Pak. J. Bot., 37(2): 299-306, 2005.

PURIFICATION AND CHARACTERIZATION OF DIHYDROPYRIMIDINASE FROM *ALBIZZIA JULIBRISSIN*

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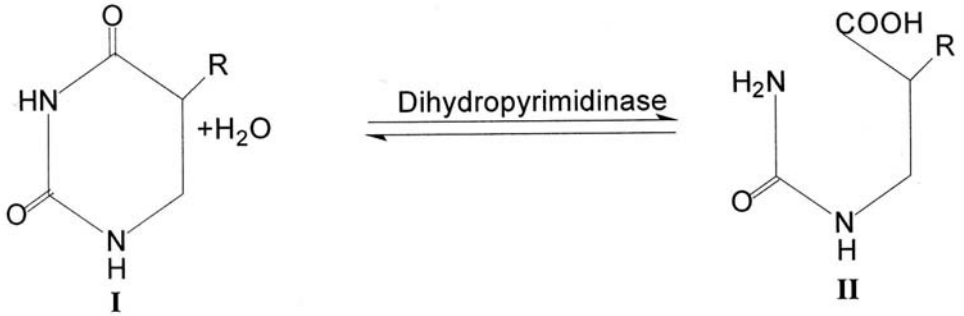
Abstract

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 Zn^{2+}-metalloenzyme, containing four tightly bound Zn^{2+} ions/molecule of active enzyme (Brooks *et al*., 1983).

The reaction catalysed is:



where R represents either CH_3 (I:dihydrothymine;II: N-carbamoyl-β-aminobuthyrate), H (I: dihydrouracil; II: N-carbamoyl-β-alanine), NH_2 (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 (Mazus & Buchowicz, 1968). Wallach & Grosilia (1957) purified this enzyme 200-fold from calf liver. The first study about DHPase in plants was reported by Mazus & 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 *Albizzia 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 *Albizzia 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^{o}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 *Albizzia 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 (tripsinojen), 18.4 kDa (β-Lactoglobulin), and 14.3 kDa (Lysozyme).

Results and Discussion

Dihydropyrimidinase from *Albizzia 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 *Albizzia 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 to 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 60ºC, while it was 55ºC for DHT (Fig. 3). On the other hand, the purified plant DHPase was reported to be higher value as 70ºC (Mazus & Buchowics, 1968).

Table 1. The purification steps of DHPase.

Purification Step	Volume (ml)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (Fold)	Yield (%)
Crude extract	125	3862	27	0.007	1.0	100
Heat treatment (60°C 10 min)	115	1442	25.95	0.018	2.6	96
Ammonium sulphate (35-55%)	5	436	24.85	0.057	8.0	92
Sephadex G-200 column	2.7	21	10.94	0.521	74.4	40

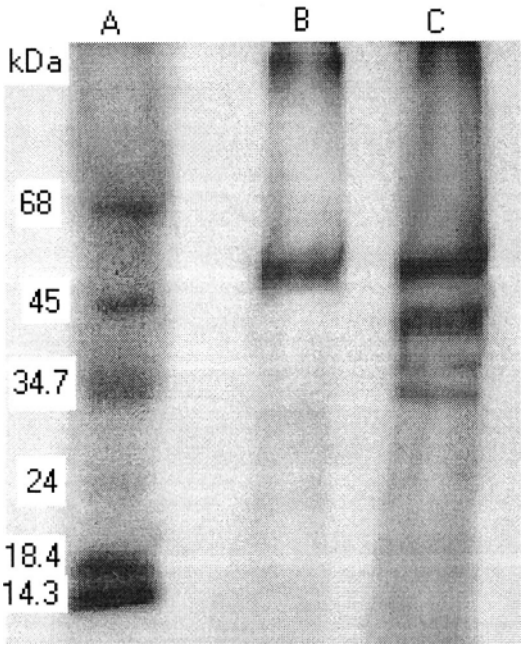


Fig. 1. SDS-PAGE of purified DHPase: A; molecular weight standards, B; purified DHPase, C; Ammonium sulphate precipitation.

