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PARTIAL PURIFICATION AND PROPERTIES OF L- ALANINE DEHYDROGENASE OF *Aspergillus terreus*

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ABSTRACT

L-Alanine dehydrogenase was partially purified about 13-fold in a two-step purification from the cell free extracts of *Aspergillus terreus*. Maximal enzyme activity occurred at pH 8.6 for reductive amination of pyruvate, where pH 10.4 for oxidative deamination of L-alanine and at a temperature of 25 °C. The K_m values for pyruvate, NH_4^+, NADH, L-alanine and NAD^+ were 4, 175.4, 0.526, 16.13 and 3.3 mM respectively. The enzyme activity was activeted by Co^{2+},Fe^{2+}, Zn^{2+}, Ca^{2+}, and Cu^{2+}. SH groups don't seem to play a role in the catalytic action of the enzyme as addition of iodoacetate or dithiothreitol did not effected the enzyme activity. Stability of the enzyme under different conditions was investigated.
Keywords : *Aspergillus terreus*. L- alanine dehydrogenase purification and properties.

INTRODUCTION

L-Alanine dehydrogenase (L–alanine NAD $^+$ oxidoreductase, EC 1.4.1.1) purification and properties have been elucidated in microorganisms including bacteria (Crow,1987 ; Porumb *et al.*,1987 ; Elfimova *et al.*, 1997 ; Chowdhury *et al.*, 1998 ; Kim *et al.*, 2000; Laue and Cook, 2000 ; Usha *et al.*, 2002 ;Kato *et al.*, 2003 ; Lodwig *et al.*,2004 and Mihara *et al.*,2005) , actinomycetes (Vancurova *et al.*, 1988 ; Vancura *et al.*, 1989 ; Diao and Jiao, 1991) , and fungi (EL–Awamry & EL–Rahmany 1989, Al-Kadeeb, 2001 and El–Awamry& Al- Kadeeb, 2003).

EL–Awamry & EL–Rahmany (1989) showed that L-alanine dehydrogenase was partially purified from the mycelial extracts of *Cunninghamella elegans* and the purified enzyme was fractionated by TEAE-cellulose column chromatography into two fractions. The activity of both fractions in the aminating reaction was 8 times higher than the activity of the deaminating reaction. Some of the kinetic properties of the enzyme (optimal pH, effect of heat, Michaelis constants, substrate specificity, effect of sulfhydryl reagents, effect of divalent metal ions and stability) were also demonstrated.

Al-Awamry and Al-Kadeeb (2003) reported that L- alanine dehydrogenase of *Trichoderma viride* was thermolable and some of the kinetic properties of the enzyme were also studied.

Kato *et al.*(2003) reported that L- alanine dehydrogenase from a marine bacterium ,*Vibrio proteolyticus* has been purified to homogeneity with ayield of 46% and some of its enzymological properties were also elucidated.

The present study describes the partial purification and enzymological and physiochemical properties of L-alanine dehydrogenase from *A. terreus*. This might contribute to the comprehensive picture of this enzyme in different microorganisms.

MATERIAL AND METHODS

Organism

The filamentous fungus *Aspergillus terreus* were isolated from Red Sea Coast (Al-Qunfidah Region) and identified by Al-Hazmi(2005).

Media and culture

The organism was grown on glucose Czapek - Dox liquied medium with L-alanine replacing $NaNO_3$ on nitrogen equivalent basis to induce the formation of L-alanine dehydrogenase. Five ml aliquots of spore suspension of *Aspergillus terreus* were used to inoculate 250 ml Erlenmeyer flasks, each containing 50 ml sterile medium. The inoculated flasks were incubated at 28 °C for 3 days, then the fungal mycelia were harvested by culture filtration, washed thoroughly with distilled water, and finally blotted dry with absorbent paper.

Chemical analysis methods

Pyruvate was estimated by the method of Friedmann and Haugen (1943). L-alanine was determined by quantitative paper chromatography, using Whatman No.1 filter paper and water- saturated phenol as a solvent system (Kay *et al* .,1956). Protein was determined according to the method of (Lowery *et al.*, 1951).

