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Isolation and Characterization of *Thielaviopsis paradoxa* L-alanine Dehydrogenase

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ABSTRACT

Alanine dehydrogenase has not yet been purified from any fungal sources. The aims of this study was to isolate alanine dehydrogenase from *Thielaviopsis paradoxa*. The biochemical kinetic properties of the enzyme like Km, Vmax, optimum Temperature and pH were also determined. In this study, effect of certain inhibitors on the enzyme was tested. Alanine dehydrogenase (EC 1.4.1.1), an enzyme that catalyzes the reversible deamination of L-alanine in the presence of NAD^+, was extracted and characterized from *Thielaviopsis paradoxa,* a pathogenic fungi for the date palm (*Phoenix dactylifera*). The enzyme showed maximal activity at pH 9.5 for the deamination of L-alanine and at pH 8.5 for the amination of pyruvate. It was active in the presence of both NAD^+ and $NADP^+$ as coenzymes for the deamination of L-alanine. In addition, the enzyme was not absolutely specific to L-alanine as a substrate in the deamination of L-alanine. L-alanine dehydrogenase had Km value for L-alanine (1.35 mM), for NAD^+ 0.274 mM, NADH (0.197 mM), pyruvate (8.16 mM) and ammonia (33.5 mM). It was inhibited by Zn^{2+}, CO^{2+} and by iodoacetate. Activation by using reduced glutathione and DTT are suggestive of sulfhydryl group participation in enzyme activity.

Key words: L-alanine, NAD^+, *Thielaviopsis paradoxa,* sulfhydryl group, *Phoenix dactylifera,* deamination

INTRODUCTION

Thielaviopsis is small genus of fungi belonging to the order Microascales. The genus includes several important agricultural pathogens. One of these is *Thielaviopsis paradoxa,* synonym: *Ceratocystis paradoxa* (Paulin-Mahady *et al*., 2002). *Thielaviopsis paradoxa* is a soil borne plant pathogen and has a wide host range of palms. Palms are considered to be the most important tree in Saudi Arabia, specifically the date palms. *T. paradoxa* causes disease in the date tree in Saudi Arabia (Al-Rokibah *et al*., 1998; Molan *et al*., 2004) and the most date palm producing countries including Iraq, Kuwait (Suleman *et al*., 2001), Qatar (Abbas and Abdulla, 2003) and in the United States (Garofola and McMillan, 2004). The *T. paradoxa* can also attack pineapple (*Ananas comosus*) in Brazil and coconut (*Cocos nucifera*) in Venezuela (Sanchez *et al*., 2007). *T. paradoxa* is the causal fungus of a range of diseases including bud rot, leaf spot, heart rot, bleeding trunk canker and root decay of coconut and pineapple butt rot, black rot and white leaf spot (Al-Rokibah *et al*., 1998; Suleman *et al*., 2001; Garofola and McMillan, 2004).

Alanine dehydrogenase plays an important role in the carbon and nitrogen metabolism of microorganisms and is a key factor in assimilation of L-alanine as an energy source through TCA cycle. L-alanine dehydrogenase (L-alanine-NAD^+ oxidoreductase, (deaminating), EC 1.4.1.1, AlaDH), catalyzes the reversible deamination of L-alanine to pyruvate in the presence of NAD^+. The enzyme is found in various microorganisms including bacteria, archaea and eukaryotes but not in higher plants and animals (Ohashima and Soda, 1990). It was first isolated from *Bacillus subtilis* itelic (Wiame and Pierard, 1955; Pierard and Wiame, 1960) and was shown to catalyse the reversible, NAD+-dependent, oxidative deamination of L-alanine:

$$CH_3\text{-}CH\ (NH_2)\text{-}CO_2 + H_2O + NAD^+ \rightleftharpoons CH_3\text{-}CO\text{-}CO_2^- + NH_4^+ + NADH$$

Alanine dehydrogenase has been isolated and purified from several microorganisms in which it has different metabolic uses. For example in *Bacillus* it has a role in spore germination it plays an important role in the carbon and nitrogen metabolism of microorganisms and is a key factor in the assimilation of L-alanine as an energy source through the tricarboxylic acid cycle (Brunhuber and Blanchard, 1994). The function of L-Ala dehydrogenase in germinating spores is the production of energy, both directly via formation of NADH and indirectly since pyruvate enters the TCA cycle and can be oxidized with the production of NADH (Siranosian *et al*., 1993). In the present study alanine dehydrogenase was isolated from *Thielaviopsis paradoxa*. The inhibitory effect of certain inhibitors for this enzyme was analysed. We also determine the biochemical kinetic properties like Km, Vmax, optimum Temperature and pH for the isolated enzyme.