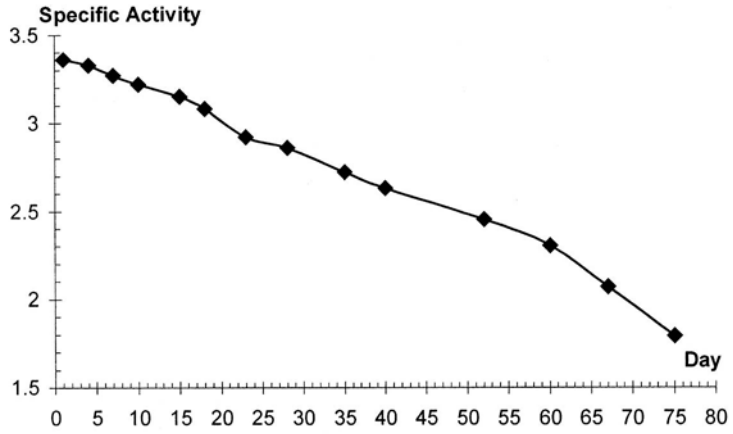


Fig. 2. The stability plot of DHPase.

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.

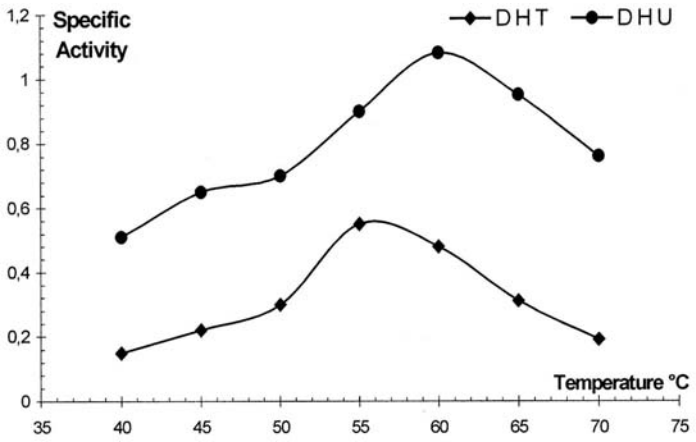


Fig. 3. The effect of temperature on DHPase activity.

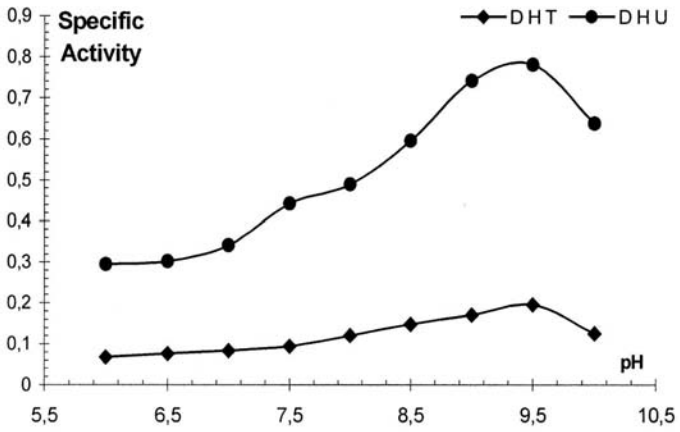


Fig. 4. The effect of pH on DHPase activity.

K_M and Vmax 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 *Albizzia 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.

Table 2. The effect of metal ions on DHPase activity.

Addition metal Ion (1 mM)	Relative activity (%) enzyme
Control	100
$CoCl_2$	76
$AgNO_3$	6
$HgCl_2$	13
$CuSO_4$	93
$FeSO_4$	20
$SnCl_2$	118
$MgCl_2$	109
$MnSO_4$	114
$PbNO_3$	103
$ZnCl_2$	107
$NiCl_2$	105

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^{1+} ions in 1 mM final concentration. Of the other metal ions tested, Cu^{2+}, Mg^{2+}, Pb^{1+}, 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).

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(Received for publicaiton 17 April 2004)