Assay of L-alanine dehydrogenase

L-Alanine dehydrogenase activity was routinely assayed by following the formation of pyruvate from alanine (oxidative deamination). One unit of enzyme activity is defined as the amount of protein which catalyzes the formation of one µmole pyruvate in 15 min at 25 °C. The forward reaction (reductive amination) was assayed by following the formation of alanine from pyruvate.

Partial pufrication of *A. terreus* L-alanine dehydrogenase

The harvested mycelia were ground with cold sand in a cold mortar and extracted with cold 0.05 M Na_2CO_3-$NaHCO_3$ (pH 10.4) buffer. The obtained slurry was then centrifuged at 12.000 xgm for 10 min and the supernatant was used for enzyme purification. A summay of the procedure is given in table 1.

A. Ammonium sulfate precipitation

1- Beaker of the extracts placed within another beaker containing a water-ice slurry on top of magnetic stir plate.
2- While agitating gently on magnetic stirrer, more slowly added ammonium sulfate to achieve 40% saturation.
3- Continue stirring for 10-30 minutes after all salt has been added.
4- Spin at 12.000 x g for 20 min.
5- Decant supernatant and resuspend precipitate in 2 pellet volumes of extract buffer.

B. Sephadex G-100 chromatography

The enzyme solution was applied to Sephadex G-100 column (3 x 50 cm) preequilibrated with 0.2 M Na_2CO_3-$NaHCO_3$ buffer (pH 10.4). The enzyme was eluted with buffer containing (0-0.3 M) Linear NaCl gradient. The fractions containing L- alanine dehydrogenase activity of 4.5 ml was collected. The flow rate was 2ml / min.

RESULTS AND DISCUSSION

Properties of L- alanine dehydrogenase

The partially purified fractions was used in studying the properties of the enzyme.

Table 1. Partial pufrication of L-alanine dehydrogenase from *A. erreus*.

Steps	Total protein (mg)	Total activity (U/mg)	Specific activity (U/mg)	Purification fold	Yield(%)
Crud exracts	178	15.2	0.085	1	100
40%$(NH_4)_2SO_4$	64	9.4	0.15	1.73	61.8
SephadexG-100	2.3	2.59	1.13	13.3	17

Rate of reductive aminatin of pyruvate and oxidative deamination of L – alanine

Figure 1 (A & B) show the rate of reductive amination of pyruvate and oxidative deamination of L–alanine by *A. terreus* L-alanine dehydrogenase. It is clear that the maximal velocity of reductive amination at pH 8.6 was 14 times greater than that of the oxidative deamination at pH 10.4.

Table 2. Apparent K_m values for substrates of L-alanine dehydrogenase.

Substrate	Km (mM)
pyruvate	4
NH_4^+	175.4
NADH	0. 526
L- alanine	16.13
NAD^+	3.3

- **Reaction mixture for reductive amination contained (total volume 1 ml) : (2.5–40 µmoles) pyruvate, (25-400) µmoles; NH_4Cl, (0.25 – 4 .0) µmoles NADH, 80 µmoles Tris–HCl buffer (pH 8.6) and 267 µg partial purified extract. The reaction mixture was incubated at 25 ° C for 15 min. When concentration of one substrate was changed the other substrates were added at saturating levels .**
- **Reaction mixture for oxidative deamination contained (total volume 1 ml) : (5 –40) µmoles L-alanine, (1. 25 – 30) µmoles NAD^+, 80 µmoles Na_2CO_3-$NaHCO_3$ buffe(pH 10.4) and 580 µg partial purified extract. The reaction mixture was incubated at 25 ° C for 15 min.**

