PURIFICATION AND CHARACTERIZATION OF DIHYDROPYRIMIDINASE FROM *ALBIZZIA JULIBRISIN*

Y. TURAN AND O. SINAN

Department of Biology, Faculty of Science and Art, Balikesir University, Balikesir-10100, Turkey.

Abstract

Dihydropyrimidinase (DHPase) was purified 74-fold over the initial Albizzia extract using heat treatment, ammonium sulphate precipitation and sephadex G-200 column chromatography. Its molecular mass, determined by SDS-PAGE, was approximately 56 kDa. The optimum temperatures of DHPase were 60°C and 55°C for dihydrouracil (DHU) and dihydrothymine (DHT), respectively. Optimum pH value of DHPase for two substrates was found to be 9.5. The stability of DHPase was determined both in crude enzyme extract and in the sample obtained from ammonium sulphate precipitation. The effect of some metal ions on this enzyme was also examined. Km values of the enzyme for DHU and DHT were 0.33 mM and 0.37 mM, respectively. Vmax values were found as 0.15 U/mL min⁻¹ and 0.092 U/mL min⁻¹ for DHU and DHT, respectively.

Introduction

Dihydropyrimidinase (EC 3.5.2.2) is the second enzyme in pyrimidine catabolic metabolism, catalysing the reversible hydrolysis of 5,6-dihydrouracil to N-carbamoyl-β-alanine (Fink et al., 1952). This enzyme also hydrolyses a variety of other 5,6-dihydropyrimidines such as dihydrothymine, 5-amino dihydrouracil (Wallach & Grisolia, 1957) as well as hydantoins and succinimides (Dudley et al., 1974). DHPase is a tetramer and a Zn²⁺-metalloenzyme, containing four tightly bound Zn²⁺ ions/molecule of active enzyme (Brooks et al., 1983).

The reaction catalysed is:

where R represents either CH₃ (I: dihydrothymine; II: N-carbamoyl-β-aminobutyrate), H (I: dihydrouracil; II: N-carbamoyl-β-alanine), NH₂ (I: 5-aminodihydrouracil; II: albizzine).
Most of the early data on dihydropyrimidinase, previously termed DHU- or DHT-hydrase, came from studies on reductive degradation of uracil and thymine (Mazuś & Buchowicz, 1968). Wallach & Grosilia (1957) purified this enzyme 200-fold from calf liver. The first study about DHPase in plants was reported by Mazuś & Buchowicz (1968) in pea seedlings. Tintemann et al., (1987) studied the intracellular location of the enzyme in tomato cell suspension cultures.

Pyrimidine degradation is important for a variety of biological processes such as production of putative neurotransmitter β-alanine, regulation of pyrimidine base concentration (Sandberg & Jacobson, 1981; Naguib et al., 1985) and the catabolism of clinically applied pyrimidine base analogues. Patients with familial pyrimidinemia and pyrimidinuria were reported to develop neurological abnormalities when treated with 5-fluorouracil (Tuchman et al., 1985) a pyrimidine base analogue. The severe 5-fluorouracil toxicity in these patients may be caused by a genetic defect in the pyrimidine metabolism (Diasio et al., 1988). Also, regulation of the secondary product formation by dihydropyrimidinase activity level in some leguminous plants was reported. In *Albizia julibrissin*, 5-aminodihydrourasil generated by dihydrourasil dehydrogenase is quickly converted to a uracil derived secondary compound albizzine by the catalysis of dihydropyrimidinase (Turan, 1995). According to published reports, 5-aminouracil blocks the mitotic cycle (Prensky & Smith, 1965), depresses the rate of DNA synthesis (Wagenaar, 1966; Eriksson, 1966), and inhibits incorporation of guanosine into RNA of meristematic cells (Jakob, 1968). Since neither albizzine nor its further degradation product 2,3-diaminopropanoic acid have any significant toxic activity in the tissues of higher plants, enzymic degradation of 5-aminouracil by the pyrimidine catabolic pathway can also be regarded as a detoxification mechanism (Turan, 1995).

In the present study, we describe the partial purification and determine some physical properties and kinetic parameters of DHPase from *Albizia julibrissin* not hitherto reported.

**Materials and Methods**

**Materials:** Dihydrouracil (DHU), dihydrothymine (DHT), N-carbamoyl-β-alanine (NCβA), N-carbamoyl-β-aminoizobutiric acid (NCβAIBA), Sephadex G-200, acrylamide and bisacrylamide were purchased from Sigma. All other reagents were of the highest purity available from commercial sources.

