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Original Paper

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Properties of L–Alanine Dehydrogenase of *Trichoderma viride*

Abstract: A study of the properties of L-alanine dehydrogenase of *Trichoderma viride* was undertaken. Maximal enzyme activity occurred at pH 8 for reductive amination of pyruvate, and at pH 9.5 for oxidative deamination of L-alanine at a temperature of 50°C. Maximum velocity of the reductive amination reaction was ten times greater than that of the oxidative deamination reaction. The K_m values for pyruvate, NH_4^+, NADH, L-alanine and NAD^+ were 17.2, 166, 0.7, 6.06 and 7.14mM respectively. The enzyme was not inhibited by EDTA, suggesting that no metal cation is participation in enzyme catalysis. This is supported by the finding that none of the tested metal salts activated the enzyme. Alternatively, the enzyme activity was inhibited by Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, and Mn^{2+}. SH groups don't seem to play a role in the catalytic action of the enzyme, as addition of iodoacetate or reduced glutathione did not effect the enzyme activity. Stability of the enzyme under different conditions was investigated.

Keywords: *Trichoderma viride,* L-alanine dehydrogenase, puruvate

خواص إنزيم ل – ألانين يديهيدروجينيز لفطرة ترايكوديرما فيريدي

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المستخلص: تمت دراسة خواص إنزيم ل– ألانين ديهيدروجينيز لفطرة ترايكوديرما فيريدي. وبرهنت النتائج على أن تفاعل إضافة المجموعة الأمينية بالإختزال قد بلغ أقصى معدل له، عند الرقم الهيدروجيني 8. بينما كان أقصى معدل لتفاعل نزع المجموعة الأمينية بالأكسدة، عند الرقم الهيدروجيني 9.5 وفي درجة حرارة 50°م. كما أثبتت النتائج أيضاً أن الإنزيم ليس ثابت حرارياً. إذ وجد أن قيمة ثابت ميكالس للأنزيم، مع كل من لبيروفات، كلوريد الأمونيوم، NADH، ل – ألانين و NAD+ قد بلغت 17.2، 166، 0.27، 6.06 و 7.14 مليمولار على التوالي. برهنت النتائج أن مركب رابع خلات ثنائي أمين الأثيلين لم يستطع تثبيط نشاط الإنزيم مما يشير إلى عدم مشاركة أيون معدني في الحفز الإنزيمي. ومما يدعم هذا الاستنتاج أن أياً من الأملاح المعدنية المختبرة، لم يؤد إلى زيادة نشاط الإنزيم. و على العكس من ذلك فقد تسبيت أيونات Mn^{2+}، Cu^{2+}، Ca^{2+}، Mg^{2+} و Fe^{2+} في تثبيط نشاط الإنزيم.كما تبين أن مجموعة الثيول ليس لها دور في الفعل الحفزي لإنزيم ل– ألانين ديهيدروجينين، حيث أن إضافة كل من أيود والخلات والجلوتاثيون المختزل لم تؤثر على نشاط الإنزيم. وقد تم دراسة مدى ثبات نشاط الإنزيم تحت الظروف المختلفة.

كلمات مدخلية: ترايكوديرمافيردي، ل – الانين، يديهيدروجينين، خواص

Introduction

The properties of L-alanine dehydrogenase (L-alanine NAD^+ Oxidoreductase, EC 1.4.1.1) have been studied in various microorganisms including bacteria (Goldman, 1959; Pierard and Wiam, 1960; McCromic and Halvorson, 1964; Yoshida and Freese, 1964 and 1956; Nitta, *et al.* 1974; McCowen and Phibbs, 1974; Kim and Fitt, 1977; Ohshima and Soda, 1979; Crow, 1987; Porumb, *et al.* 1987; Elfimova, *et al.* 1997; Chowdhury, *et al.* 1998; Laue and Cook, 2000), actinomycetes (Aharonowitz and Friedrich, 1980; Itoh and Morikawa, 1983; Vancura, *et al.* 1989; Vancurova, *et al.* 1989; Diao and Jiao, 1991), algae (Rowell and Stewart, 1976) and fungi (El-Awamry and El-Rahmany, 1989 and Al-Kadeeb, 2001).

