally, so *Candida* asparaginase was relatively less effective than *E. coli*.

**Stability of asparaginase in blood**

1) **Stability in vitro.** Each 0.2 ml of *Candida* and *E. coli* asparaginases were in-
cubated with 1.8 ml of human plasma at 37°C for 26 hr and as the result their remaining activity were 102% and 99%, respectively.

2) Stability in vivo (clearance rate). *Candida* and *E. coli* asparaginases were injected into peritoneal cavities of DDD mice, and after the injection, enzyme levels in blood were determined at various times. As shown in Fig. 12, *E. coli* asparaginase activities reached maximum in 2 hr and reduced slowly. On the other hand, *Candida* asparaginase was low levels at early time and was cleared rapidly from blood. Half-life of the enzyme was 3 hr in *E. coli* and shorter than 2 hr in *Candida*.

![Fig. 12. Clearance of Asparaginase from Blood.](image)

DISCUSSION

The purified preparation of *Candida* asparaginase was proved to contain relatively large amount of mannose. Its specific activity was 5500 unit per mg of protein. This value is lower than 18000 to 24000 in *E. coli* and 15000 in *Serratia*. The molecular weight of this enzyme was found to be about 480,000 in appearance. But Andrews reported that the molecular weight of glycoprotein was estimated higher than the true value in the method of gel filtration. So, the molecular weight of *Candida* enzyme is probably lower than 480,000. While, the molecular weight of *Saccharomyces* asparaginase is reported to be about 800,000, that of bacteria asparaginases are nearly 130,000 to 150,000 or lower. *E. coli* asparaginase (EC-2) is able to exist as polymer, 270,000 and 520,000. Then those polymers were dissociated to various subunits with guanidine or urea. The dissociation to subunits could not be recognized in regard to *Candida* asparaginase. Isoelectric point of *Candida* asparaginase was a little lower than one of *E. coli* enzyme (pH 4.85).

Till today there have been various enzymes which are proved to be glycoprotein, for example Taka-amylase A from *Aspergillus*, glucose oxidase from *Aspergillus*, invertase from *Neurospora* and *Saccharomyces*. External invertase from *Saccharomyces* purified by Lampen *et al.* was found to be glycoprotein containing mannan at 50% of molecular weight. *Candida* asparaginase containing mannan resemble to this one with high content of carbohydrate. Then this result suggests that *Candida* asparaginase localizes in mannan layer of yeast cell wall and is released into the culture medium by some mechanism. A-

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**Table I. Anti-tumor Effect of Asparaginase on Lymphoma D-28**

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Controlb)</th>
<th>CUc) 1400 unit ip.</th>
<th>CU 1400 unit iv.</th>
<th>EC-2d) 600 unit ip.</th>
<th>Intact mice</th>
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<tbody>
<tr>
<td>Exp. 1</td>
<td>1.20</td>
<td>0.70</td>
<td>0.35</td>
<td>0.12</td>
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<tr>
<td>Exp. 2</td>
<td>1.28</td>
<td>1.20</td>
<td>0.37</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Exp. 3</td>
<td>0.85</td>
<td>1.05</td>
<td>0.40</td>
<td>0.13</td>
<td></td>
</tr>
</tbody>
</table>

a) 24 hr (exp. 1,2) and 40 hr (exp 3) after injection of asparaginase.
b) No injection of asparaginase.
c) *Candida* asparaginase.
d) *E. coli* asparaginase.
about 30% of the mannan moiety of Candida asparaginase was removed without diminishing asparaginase activity by the enzymic reaction of Arthrobacter mannosidase.17)

It was found that Candida asparaginase possessed less antitumor activity than E. coli asparaginase. Schwartz18) and Broome19) have demonstrated two factors on effective asparaginase against tumor: first, the affinity of the enzyme for asparagine, and secondary the rate of removal of the asparaginase from the blood of treated animals. The affinity of Candida asparaginase for asparagine is shown as Km value. Km value, 7.7 x 10^{-5} M, of Candida enzyme was a little larger than 1.25 x 10^{-6} M of E. coli EC-2.19) Asparagine concentration is about 2.5 x 10^{-5} M in mouse blood.18) So, Candida asparaginase is disadvantageous against tumor inhibition. The result on the stability of enzyme in the blood (Fig. 12) suggest that the rate of clearance of Candida asparaginase is not only more rapid than E. coli asparaginase, but also it is difficult for Candida enzyme to move from peritoneal cavity into blood vessel. These possibly are due to high molecular enzyme which has high content of carbohydrate.

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17) Unpublished data.