**Enzyme assay:** Enzyme activity was determined by the colorimetric method of West et al., (1982). The reaction mixture contained 0.1 M Tris-HCl pH: 9.5, 3 mM Dihydropyrimidine (DHU and DHT), and enzyme solution in a total volume of 1 mL. Reaction mixture was incubated at 37°C for 1 hour. After incubation, the color mix was added and West et al., (1982) method was followed by modification of time. One unit of enzyme activity was defined as the amount of enzyme that catalyses the conversion of 1µmol DHU and DHT to NCβA and NCβAIBA, respectively, in the assay. The protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as the standard.

**Enzyme purification:** DHPase was purified from *Albizia julibrissin*. Fifteen days old seedlings were ground using a pestle and mortar with 0.1 M Tris buffer (2 ml per g of seedling), pH 9.5. Homogenizations and subsequent applications were performed at 0-
4°C. The homogenate was filtered through double-layer cheese cloth and centrifuged at 12000g for 20 min. The supernatant was heated to 60°C for 10 min., cooled to 4°C and then centrifuged at 6000g for 20 min. The precipitate was discarded and the supernatant was fractionated with ammonium sulphate. The precipitate obtained at 35-55% of saturation was dissolved in a minimum volume of 0.1 M Tris buffer pH 9.5 and desalted by dialysis against the same buffer overnight at 4°C. After dialysis, 5 mL of sample was applied to a column (1x34cm, flow rate 0.3 mL/min) of Sephadex G-200 equilibrated with Tris buffer pH 9.5. Fractions (2 mL) from the column were collected. The fractions displaying enzyme activity were combined and concentrated by ultrafiltration (Centriprep 3, Amicon). Table 1 shows the purification process.

**SDS-Polyacrylamide gel electrophoresis:** SDS-PAGE was performed as described by Laemmli (1970). Size markers for molecular mass determination were 68 kDa (Bovine serum albumin), 45 kDa (ovalbumin), 34.7 kDa (pepsin), 24 kDa (tripsinogen), 18.4 kDa (β-Lactoglobulin), and 14.3 kDa (Lysozyme).

**Results and Discussion**

Dihydropyrimidinase from *Albizia julibrissin* was purified 74-fold with a 40% recovery as per purification procedure summarized in Table 1. Heat treatment was used for the first time for the purification step of dihydropyrimidinase from plants. Various salt percentages for the precipitation of dihydropyrimidinase have been reported from different organisms (Brooks et al., 1983; Mazus & Buchowicz, 1968; Kautz & Schnackerz, 1989). We have determined the maximum salt percentage range for the precipitation of dihydropyrimidinase as 35% to 55%. In this range the enzyme was purified and concentrated, which enhanced the yield of the gel filtration chromatography step in the purification procedure.

As shown in Table 1, gel filtration chromatography was utilized and yielded significantly higher degree of purification than other purification steps in the protocol. Mazus & Buchowics (1968) have reported 3 fold purification using a Sephadex G-100 gel, while we were able to obtain 66-fold purification of the enzyme by this step in *Albizia julibrissin*.

The purified DHPase migrated as a single band during SDS-PAGE. On SDS-PAGE, subunit mass of DHPase produced a single band of 56 kDa (Fig. 1). These values are in good agreement with reports of Kautz & Schnackerz (1989) and Jahnke et al., 1983).

When the purified DHPase was stored at 4°C, 35% decrease in the specific activity was observed within two months (Fig. 2). Dihydropyrimidinase has been widely reported to be a stable enzyme in appropriate buffers (Brooks et al., 1983; Mazus & Buchowics, 1968; Kautz, & Schnackerz, 1989; Jahnke et al., 1983; Maguire & Dudley, 1978). Mazus & Buchowics (1968) reported 50% preservation of the activity in crude extract after 60 days at 2°C in a diethanolamine-acetate (pH 10) buffer. Brooks et al., (1983) also reported a similar activity preservation of dihydropyrimidinase isolated from calf liver.