MATERIALS AND METHODS

This study was conducted from 1-1-2008 to 9-6-2009. In this study we extracted and characterized Alanine dehydrogenase (EC 1.4.1.1), an enzyme that catalyzes the reversible deamination of L-alanine in the presence of NAD^+, from *Thielaviopsis paradoxa*.

Fungal organism: The Fungal organism *Thielaviopsis paradoxa* was obtained from the College of Agriculture, King Saud University, Riyadh, Saudi Arabia.

Chemicals: NAD^+, NADH, $NADP^+$ and Acrylamide/bis-acrylamide were obtained from Sigma-Aldrich Chemical Company, Germany. L-alanine was supplied by MP Biomedicals.

Culture: The fungus was grown on PDA (Potato dextrose agar) plates, at 25°C, circular plugs (5 mm in diameter) made with a sterile metal cork borer and then transferred to 250 mL Erlenmeyer flasks, each containing 50 mL sterile potato dextrose broth medium, then incubated at 26°C for 4 days. The fungal mycelia were harvested by culture filtration, washed thoroughly with distilled water and finally blotted dry with absorbent paper.

Preparation of crude extract: The harvested mycelia were ground with cold sand in a cold mortar and extracted with cold distilled water. The obtained slurry was then centrifuged at 5000 rpm for 10 min and the supernatant was used as the crude enzyme preparation.

Determination of protein concentration: The Bradford (1976) method was used to determine the protein concentration.

Enzyme assays: On oxidative deamination L-alanine dehydrogenase was assayed spectrophotometrically, using Ultrospec 2100 pro Amersham Biosciences, by following formation of pyruvate from alanine using 2, 4-dinitrophenylhydazine at alkaline pH, a method originally described by Friedemann and Haugen (1943). The standard reaction mixture (1 mL) was obtained by mixing 10 μmole L-alanine, 3 μmole NAD^+, 0.05 M Na_2CO_3/$NaHCO_3$ buffer (pH 9.5) and finally the enzyme was added in a final volume of 1 mL. A unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1μmole pyruvate for 1 min at 60°C.

The forward reaction (reductive amination) L-alanine dehydrogenase was assayed by following the reduction of Nicotinamide Adenine Dinucleotide (NADH) at 340 nm via the decrease in absorbance per min.

A standard reaction mixture contained: (10 μmole) pyruvate, (100 μmole) NH_4Cl, (1 μmole) NADH, 0.05 M Tris-HCl buffer at pH 8.5 and the enzyme was added in a final volume of 1 mL.

A unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmole of NADH min^{-1}. The extinction coefficient of NADH is 6.22×10^3 L/moL/cm (Horecker and Kornberg, 1948).

Statistical analysis: The data and graphs were plotted by using Prism 2.01 and SPSS 13. The values of the slope and intercept were obtained by linear regression analysis of the Prism 2.01.

RESULTS

Enzymatic properties: A detailed study of the properties of the enzyme was made for both the reductive amination of pyruvate and the oxidative deamination of alanine. In this study different media, under different conditions, were used to reach the optimum condition for *T. paradoxa* growth. Two types of media were used: firstly, glucose-Czapek-Dox and secondly, potato dextrose media. In both media, different conditions were applied, one with shaking and another without to see the differences in growth. After optimizing the media conditions, the inoculated flasks were incubated at 25°C for different incubation periods starting from day 2 up to day 5 for the mycelium stage. For each day, the mycelia were harvested and washed thoroughly with distilled water, the enzyme was excited and its activity was measured. Table 1 demonstrate L-alanine dehydrogenase activity at different stages of *T. paradoxa* growth. Minimum activity was found with Glucose-Czpak Dox in static condition and maximum with Potato dextrose with L-alanine under shaking conditions and were 0.092±0.018 to 0.3±0.019, respectively.