El-Awamry and El-Rahmany (1989) reported 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, stability) were also demonstrated.

Al-Kadeeb (2001) reported that L-alanine dehydrogenase of *thielaviopsis paradoxa* was thermostable and some of the kinetic properties of the enzyme were also studied.

The present study aimed to demonstrate some enzymic properties of L-alanine dehydrogenase of *T. viride.* This might contribute to the comprehensive picture of this enzyme in different microorganisms.

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Material and Methods

Organism

The filamentous fungus *Trichoderma viride* was obtained form Cairo Mircen, Ain Shams University, Egypt.

Media and culture

The organism was grown on Czapek–Kox liquid medium with L-alanine replacing $NaNO_3$ on a nitrogen equivalent basis to induce the formation of L-alanine dehydrogenase. 5 ml aliquots of spore suspension of *Trichoderma viride* were used to inoculate 250 ml Erlenmeyer flasks, each containing 50 ml of sterile medium. The inoculated flasks were incubated at 28°C for 4 days. Then the fungal mycelia were harvested by culture filtration, washed thoroughly with distilled water, and finally blotted dry with absorbent paper.

Preparation of cell-free 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 10,000 gm for 10 min and the supernatant was used as the crude enzyme preparation.

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 (Sutherland, *et al.* 1949).

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 6 min at 50°C. The forward reaction (reductive amination) was assayed by following the formation of alanine from pyruvate.

Results and Discussion

Properties of L-alanine dehydrogenase

Crude extract was used in studying the properties of the enzyme.

Rate of reductive amination of pyruvate and oxidative deamination of L-alanine

Fig 1 (A & B) shows the rate of reductive amination of pyruvate and oxidative deamination of L-alanine by *T. viride* L-alanine dehydrogenase. It is clear that the maximal velocity of reductive amination at pH 8 was ten times greater than that of the oxidative deamination at pH 9.5.

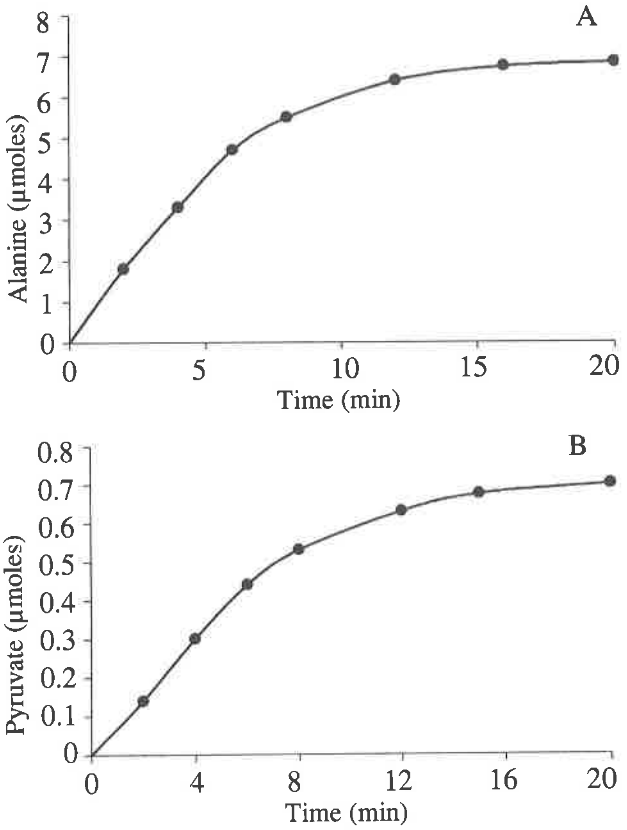
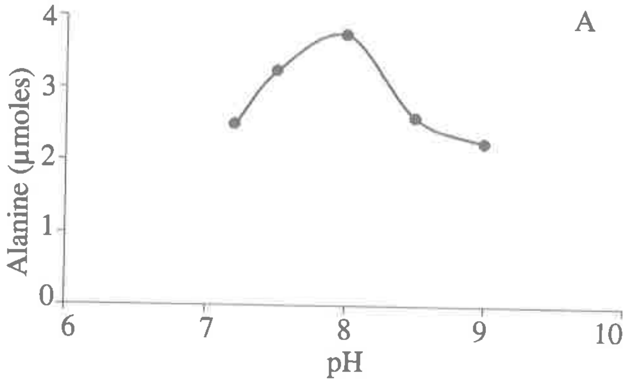