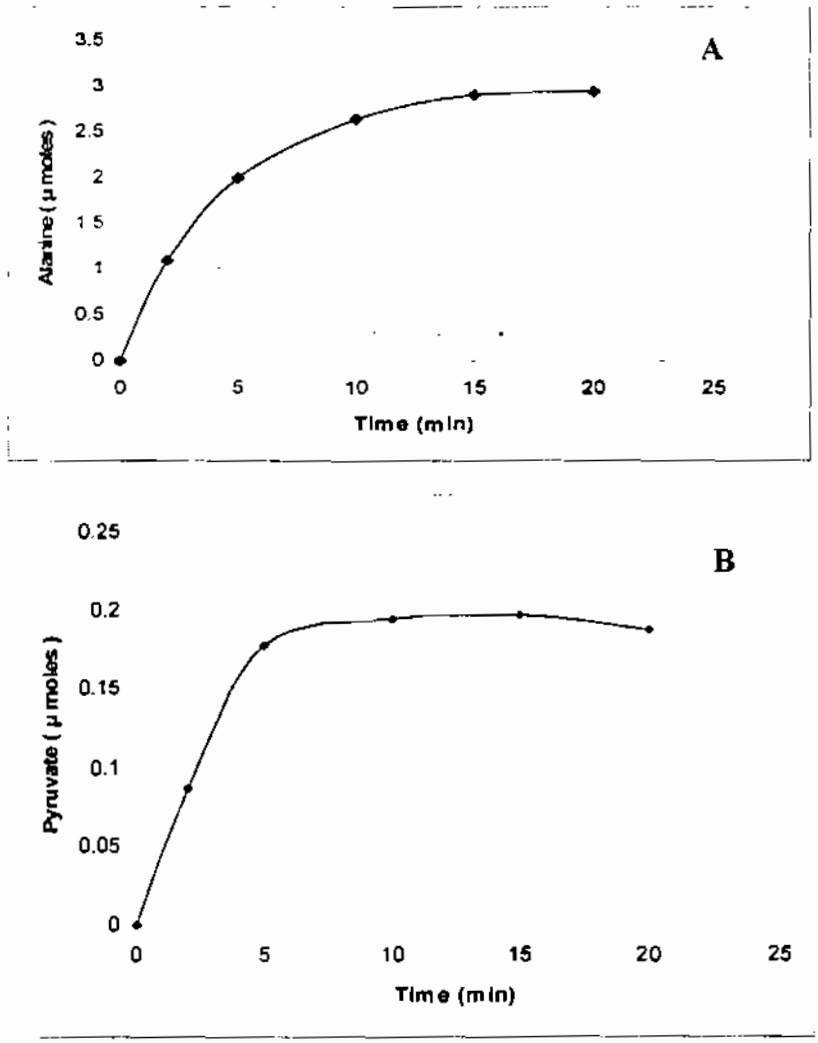

Fig. 1. Time course of reductive amination of pyruvate (A) and oxidative deamination of L-alanine (B) by *A. terreus* L-alanine dehydrogenase. Reaction mixture for reductive amination contained (total volume 1 ml): 10 µmoles, pyruvate 100 µmoles NH_4Cl, 1 µmoles NADH, 80 µmoles Tris-- HCl buffer (pH 8.6) and 254 µg partial purified extract. The reaction mixture was incubated at 25 °C for time as indicated.
Reaction mixture for oxidative deamination contained (total volume1 ml): 10 µmoles L-alanine, 3 µmoles NAD^+, 80 µmoles Na_2CO_3-$NaHCO_3$ buffer (pH 10.4) and 267 µg partial purified extract. The reaction mixture was incubated at 25 °C for time as indicated.