Table 1: *Thielaviopsis paradoxa* growth in different media and different incubation culture states

Media	Culture medium	Enzyme activity (U mL^{-1})
Glucose-Czpak Dox	Static	0.092±0.018*
Glucose-Czpak Dox	Shaking	0.212±0.012*
Potato dextrose	Static	0.162±0.016**#
Potato dextrose with L-alanine	Static	0.240±0.02**^
Potato dextrose	Shaking	0.170±0.014***#
Potato dextrose with L-alanine	Shaking	0.300±0.019***^

Activity is Mean±SD of three-independent harves [p*: 0.00918] [p**: 0.03112] [p***: 0.0098] [p#: NS] [p^: NS]

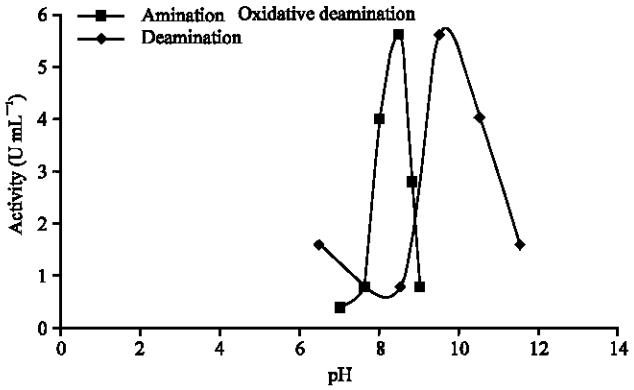

Fig. 1: Effect of pH on deamination and amination catalyzed by L-alanine dehydrogenase

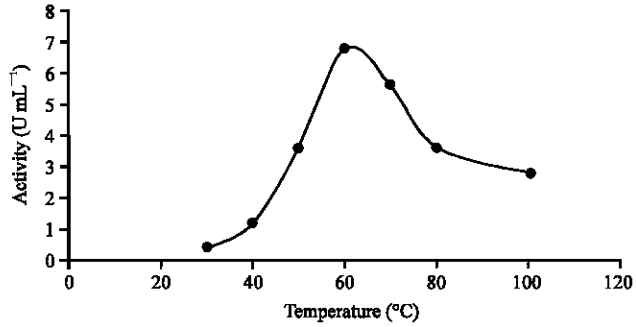

Fig. 2: Effect of temperatures on the oxidative deamination reaction catalyzed by L-alanine dehydrogenase. The enzyme activity was assayed at different temperatures (30-100°C) in the oxidative deamination direction using a reaction mixture containing, 0.05 M sodium bicarbonate buffer Na_2CO_3-$NaHCO_3$ (pH 9.5), alanine (10 μmole), NAD^+ (3 μmole) and enzyme in a final volume of 1 mL, (2 min incubation time)

Determination of pH optimum of L-alanine dehydrogenase in oxidative deamination and reductive amination: The *T. paradoxa* L-AlaDH in oxidative deamination and reductive amination exhibited pH optimums of 9.5 and 8.5, respectively (Fig. 1). The optimum pH for oxidative deamination and reductive amination are at the alkaline pH range.

Effect of temperature on L-alanine dehydrogenase activity: The L-AlaDH was assayed at different temperatures to determine the optimum temperature for the reaction. The enzymatic activity was plotted as a function of temperature. The optimum temperature for L-AlaDH was 60°C (Fig. 2).

Thermal stability of L-alanine dehydrogenase: The thermal stability of the enzyme was examined. The enzyme in solution bicarbonate buffer (pH 9.5) was heated at 60°C for different time periods from 10 to 60 min. The solution was immediately cooled on ice and assayed for oxidative deamination activity under conditions of the enzyme activity assay. The results are expressed as a percentage of the activity of the unheated enzyme assayed under the same conditions in Fig. 3.

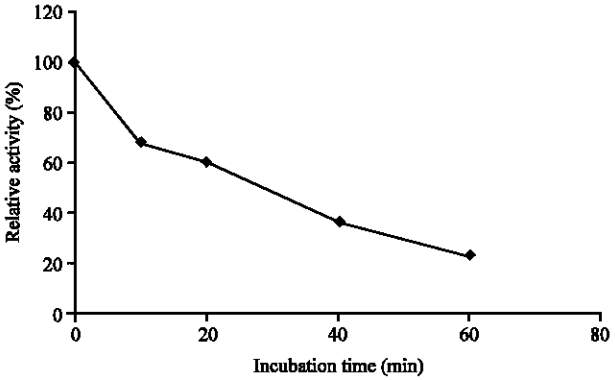

Fig. 3: Thermal stability of L-alanine dehydrogenase. The enzyme activity was assayed at a different time periods in the oxidative deamination of the reaction mixture: the 0.05 M sodium bicarbonate buffer Na_2CO_3-$NaHCO_3$ (pH 9.5), alanine (10 µmole), NAD^+(3 µmole) and enzyme in a final volume of 1 mL, at 60°C. Thermal stability was measured for alanine dehydrogenase to find out the stability of the enzyme, the enzymatic activity from the sample (unheated) assay as oxidative deamination was set as 100%