Fig. 1. Time course of reductive amination of pyruvate **(A)** and oxidative deamination of L-alanine **(B)** by *T. viride* L-alanine dehydrogenase. Reaction mixture for reductive amination contained (total volume 1 ml): 10 µmole pyruvates, 100 µmoles NH_4Cl, 1 µmoles NADH, 80 µmoles Tris-HCl buffer (pH 8) and 4.8 mg extract. The reaction mixture was incubated at 40°C for time as indicated. Reaction mixture for oxidative deamination contained (total volume ml): 10 µmoles L-alanine, 3 µmoles NAD^+, 80 µmoles Na_2CO_3-$NaHCO_3$ buffer (pH 9.5) and 4.8 mg extract. The reaction mixture was incubated at 40°C for time as indicated.

pH profile

Fig 2 (A & B) shows the pH profile for amination and deamination catalyzed by L-alanine dehydrogenase. Reductive amination of pyruvate was maximal at pH 8.0 in Tris-HCl buffer, while the oxidative deamination of L-alanine was optimal at pH 9.5 in Na_2CO_3-$NaHCO_3$ buffer. These pH optima are in close agreement with those reported for L-alanine dehydrogenase of *Desulfovibrio desulfuricans* (Germano and Anderson, 1968), a thermophilic bacillus (Epstein and Grossowicz, 1976), *Bacillus natto* KMD 1126 (Matsui, *et al.* 1977), *Streptomyces clavuligerus* (Aharonowitz and Friedrich, 1980), *Sreptomyces Phaeochromogenes* (Itoh and Morikawa, 1983), *Cunninghamella elegans* (El-Awamry and El-Rahmany, 1989) and *Thielaviopsis paradoxa* (Al-Kaddeb, 2001). On the other hand, the reaction catalyzed by L-alanine dehydrogenase of *Bacillus subtilis* (Yoshida and Freese, 1965), *Bacillus sphaerticus* (Ohshima and Soda, 1979) reported optimal pH higher than that for reactions activated by the *T. viride* enzyme, while pH 9.0 was reported for both reactions catalyzed by L-alanine dehydrogenase of *Halobacterium cutirubrum* (Kim and Fitt, 1977). Also Laue and Cook (2000) found the pH optimum was pH 9 for reductive amination of pyruvate and pH 9.0-11.5 for oxidative deamination catalyzed by L-alanine dehydrogenase of *Bilophila wadsworthia*. The study done by Diao and Jiao (1991) on L-alanine dehydrogenase of actinomycetes *Nocardia mediterranei* showed that, the optimal pH for oxidative deamination was 11.5, while the optimum activity of the reductive amination reaction was found to be 8.5. Chowdhury, *et al.* (1998) reported that the enzyme of *Enterobacter aerogenase* showed maximal activity at about pH 10.9 for the deamination of L-alanine and about pH 8.7 for the amination of pyruvate.