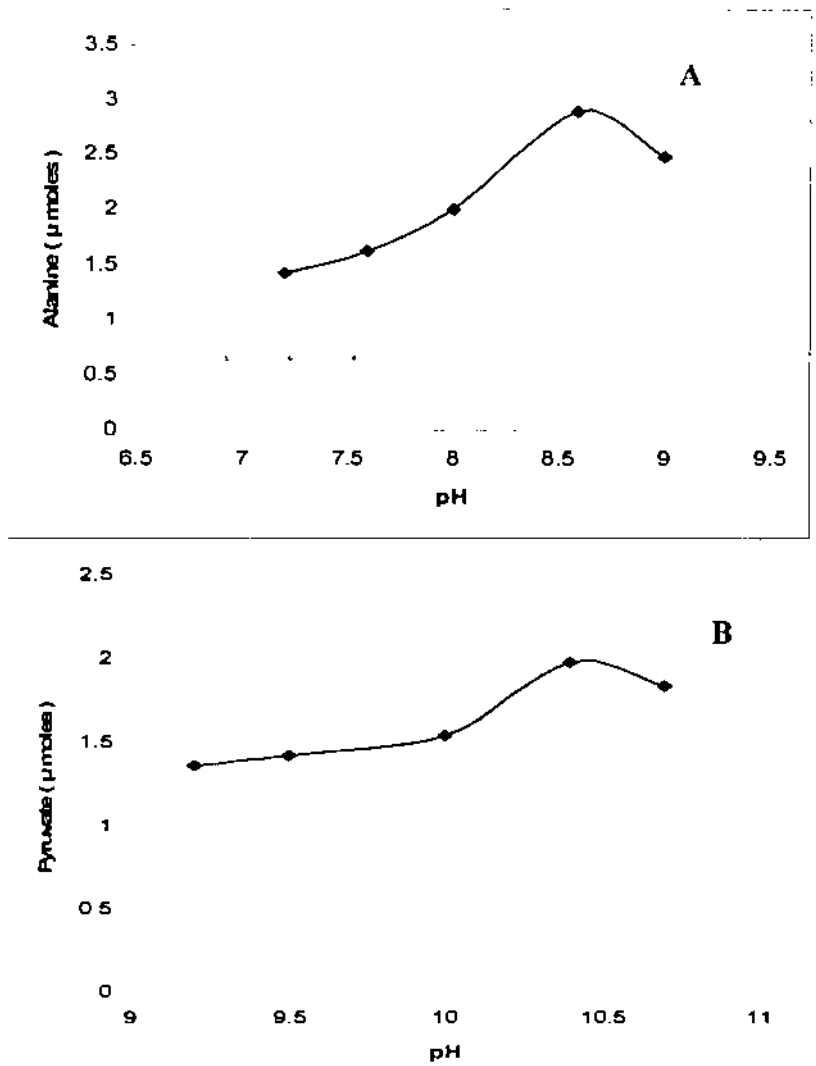

Fig . 2 . pH profile of reductive amination of pyruvate (A) and oxidative deamination of L-alanine(B) by *A.terreus* L-alanine. Reaction mixture for reductive aminationcontained (total volume1 ml) : 10 µmoles pyruvate, 100 µmoles NH_4Cl, 1 µmoles NADH, 80 µmoles Tris– HCl buffer (pH as indicated) and 580 µg partial purified extract. The reaction mixture was incubated at 25 °C for 15 min.

Reaction mixture for oxidative deamination contained (total volume 1 ml): 10 µmoles L- alanine, 3 µmoles NAD^+,80 µmoles buffer (pH as indicated) and 254 µg partial purified extract. The reaction mixture was incubated at 25 °C for 15 min.

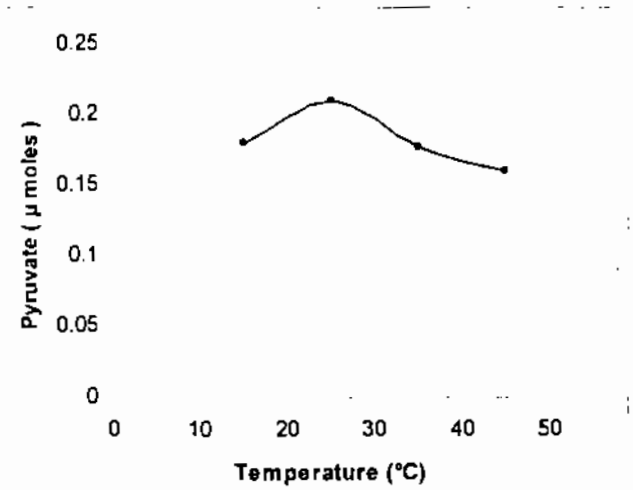

Fig . 3. Temperature dependence of oxidative deamination of L-alanine by *A. terreus* partial purified extracts .
Reaction mixture contained (total volume 1 ml): 10 µmoles L-alanine, 3 µmoles NAD^+, 80 µmoles Na_2CO_3 - $NaHCO_3$ buffer (pH10.4) and 207µg partial purified. extract The reaction mixture was incubated at temperature as indicated for 15 min.