The effect of temperature on DHPase activity was determined between 35-70°C. The optimum temperature for DHPase activity for DHU was determined as 60°C, while it was 55°C for DHT (Fig. 3). On the other hand, the purified plant DHPase was reported to be higher value as 70°C (Mazus & Buchowics, 1968).
Table 1. The purification steps of DHPase.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (U)</th>
<th>Specific Activity (U/mg)</th>
<th>Purification (Fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>125</td>
<td>3862</td>
<td>27</td>
<td>0.007</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Heat treatment (60°C 10 min)</td>
<td>115</td>
<td>1442</td>
<td>25.95</td>
<td>0.018</td>
<td>2.6</td>
<td>96</td>
</tr>
<tr>
<td>Ammonium sulphate (35-55%)</td>
<td>5</td>
<td>436</td>
<td>24.85</td>
<td>0.057</td>
<td>8.0</td>
<td>92</td>
</tr>
<tr>
<td>Sephadex G-200 column</td>
<td>2.7</td>
<td>21</td>
<td>10.94</td>
<td>0.521</td>
<td>74.4</td>
<td>40</td>
</tr>
</tbody>
</table>
Fig. 1. SDS-PAGE of purified DHPase: A; molecular weight standards, B; purified DHPase, C; Ammonium sulphate precipitation.

Fig. 2. The stability plot of DHPase.

The pH profile of DHPase was determined between 5.5 and 10.5. The optimum pH value of DHPase for both substrates (DHU and DHT) was 9.5 (Fig. 4) which is similar with the previous reports (Brooks et al., 1983; Mazus & Buchowics, 1968; Kautz & Schnackerz, 1989; Jahnke et al., 1983; Maguire & Dudley, 1978; Kikugava et al., 1994). These results suggest a relatively basic environment for maximum DHPase activity.
K_M and V_max values were determined by means of Lineweaver–Burk graphs. The values of K_M for DHU and DHT were 0.33 mM and 0.37 mM, respectively. Therefore, DHU served as better substrate than DHT. The V_max and K_M values for dihydropyrimidinase in *Albizia julibrissin* appear to be considerably different with previously reported values for the same enzyme from different source tissues. Our values for DHU and DHT are lower than for DHPase from pea plant (Mazus & Buchowics, 1968), while Kautz & Schnackerz (1989) reported 0.025 mM K_M value for DHU, and 0.085 mM for DHT, similar results were reported elsewhere (Brooks et al., 1983; Naguib et al., 1985; Jahnke et al., 1983). These reports suggest a usually lower K_M value for DHU than that of DHT. However, dihydropyrimidinase has been reported to have affinity for various substrates such as 5-aminouracil, 5-iodouracil, 5-fluorouracil, and hydantoin in addition to DHU and DHT (Brooks et al., 1983, Kautz & Schnackerz, 1989; Jahnke et al., 1983; Kikugava et al., 1994; Brown & Turan, 1995). The kinetic constant V_max for DHU and DHT were 0.15 U/mL and 0.092 U/mL, respectively.
The effect of metal ions on DHPase activity was also examined (Table 2). The enzyme is strongly inhibited by Fe$^{2+}$, Hg$^{2+}$ and Ag$^{+}$ ions in 1 mM final concentration. Of the other metal ions tested, Cu$^{2+}$, Mg$^{2+}$, Pb$^{2+}$, Zn$^{2+}$ and Ni$^{2+}$ caused no important effect on enzyme activity. The common inhibitors for most enzymes, AgNO$_3$ and HgCl$_2$, significantly inhibited the DHPase activity. FeSO$_4$, which was not widely tested for inhibition, also significantly inhibited the enzyme activity. On the contrary to Mazus & Buchowics (1968) findings that CuSO$_4$ slightly increased DHPase activity, our results displayed a slight inhibition of DHPase activity in the presence of CuSO$_4$. We also determined a 25% activity decrease of DHPase by CoCl$_2$, while it was reported to increase the activity of the enzyme in pea plant (Mazus & Buchowics, 1968). Among other metal ions tested, SnCl$_2$, MgCl$_2$, MnSO$_4$, ZnCl$_2$, PbNO$_3$ and NiCl$_2$ slightly increased DHPase activity, while SnCl$_2$ was most effective.

Dihydropyrimidinase is one of the most important enzyme in possible detoxification process of uracil and 5-aminouracil in plants. The results with purified dihydropyrimidinase activity in our study are consistent with the previous findings Brown & Turan (1995) and Turan & Konuk (1999).

### References


Turan, Y. 1995. Pyrimidine primary and secondary metabolism in plants. A thesis presented for the Degree of Doctor of Philosophy in the University of Wales Swansea, *Department of Biochemistry School of Biological Science University of Wales Swansea*, November. 172 PP.


(Received for publication 17 April 2004)