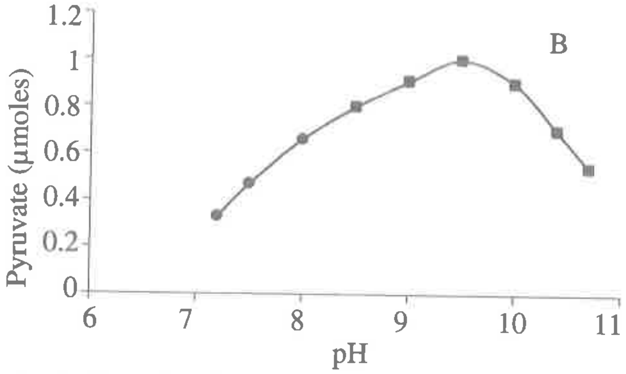


Fig. 2. pH profile of reductive amination of pyruvate **(A)** and oxidative deamination of L-alanine **(B)** by *T. viride* L-alanine. Reaction mixture for reductive amination contained (total volume 1 ml): 10 μmoles pyruvate, 100 μmoles NH_4Cl, 1 μmole NADH, 80 μmoles Tris-HCl buffer (pH as indicated) and 3.9 mg extract. The reaction mixture was incubated at 40°C for 6 min. Reaction mixture for oxidative deamination contained (total volume 1ml): 10 μmoles L-alanine, 3 μmoles NAD^+, 80 μmoles buffer (pH as indicated) and 1.12 mg extract. The reaction mixture was incubated at 40°C for 6 min. (l) Tris –HCl buffer. (n) Na_2CO_3 – $NaHCO_3$ buffer.

Effect of temperature

Fig 3 illustrates the effect of temperature on the oxidative deamination of L-alanine by L-alanine dehydrogenase of *T. viride*. As seen the temperatures used ranged from 20°C to 60°C. The results demonstrated that the optimum activity of enzyme was obtained at 50°C. This result agreed with that reported for L-alanine dehydrogenase of *Nocardia mediterrani* (Diao and Jaio, 1991). In addition, exposure of the enzyme to 60°C for 5 and 15 min resulted in about 66% and 82% loss of its activity, respectively. This shows that the enzyme may be denatured at 60°C and the enzyme is thermolabile.

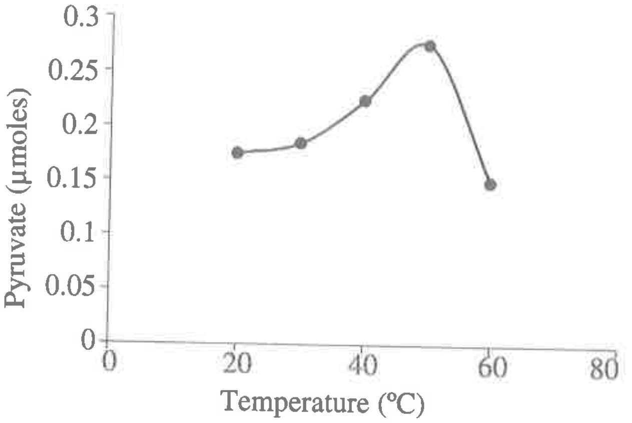


Fig. 3. Temperature dependence of oxidative deamination of L-alanine by *T. viride* extracts. Reaction mixture contained (total volume 1 ml): 10 μmoles L-alanine, 3 μmoles NAD^+, 80 μmoles Na_2CO_3 – $NaHCO_3$ buffer (pH 9.5) and 3.4 mg extract. The reaction mixture was incubated at the temperature indicated for 6 min.

Thermal stability

The enzyme activity was studied as a function of incubating the enzyme at 60°C for different time intervals (0-30 min) in the presence of Na_2CO_3-$NaHCO_3$ buffer, at pH 9.5. Results obtained and presented in Fig 4 clearly show that the activity of L-alanine dehydrogenase of *T. viride* decreased. It is shown that exposure to 60°C for 5 and 15 min resulted in about 66% and 85% loss of activity, respectively. On the contrary, L-alanine dehydrogenase extracts from *B.* subtilis (Hermier, *et al.* 1965; Nagata, *et al.* 1989 and Ohshima, *et al.* 1990) and from *T. paradoxa* (Al-Kadeeb, 2001) were thermostable.