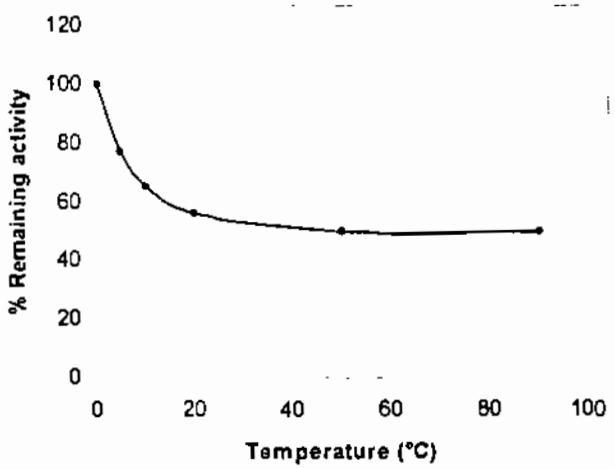

Fig. 4. Thermal stability of L-alanine dehydrogenase of *A. terreus*. 1212 µg partial purified extract was incubated at 35 ° C with an equal volume of 0.2 MNa_2CO_3- $NaHCO_3$ buffer (pH10.4). Samples were withdrawn at different time intervals and assayed for L–alanine dehydrogenase activity (oxidative deaminating reaction).

Substrate specificity

Compounds structurally related to L-alanine were used at concentration of 10 µmoles to determine the substrate specificity of L-alanine dehydrogenase in the oxidative deamination reaction. The results (Table 3) demonstrate that L-alanine dehydrogenase catalyzed the oxidative deamination reaction of β-alanine, DL–alanine, L–glutamic acid,L-theronine and L- serine. These results demonstrate that L-alanine dehydrogenase of *A. terreus* was not specific for L-alanine. Also Nagata *et al* . (1989) illustrated that L-alanine dehydrogenase of *Bacillus* sp. DSM730 catalyzed oxidative deamination reaction from L-alanine and L–serine, while Al-Kadeeb (2001) reported that L-alanine dehydrogenase of *T. paradoxa* catalyzed the oxidative deamination reaction of DL–alanine, L-serine, L–isoleucine and L–theronine at quiker rates than L–alanine, these results demonstrate that L-alanine dehydrogenase of *T . paradoxa* was not specific for L-alanine and Al – Awamry and Al- Kadeeb (2003) showed that L-alanine dehydrogenase of *T. viride* catalyzed the oxidative deamination reaction of D–alanine, L–glutamic acid, L–isoleucine and L- serine, but at slower rates than that found for L–alanine. DL–alanine was oxidatively deamination at the same rate of L-alanine deamination.

Table3. Substrate specificity of *A. terreus* L-alanine dehydrogenase.

Substrate	Relative activity %
L - Alanine	100.00
β - Alanine	89
DL - Alanine	76.3
L – Glutamic acid	74.4
L-Theronine	75.6
L - Serine	88.9

Reaction mixture contained (total volume 1 ml): 10 µmoles substrate, 3 µmoles NAD^+, 80 µmoles Na_2CO_3-$NaHCO_3$ buffer (pH10.4) and 580 µg partial purified extract. The reaction mixture was incubated at 25 °C for 15 min.

Effect of some activators or inhibitors on L - alanine dehydrogenase activity of *A. terreus*

Table 4 shows the effect of various compounds on oxidative deamination of L-alanine dehydrogenase. Addition of iodoacetate and dithiothreitol at concentration of 20 mM did not effect the enzyme activity, this suggest that the sulfhydryl group do not seem to play a role in the catalytic action of *A. terreus* L-alanine dehydrogenase. The same results reported by Al –Kadeeb (2001) and El-Awamry & Al-Kadeeb(2003).

As also shown in Table 4 that the activity of L-alanine dehydrogenase not affected by addition of ethylenediaminetetracetate at the two concentrations 20 and 40 mM, this suggests that metal cations not participated in enzyme activity. This is supported by the finding that the activity of the enzyme was increased , but with different rates by addition of Co^{2+},Fe^{2+}, Zn^{2+} , Ca^{2+}, and Cu^{2+} ions. Mn^{2+} had no effect enzyme activity. These results are in close agreement with that reported for L-alanine dehydrogenase of *C. elegans* (EL-Awamry and EL-Rahmany, 1989).

