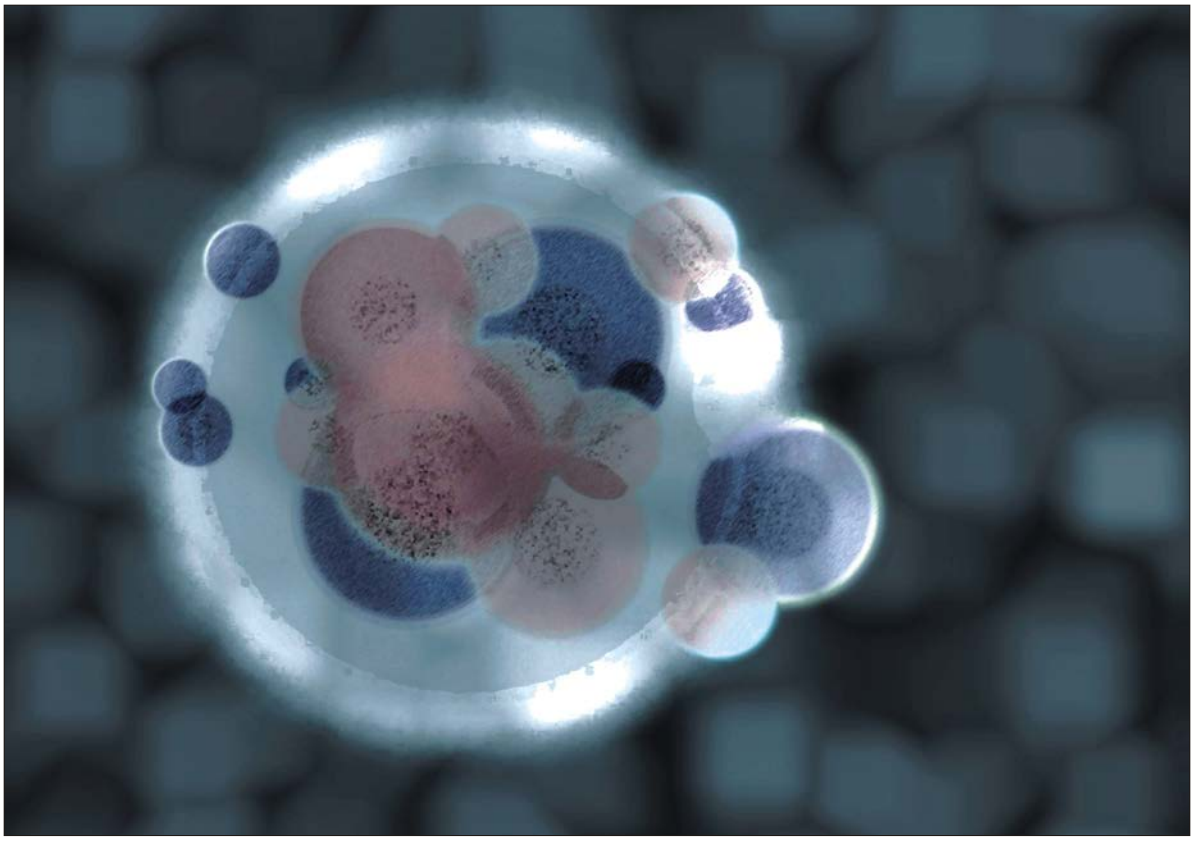

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# Occurrence of L-Serine (L-Threonine) Deaminating Enzyme in Filamentous Fungi

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**Abstract:** Extracts of nitrate-grown mats of some filamentous fungi namely, *Aspergillus terreus* DSM 826, *Aspergillus niger* NRRL3, *Aspergillus terricola* strain and *Penicillium politans* NRC-510 could deaminate each of L-serine and L-threonine leading to formation of equimolar amounts of the corresponding α-keto acid and ammonia. *Aspergillus terreus* extracts showed somewhat higher activity. This activity did not increase when the organism was grown on medium containing L-serine, instead of nitrate, as nitrogen source. The deaminating activities of *Aspergillus terreus* extracts with L-serine and L-threonine responded similarly to changes in the conditions of the reactions. Both activities were optimum, with almost the same profile, at pH 8 of 0.04-0.06 M Tris-HCl buffer and 50°C. Exposure of the extracts to 50°C or above, in absence of the substrate, revealed almost similar degrees of inactivation with the two substrates. The activity in presence of the two substrates in the same reaction mixture was much less than additive. The substrate saturation kinetics with each of L-serine and L-threonine was hyperbolic and the apparent $k_m$ values for L-serine and L-threonine was $2.7\times10^{-4}$ and $5\times10^{-4}$ M, respectively. On fractionation of extract proteins using DEAE Sephadex G A-25 column chromatography, the two activities were eluted in the same protein fractions, with the same activity peaks and with almost constant ratios. The ratio of activity with L-threonine to that with L-serine was about 0.6:1.0 in all experiments carried out.

**Key words:** L-serine deamination, L-threonine deamination, serine dehydratase of filamentous fungi, *Aspergillus terreus* serine dehydratase, L-serine (L-threonine) deamination

## INTRODUCTION

To our knowledge, neither serine nor threonine degrading enzymes has been previously recognized in the filamentous fungi. However, inspite of absence of such information, extensive studies on bacterial enzymes, capable of degrading serine and threonine, have appeared in the literature since 1938 (Gale and Stephenson, 1938). Also, yeast enzymes having this ability were reported since 1963 (Holzer *et al*., 1963).