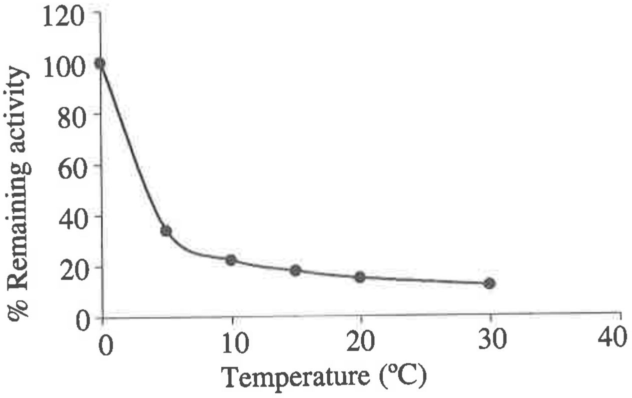


Fig. 4. Thermal stability of L-alanine dehydrogenase of *T. viride.* 28.8 mg extract was incubated at 60°C with an equal volume of 0.2M Na_2CO_3-$NaHCO_3$ buffer (pH 9.5). Samples were withdrawn at different time intervals and assayed for L-alanine dehydrogenase activity (oxidative deaminating reaction).

Michaelis constant

The apparent K_m values of L-alanine dehydrogenase for L-alanine and NAD^+ in the oxidative deamination and pyruvate, NH_4^+ and NADH in the reductive amination were determined from Linweaver-Burk plots of the reciprocal of initial velocities and substrate concentrations. The results are shown in Table 1. As commonly observed for reductive aminations, the K_m values for ammonia were high as compared to other substrates. Similar data were obtained for enzyme from *D. desulfuricans* (Germano and Anderson, 1968), *Anabaena cylindrical* (Rowell and Stewart, 1976), *St. clavuligerus* (Aharonowitz and Friedrich, 1980), *C. elegans* (El-Awamry and El-Rahmany, 1989), *B. sphaericus* DSM 462 (Ohshima, *et al.* 1990), *Enterobacter aerogennes* (Chowdhury, *et al.* 1998), *Bilophila wadsworthia* (Laue and Cook, 2000) and *T. paradoxa* (Al-Kadeeb, 2001). There was no indication for substrate (pyruvate) inhibition. This result agreed with that reported for L-alanine dehydrogenase of *St. clavuligerus* (Aharonowitz and Friedrich, 1980), *C. elegans* (El-Awamry and El-Rahmany, 1989) and *T. Paradoxa* (Al-Kadeeb, 2001), while the enzyme from *M. bacterium* (Goldman, 1959) and *D. desulfuricans* (Germano and Anderson, 1968) was inhibited with pyruvate. Also, there was no indication for cosubstrate (NADH) inhibition. Similar data were obtained for the enzyme from *T. paradoxa* (Al-Kadeeb, 2001), while the enzyme from *St. clavuligerus* (Aharonowitz and Friedrich, 1980) was inhibited with NADH at a concentration of more than 0.25 mM. In the deamination reaction, the K_m value for L-alanine was in close agreement with that reported for L-alanine dehydrogenase extracts from other microorganisms (McCormick and Halvorson, 1964; McCowen and Phibbs, 1974; Epstein and Grossowicz, 1976; Kim and Fitt, 1977; Vancurova, *et al.* 1988 and El-Awamry and El-Rahmany, 1989). On the other hand, Ohshima and Soda (1979) reported a high value K_m for L-alanine of L-alanine dehydrogenase of *Bacillus sphaericus*. It was found to be 18.9 mM.

Table 1. Apparent K_m values for substrates of L-alanine dehydrogenase.

Substrate	Km (mM)
Pyruvate	17.2
NH_4^+	166
NADH	0.27
L-alanine	6.06
NAD^+	7.14

Reaction mixture for reductive amination contained (total volume 1 ml): (10-100 μmoles) pyruvate, (50—1000) μmoles NH_4C1, (0.2–4.0) μmoles NADH, 80 μmoles Tris-HC1 buffer (pH 8) and 3.6 mg extract. The reaction mixture was incubated at 50° for 6 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, (1.25–20) μmoles NAD^+, 80 μmoles Na_2CO_3-$NaHCO_3$ buffer (pH 9.5) and 3.48 mg extract. The reaction mixture was incubated at 50°C for 6 min.