Table 2: The kinetic parameters of substrates of L-alanine dehydrogenase

Substrate	Km (mM)	Vmax (µmole/min/mL)
L-alanine	1.35	0.429
NAD^+	0.274	0.135
pyruvate	8.16	0.50
NADH	0.197	8.56
NH_4^+	33.50	15.72

Kinetic characterizations: Kinetic parameters were determined for AlaDH. Initial velocities were obtained by varying the concentration of one substrate or coenzyme at several fixed concentrations of other substrate(s). In oxidative deamination, a range of concentration of L-alanine (0.4-10 mM), NAD^+ (0.4-4 mM) were used. A range of concentration of pyruvate (0.1-10 mM); NH_4Cl (5-500 mM) and NADH (0.1-1.4 mM) were used for reductive amination. The Lineweaver-Burk plots were used to calculate the Km values of the substrates and coenzyme. Table 2 present the Km and Vmax values of L-alanine dehydrogenase towards different substrates and coenzymes in both directions.

The values obtained via Line-weaver-Burk plot, were 1.35 mM for L-alanine, 0.274 mM for NAD^+, 8.16 mM for pyruvate, 0.197 mM for NADH and 33.5 mM for NH_4^+.

Substrate and coenzyme specificity of L-alanine dehydrogenase from *T. paradoxa*: Compounds structurally related to L-alanine like DL-alanine, L-serine, L-isoleucine, L-threonine and Glycine were used at concentration of 100 mM to determine the substrate specificity of L-AlaDH in the oxidative deamination reaction. Table 3 shows that the enzyme exhibited activity towards several of these substrates in which maximum activity was found with L-serine (0.4±0.025) and minimum with L-threonine (0.231±0.006). The enzyme activity was also measured using 3 µmole of NAD^+ and $NADP^+$ and was found to be 0.287±0.019 and 0.347±0.013, respectively. Table 4 displays that the enzyme exhibited activity with both NAD^+ and $NADP^+$.

Table 3: The substrate specificity of L-alanine dehydrogenase

Substrate	Activity (U mL^{-1})
L-alanine	0.284±0.019*
DL-alanine	0.337±0.012#
L-serine	0.400±0.025*
L-isoleucine	0.260±0.079#
L-threonine	0.231±0.006#
Glycine	0.365±0.008**

Reaction mixture contained: substrate (10 μmole); NAD^+ (3 μmole); bicarbonate buffer at pH 9.5, 0.05 M and enzyme in a final volume of 1 mL, at 60°C for 2 min. Results are expressed as Mean±Standard deviation (SD) of three-independent experiments. The activity using L-serine and glycine compared to L-alanine is higher and statistically significant [p*= 0.02134; p**= 0.01391, respectively] but activity using DL-alanine, L-isoleucine and L-threonine are not significantly different [p# = NS]

Table 4: The coenzyme specificity of L-alanine dehydrogenase

Coenzyme	Activity (U mL)
NAD^+	0.287±0.019
$NADP^+$	0.347±0.013

Table 5: Effect of various compounds on L-alanine dehydrogenase activity

Compound	Concentration (mM)	Relative activity (%)
None		100.00
$COCl_2$	10	83.00
$COCl_2$	20	76.50
$ZnCl_2$	10	43.00
$ZnCl_2$	20	26.60
Iodoacetate	10	76.25
Reduce glutathione	10	116.50
Dithiothreitol (DTT)	10	131.00

Reaction mixture contained: L-alanine (10 μmole); NAD^+ (3 μmole), bicarbonate buffer at pH 9.5, 0.05 M and enzyme in a final volume of 1 mL, at 60°C for 2 min

In this study, the effects of various compounds on oxidative deamination of L-AlaDH were traced. Table 5 presents the effect of CO^{+2} (10, 20 mM), Zn^{+2} (10, 20 mM), iodoacetate 10 mM, reduced glutathione 10 mM and Dithiothreitol (DTT) 10 mM. It is clear from the table that CO^{2+}, Zn^{+2} and iodoacetate show inhibitory effects while both reduced glutathione and Dithiothreitol (DTT) activate the enzyme. Figure 4 demonstrates the non-competitive nature of inhibition caused by Zn^+ The effect of various compounds on oxidative deamination of L-AlaDH is demonstrated in Table 5. It could be observed that the enzyme was inhibited by addition of iodoacetate at a concentration of 10 mM (23.75%). On the other hand, the enzyme was activated by either Dithiothreitol (DTT) or reduced glutathione at a concentration of 10 mM for (131 and 116.5%) higher activity, respectively.