Table 4. Effect of some activators or inhibitors on L-alanine dehydrogenase activity.

Addition	Concentration(mM)	Relative activity (%)
None		100.00
Iodoactate	20	100.00
Dithiothreitol	20	100.0
EDTA	20	90
	40	94.3
$CaCl_2$	10	133
$CuSO_4$	10	122
$FeSO_4$	10	175
$CoCl_2$	10	218
$MnCl_2$	10	100
$ZnCl_2$	10	118

Reaction mixture contained (total volume 1 ml): 10 µmoles L-alanine , 3 µmoles NAD^+ , 80 µmoles Na_2CO_3-$NaHCO_3$ buffer (pH10.4), addition as indicated and 400 partial purified extract. The reaction mixture was incubated at 25 °C for 15 min.

Enzyme stability

L-alanine dehydrogenase was relatively stable when extracts of *A. terreus* was stored at either 4 °C or – 15 °C for one month. Frequent freezing and thawing of the extracts had no appreciable effect on enzyme activity. Dialysis for 24 hr against H_2O or 0.05 M Na_2CO_3 -$NaHCO_3$ buffer caused 50% loss in enzyme activity.

CONCLUSION

L- Alanine dehydrogenase was partially purified about 13-fold in two-step purification from *Aspergillus terreus*. Maximal activity of enzyme occurred at pH 8.6 for redutive amination of pyruvate, where pH 10.4 for oxidative deamination of L-alanine and at a temperature of 25°C. Maximal velocityof the redutive amination reaction was14 times greater than that of the oxidative deamination reaction. The K_m for pyruvate , NH_4^+ , NADH , L-alanine and NAD^+ were 4, 175.4, 0.526, 16.13 and 3.3 mM respectively. The enzyme was not inhibited by EDTA, while it was activeted by Co^{2+},Fe^{2+}, Zn^{2+} , Ca^{2+}, and Cu^{2+}. SH groups don't seem to play a role in the catalytic action of the enzyme. L-alanine dehydrogenase was relatively stable when stored at either 4 °C or – 15 °C. Dialysis for 24 hr against H_2O or 0.05 M Na_2CO_3-$NaHCO_3$ buffer caused 50% loss in enzyme activity.

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خواص إنزيم ل – ألانين ديهيدروجينيز لفطرة اسبرجلس تيريس

سهام عبدالمحسن القضيب

كلية التربية للبنات بالرياض / الأقسام العلمية (قسم النبات) – المملكة العربية السعودية

تمت تنقية إنزيم ل– ألانين ديهيدروجينيز لفطرة أسبيرجلس تيريس تنقية جزئية إلى حـــوالي ١٣ ضعف . وبرهنت النتائج على أن تفاعل إضافة المجموعة الأمينية بالاختزال بلغ أقصى معدل له عند الـــرقم الهيدروجيني ٨،٦ ، بينما كان أقصى معدل لتفاعل نزع المجموعة الأمينية بالأكسدة عند الرقم الهيـــدروجيني ١٠،٤ وعند درجة حرارة ٢٥ م° .

كما أثبتت النتائج أن الإنزيم ليس ثابت حراريا ، ولقد وجد أن قيمة ثابت ميخائيل للأنزيم مع كل من البيروفات، كلوريد الأمونيوم ، NADH ، ل – ألانين و NAD^+ قد بلغـــت ٤ ، ٤، ١٧٥ ،٥٢٦ ، ٠ ، ١٦،١٣ و ٣،٣ مليمولار على التوالي. تم تنشيط الإنزيم بواسطة أيونات كل مـــن الكوبالـــت والحديـــدوز والزنك والكالسيوم والنحاس.

كما تبين أن مجموعة الثيول ليس لها دور في الفعل الحفزي لإنزيم ل–ألانين ديهيدروجينيز حيث أن إضافة كل من أيودوالخلات و ثنائي ثيوثيرتول لم تؤثر على نشاط الإنزيم . وقد تم دراسة مـــدى ثبـــات نشاط الإنزيم تحت الظروف المختلفة .