Substrate specificity

Compounds structurally related to L-alanine were used at a concentration of 100 mM to determine the substrate specificity of L-alanine dehydrogenase in the oxidative deamination reaction. The results as shown in Table 2 demonstrate that L-alaninc dehydrogenase 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 oxidative deamination was at the same rate as L-alanine deamination. These results demonstrate that L-alanine dehydrogenase of *T. viride* was not specific for L-alanine. Also Nagata, *et al.* (1989) illustrate that L-alanine dehydrogenase of *Bacillus sp.* DSM730 catalyzed the oxidative deamination reaction from L-alanine and L-serine and 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 quicker rates than L-alanine. These results demonstrate that L-alanine dehydrogenase of *T. paradoxa* was not specific for L-alanine.

Table 2. Substrate specificity of *T. viride* L-alanine dehydrogenase.

Substrate	Relative activity
L-alanine	100.00
D-alanine	54.74
DL-alanine	100.00
L-glutamic acid	61.05
L-isoleucine	30.53
L-serine	54.74

Reaction mixture contained (total volume 1 ml): 10 µmoles Substrate, 3 µmoles NAD$^+$, 80 µmoles Na_2CO_3-$NaHCO_3$ buffer (pH 9.5) and 1 mg extract. The reaction mixture was incubated at 50°C for 6 min.

Effect of various compounds on L-alanine dehydrogenase activity of T. paradoxa

Table 3 illustrates the effect of various compounds on oxidative deamination of L-alanine dehydrogenase. Addition of iodoacetate at concentration of 20 mM and reduced glutation at concentration 10 and 20 mM did not affect the enzyme activity. This suggests that the sulfhydryl group do not seem to play a role in the catalytic action of *T. viride* L-alanine dehydrogenase. The same results have been reported by Al-Kadeeb (2001). On the other hand, (Goldman, 1959; O'Connor and Halvorson, 1960; Pierard and Wiamex, 1960; McCormick and Halvorson, 1964; Yoshida and Freese, 1965; Grermano and Anderson, 1968; Rowell and Stewart, 1979; Ohshima and Soda, 1979 and El-Awamry and El-Rahmany, 1989) reported the participation of the sulfhydryl group in enzyme catalysis for L-alanine dehydrogenase extracted from some microorganisms.

It is also shown in Table 3 that the activity of L-alanine dehydrogenase was not affected by addition of ethylenediaminetetraacetate at the three concentrations 10, 20 and 40 mM. This suggests that metal cations do not participate in enzyme activity. This is supported by the finding that none of the tested metal salts activated the enzyme. As well, addition of Fe^2, Mg^{2+}, Ca^{2+}, Cu^{2+} and Mn^{2+} caused inhibition in enzyme activity. This result agreed with that reported for L-alanine dehydrogenase of *H. cutirubrum* (Kim and Fitt, 1977), *B. sphaericus* (Ohshima and Soda, 1979) and *T. paradoxa* (Al-Kadeeb, 2001).

Table 3. Effect of various compounds on L-alanine dehydrogenase activity.

Addition	Concentration (mM)	Pyruvate (µmole)	Relative activity(%)
None		0.34	100.00
Iodoactate	20	0.34	100.00
Reduced glutothione	10	0.36	105.88
	20	0.36	105.88
EDTA	10	0.32	96.97
	20	0.30	90.91
	40	0.33	100.00
$CaCl_2$	10	0.18	78.26
$CuSO_4$	10	0.19	82.61
$FeSO_4$	10	0.08	34.78
$MgCl_2$	10	0.09	39.13
$MnCl_2$	10	0.20	86.96
$ZnCl_2$	10	0.24	104.35

Reaction mixture contained (total volume 1 ml): 10 µmoles L-alanine, 3 µmoles NAD$^+$, 80 µmoles Na_2CO_3-$NaHCO_3$ buffer (pH 9.5), addition as indicated and 2.06 mg extract. The reaction mixture was incubated at 50°C for 6 min.