DISCUSSION

The results of the present study on oxidative deamination and reductive amination coincide with other studies related to L-AlaDH (Brunhuber and Blanchard, 1994) which showed that the enzyme had a high pH optimum for both directions. Here the optimum pH for AlaDH for oxidative deamination and reductive animation were at the alkaline pH range. Such high pH optima are known not only for AlaDH from different species but also for other amino acid dehydrogenases such

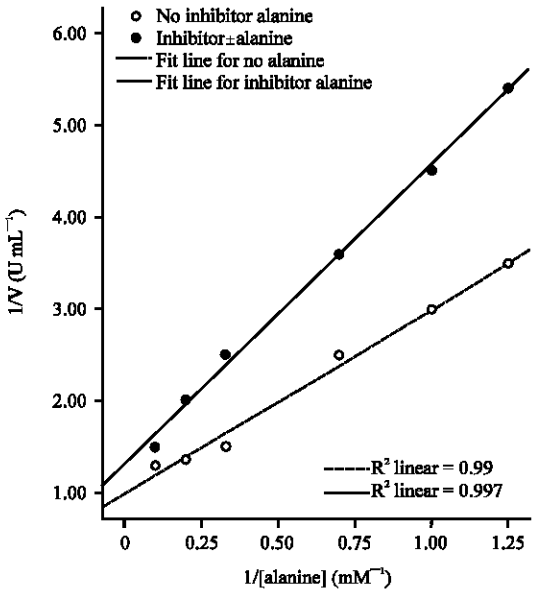

Fig. 4: Lineweaver-Burk plot (1/V versus 1/[S]) for the L-alanine dehydrogenase in both the presence or absence of Zn^{+2}

as leucine dehydrogenase and glutamate dehydrogenase. The high pH optima may reflect common catalytic mechanism or roles of amino acid dehydrogenases (Ohashima and Soda, 1979). The recorded optimum pH for L-AlaDH of *T. paradoxa* is similar to pH optima recorded for the purified enzyme from different bacterial or fungal species.

The thermal stability of enzyme found in our results are in agreement with those of Keradjopoulos and Holldorf (1979) and Vancura *et al.* (1989), who recorded high optimum temperatures (60/70°C) for purified enzyme from *Halobactrium salinarum* and *Streptomyces fradiae,* respectively. Also alanine dehydrogenase purified from *Bilophila wadsworthia* showed maximum activity at 50-60°C (Laue and Cook, 2000). Moreover, thermal-stability was conducted to determine the relative thermo-stability of AlaDH.

Figure 4 demonstrates that the enzyme retained 22% of its activity upon heating at 60°C for at least 60 min. This was suggested that L-AlaDH of *T. paradoxa* is relatively thermostable since highly thermo-stable enzymes were reported to retain their full activity upon heating at 75°C for at least 60 min and at 80°C for 20 min (Ohshima *et al.*, 1990).

Comparing the Km values obtained deriving this study with other values for AlaDH from different bacterial sources revealed that *T. paradoxa* L-AlaDH had remarkably different Km values for L-alanine and pyruvate while other substrates had relatively similar values. The Km value for L-alanine (1.35 mM) obtained in this study is lower while for pyruvate (8.16 mM) is higher than corresponding values of alanine dehydrogenase of different species (Ohashima and Soda, 1990; Laue and Cook, 2000). This was suggested that L-AlaDH of *T. paradoxa* had a much higher affinity to L-alanine compared to pyruvate which favors the oxidative deamination reaction.

The high Km value of *T. paradoxa* alanine dehydrogenase for NH_4^+ (33.5 mM) supports the suggestion that the enzyme favours the oxidative deamination direction more than the reductive amination reaction. This is in agreement with the findings of Laue and Cook (2000) who showed that AlaDH from *Bilophila wadsworthia* is apparently suitable for the direction of oxidative

deamination with a Km value of (31 mM). In contrast, the enzyme partially purified from *Cunninghamella elegans* fungi (El-Awamry and El-Rahmany, 1989) had higher activity in the aminating reaction.

The enzyme had a broad specificity in the oxidative deamination reaction since DL-alanine, L-serine, L-isoleucine, glycine and L-threonine were all deaminated by the enzyme to the corresponding keto acid. The broad specificity observed in the present study for AlaDH was previously recorded with other AlaDH such as the enzymes obtained from spores *B. cereus* and *Enterobacter aerogenes* (O'Connor and Halvorson, 1960; Chowdhury *et al.*, 1998).

The specificity of the L-AlaDH towards the coenzymes demonstrates that the enzyme can use both NAD^+, $NADP^+$ and NADH could serve as coenzyme in the oxidative deamination and reductive amination respectively. $NADP^+$ shows 26% higher activity than that with NAD^+. This observation is consistent with the findings of Chowdhury *et al.* (1998), who reported that AlaDH from *Enterobacter aerogenes* showed 7% higher activity in the deamination reaction, when $NADP^+$ was used as the coenzyme compared to NAD^+.

Iodoacetate is known to inhibit enzymes that have an SH-group in their active sites and Dithiothreitol (DTT) or reduced glutathione are known to activate sulfhydryl enzymes. It is possible that the sulfhydryl group is participating in the catalysis of the L-AlaDH from *T. paradoxa*. Similarly, L-AlaDH from *B. subtilis, M. tuberculosis, B. cereus* spores, vegetative cells of *B. cereus, D. desulfuricans,* phototrophic bacterium *R. capsulatus* and *C. elegans,* are which all known as sulfhydryl enzymes (Pierard and Wiame, 1960; Yoshida and Freese, 1964; Goldman, 1959; O'Connor and Halvorson, 1960; McCormick and Halvorson, 1964; Germano and Anderson, 1968; Caballero *et al.*, 1989; El-Awamry and El-Rahmany, 1989). Moreover, the metal ions Zn^{2+} and CO^{2+} caused inhibition of enzymatic activity. Zn^{2+} caused 57 and 73.4% inhibition at 10, 20 mM concentration, respectively while CO^{2+} shows less inhibitory action causing 17 and 23.5 % lower activity at 10 and 20 mM concentrations respectively. These are in agreement with the findings of Lee *et al.* (1991), Kim *et al.* (2000), Bae *et al.* (2003) and Kato *et al.* (2003) who reported that the enzyme activity was inhibited by Zn^{2+}. El-Awamry and El-Rahmany (1989) also recorded a slightly higher inhibition using 10 mM Zn^{2+} (88%), On the other hand, Kim and Fitt (1977) reported 60% inhibition by CO^{2+} on partially purified L-AlaDH from *C. elegans* and *H. cutirubrum,* respectively. In contrast, alanine dehydrogenase from *Enterobacter aerogenes* (Chowdhury *et al.*, 1998) was not affected by either Zn^{2+} or CO^{2+}. Figure 4 shows that Zn^{2+} acts as a non-competitive inhibitor for *T. paradoxa* L-AlaDH which is in agreement with the work of Kim *et al.* (2000). They reported that at higher pH, (pH 10) Zn^{2+} binds with the enzyme with lower affinity and non-competitively with respect to L-alanine. This type of inhibition is recognized by its characteristic effects on V_{max} whereas Km is unchanged.

CONCLUSION

The results presented above showed that *T. paradoxa* contains an active L-alanine dehydrogenase that induced by L-alanine. Its activity was maximum in the standard oxidative deamination assay at pH 9.5 incubated after two min at 60°C. The enzyme retained activity upon heating at 60°C for at least 60 min which suggested that it is a thermostable enzyme. The enzyme was purified 37-fold and the apparent Km values are the same magnitude as those alanine dehydrogenases from other species. Lower Km value of L-alanine and higher Km value for ammonia making the *T. paradoxa* enzyme apparently suitable for the direction of oxidative deamination. The enzyme not specific for L-alanine and NAD^+ in the oxidative deamination while

it showed specificity for NADH in the reductive amination. The enzyme was inhibited by heavy metal such as Zn^{+2} and CO^{+2} and sulfhydryl group is might be participating in the catalytic activity of the enzyme.

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