Enzyme stability

L-alanine dehydrogenase was relatively unstable when stored at either 4°C or -15°C. Frequent freezing and thawing of the extracts had no appreciable effect on enzyme activity. Dialysis for 24 hr caused complete loss of enzyme activity.

Conclusion

Maximal activity of L-alanine dehydrogenase of *Trichoderma viride* occurred at pH 8 for reductive amination of pyruvate and pH 9.5 for oxidative deamination of L-alanine at a temperature of 50°C. Maximal velocity of the reductive amination reaction was ten times greater than that of the oxidative deamination reaction. The K_m for pyruvate, NH_4^+, NADH, L-alanine and NAD^+ were 17.2, 166, 0.27, 6.06 and 7.14 mM respectively. The enzyme was not inhibited by EDTA or activated by metal salts. SH groups don't seem to play a role in the catalytic action of the enzyme. L-alanine dehydrogenase was relatively unstable when stored at either 4°C or -15°C. Dialysis for 24 hr caused complete loss of enzyme activity.

References

Aharonowitz Y and **Friedrich CG** (1980) Alanine dehydrogenase of the β– lactam antibiotic producer S*treptomyces clavaligerus. Arch. Microbil.* **125**: 137-142.

Al-Kadeeb SA (2001) Properties of L-alanine dehydrogenase of *Thielaviopsis paradoxa. J. Union Arab Biol.* **10 B:** Botany (Microbiology & Virology) 1-15.

Chowdhury EK, Saitoh T, Nagata S and **Ashiuchi M** (1998) Alaninne dehydrogenase from *Enterobacter aerogenes:* Purification, characterization, and primary structure. *Biosci. Biotechnol. Biochem.* **62**: 2357-2363.

Crow VL (1987) Properties of L-alanine dehydrogenase and aspartase from *Propionibacterium freudenreichii ssp. Shermanii. Environ. Micobiol.* **35**: 1885-1892.

Diao R and **Jiao R** (1991) Purification and properties of L-alanine dehydrogenase from *Nocardia mediterranei. Acta Microbiol. Sin.* **31**: 206-212.

El-Awamry ZA and **El-Rahmany TA** (1989) Partial purification and properties of *Cunninghamella elegans* L-alanine dehydrogenase. *Zentralbl. Mikrobiol.* **144**: 231-240.

Elfimova LI, Aburova EA and **Chekulaeva LN** (1997) Ammonium activation of the halophilic alanine dehydrogenase from *Halobacterium salaniarium. Microbiol. J.* **67**: 258-263.

Epstein I and **Grossowicz N** (1976) Regulatory control and function of alanine dehydrogenase from a thermophili bacillus. *Biochim. Biophys. Acta* **445**: 549-557.

Friedemann TE, Haugen GE (1943) The determination of keto acids in blood and urine. *J. Biol. Chem.* **147**: 415-442.

Germano GT and **Anderson KE** (1968) Purification and properties of L-alaine dehydrogenase from *Desulfovibrio desulfuricans. J. Bacteriol.* **96**: 55-60.

Goldman DS (1959) Enzyme systems in the mycobacteria. VII. Purification properties and mechanism of action of alanine dehydrogenase. *Biochem. Biophys. Acta* **34**: 527-539.

Hermier J, Siegenthaler P, Blondel-Queroix J and **Bergere, J** (1965) Separation and properties of leucine dehydrogenase and alanine dehydrogenase from *Bacillus subtilis. Bull. Soc. Chem. Biol.* **47**: 1217-1234.

Itoh N, Morikawa R (1983) Crystallization and properties of L-alanine dehydrogenase from *Streptomyces phaeochromogenes. Arg. Biol. Chem.* **47**: 2511-2520.

Kay RE, Harris DC and **Entenmann C** (1956) Quantification of ninhydrin color reaction as applied to paper chromatography. *Arch. Biochem. Biophys.* **63**: 14-25.

Kim EK and **Fitt PS** (1977) Partial purification and properties of *Halobacterium cutirubrum* L-alanine dehydrogenase. *Biochem. J.* **161**: 313-320.

Laue H and **Cook AM** (2000) Purification, properties and primary structure of alanine dehydrogenase involved in taurine metabolism in the anaerobic *Bilophila wadsworthia. Arch. of Microbio.* **174**: 162-167.

Matsui K, Tamegal Y, Miyano A. and **Kameda Y** (1977) Purification and properties of L-alanine dehydrogenase from *Bacillus natto* KMD1126. *Chem. Pharm. Bull.* **25**: 2061-2066.

McCormick NG and **Halvorson HO** (1964). Purification and properties of L-alanine dehydrogenase from vegetative cells of *Bacillus cereus.* J.

McCowen SM and **Phibbs PV** (1974) Regulation of L-alanine dehydrogenase in *Bacillus Licheniformis. J. Bacteriol.* **118**: 590-597.

Nagata S, Misono H, Nagasaki S, Esaki N, Tanaka H and **Soda K** (1989) Thermostable alanine dehydrogenase of *Bacillus sp.* DSM 730 gene cloning, Purification and characterization. *Biochimie* **71**: 559-564.

Nitta Y, Yasuda Y, Tochikubo K and **Hachisuka Y** (1974) L-Amino acid dehydrogenase in *Bacillus subtilis. J. Bacteriol.* **117**: 588-592.

O'Connor K and **Halvorson HO** (1960) Intermediate metabolis of aerobic spores. V. the purification and properties of L-alanine dehydrogenase. *Arch. Biochim. Biophys. Act.* **48**: 47-55.

Ohshima T and **Soda K** (1979) Purification and a properties of L-alanine dehydrogenase from *Bacillus sphaericus. Eur. J. Biochem.* **100**: 29-40.

Ohshima T, Sakane M, Yamazaki T and **Soda K** (1990) Thermostable alanine dehydrogenase from the thermophili *Bacillus sphaericus* DSM 462. Purification, characterization and kinetic mechanism. *Eur. J. Biochem.* **191**: 715-720.

Porumb H, Vancea D, Muresan L, Presecan E, Lascu I, Petrescu I, Porumb T, Pop R and **Barzu O** (1987) Structural and catalytic properties of L-alanine dehydrogenase from *Bacillus cereus. J. Biol. Chem.* **262**: 4610-4615.

Pierard A and **Wiame JJ** (1960) Properties of (+) alanine dehydrogenase. *Biochim. Biophys. Acta* **37**: 490-502.

Rowell P and **Stewart WDP** (1976) Alanine dehydrogenase of the N_2-Fixing blue green algae, *Anabaena cylindrical. Arch. Microbiol.* **107**: 115-124.

Sutherland EV, Cori F, Haynes R and **Olsen NS** (1949). Purification of hyperglycemic–glycogenolytic factor from insulin and from gastric mucosa. *J. Biol. Chem.* **108**: 825-837.

Vancurova I, Vancura A, Volc J, Neuzil Flieger, M, Basarova G and **Behal V** (1988) Purification and partial characterization of alanine dehydrogenase from *Streptomyces aureofaciens. Arch. Microbiol.* **150**: 438-440.

Vancura A, Vancurova I, Volc J, Jones SKT, Flieger M, Basarova G and **Behal V** (1989) L-Alanine dehydrogenase from *Streptomyces fradiae.* Purification and properties. *Eur. J. Biochem.* **179**: 221-228.

Yoshida A and **Freese E** (1964) Purification and chemical characterization of alanine dehydrogenase of *Bacillus subtilis. Biophys. Acta* **92**: 33-43.

Yooshida A and **Freese E** (1965) Enzymic properties of L-alanine dehydrogenase of *Bacillus subtilis. Biochim. Biophys. Acta* **96**: 248-262.

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