L-serine (L-threonine) dehydratase (deaminase) is one of the enzymes that can degrade these two amino acids. This enzyme belongs to a group of enzymes known as gluconeogenic enzymes. It converts L-serine to the amphibolic intermediate, pyruvate, and converts L-threonine to α-oxobutyrate. This conversion proceeds via elimination of water from the hydroxy amino acid, forming an unstable unsaturated amino acid. This, then rearranges to an imino acid which is spontaneously hydrolyzed to the corresponding keto acid plus ammonia (Rodwell, 1993).

L-serine (L-threonine) dehydratase has been demonstrated in certain microorganisms which include: *Escherichia coli* (Pardee and Prestidge, 1955); *Pseudomonas putida* (Cohn and Phillips, 1974); *Chloroflexus aurantiacus* (Laakmann and Klemme, 1986, 1988); *Peptostreptococcus asaccharolyticus* (Hofmeister *et al.,* 1992) and *Saccharomyces cerevisiae* (Holzer *et al*., 1963; Katsunuma *et al*., 1971; Ramos and Wiame, 1982; Bornaes *et al*., 1992).

The present research demonstrates detection of L-serine and L-threonine deaminating (dehydrating) activities in extracts of some filamentous fungi and provides data indicating that one enzyme, in *Aspergillus terreus* DSM 826 extracts, is involved in deamination of both amino acids.

## MATERIALS AND METHODS

**Organisms:** The organisms used are from the culture collection of the Department of Microbial Chemistry of the National Research Centre of Egypt.

**Medium and cultivation of the organisms:** The organisms were grown and maintained on slants of sterile solid modified Czapek Dox's medium containing (g/L tap water): glucose, 30; $NaNO_3$, 2.0; $KH_2PO_4$, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5 and agar 20.

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**Preparation of fungal extracts:** The 4-5 days old mats, grown on sterile liquid modified Czapek Dox's medium at 28°C, were harvested by filtration, washed thoroughly with distilled water and blotted dry with absorbent paper. The mats were then ground with cold washed sand in a chilled mortar and extracted with cold distilled water. The slurry so obtained was centrifuged at 5500 rpm for 10 min and the supernatant was used as the crude enzyme preparation.

**Determination of *A. terreus* mycelial dry weight:** *A. terreus* DSM 826 was cultivated on Czapek Dox's agar slants at 28°C for 7 days. From these cultures, spore suspension, using sterile distilled water, was prepared to a concentration of $10^7$ to $10^8$ spore per mL. One milliliter portions of this suspension were transferred to 250 mL vol Erlenmeyer flasks each containing 50 mL sterile liquid medium. Every 24 h, three mats were separately washed and dried and their average weight (the weight of each mat) was taken for dry weight comparison.

**Chemical analysis:** The α-keto acids, pyruvate and α-ketobutyrate were measured as described by Ramos and Wiame (1982). The method used depends on reaction of these keto acids with the 2, 4-dinitrophenylhydrazine reagent, followed by addition of NaOH. The hydrazones formed were then measured at 520 nm. Ammonia was determined by its nesslerization according to Vogel (1961). Protein of the extracts was estimated by the method of Sutherland *et al.* (1949), with bovine serum albumin as standard and that of the eluted fractions was estimated by UV absorption according to the method of Layne (1957).

**Fractionation of extract proteins using DEAE-Sephadex A-25 column chromatography:** The crude extracts were heated at 60°C for 5 min and then immediately cooled in ice, after which they were centrifuged at 12000 rpm for 10 min at -20°C. The DEAE-Sephadex A-25 column (1.5×40 cm) was equilibrated with 0.02 M Tris-HCl buffer at pH 6 and then loaded with 10 mL of the supernatant of the pre-heated extracts. Elution was carried out by batchwise addition of 40 mL portions of increasing molarities (0.0-0.4 M) of solutions of NaCl in 0.02 M Tris-HCl buffer at pH 6. Fractions of 5 mL each were collected at room temperature (25°C) at a flow rate of about 23 mL $h^{-1}$. At the end of the fractionation, the activity of each fraction was tested with L-serine and L-threonine as substrates.

The assay reaction mixture of the eluted fractions contained (in 1 mL vol.): 0.4 mL of each fraction, 50 μmoles substrate and 60 μmoles Tris-HCl buffer at pH 8. The time of the reaction was 30 min and the temperature was 50°C.

Specific activity was expressed as μmoles pyruvate or α-oxobutyrate released from 50 μmoles substrate per mg protein per min at 50°C.

Appropriate control reaction mixtures, where the enzyme source or the substrate was omitted, were used as blanks through out the research.

Each experiment cited in this research was repeated at least five times and all the results were reproducible.

## RESULTS AND DISCUSSION

**Formation of equimolar amounts of α-keto acid and ammonia from L-serine and L-threonine by extracts of some filamentous fungi:** Table 1 shows ability of extracts of nitrate-grown mats of some filamentous fungi to degrade each of L-serine and L-threonine leading to formation of equimolar amounts of the corresponding α-keto acid and ammonia. pH 8 proved to be the optimum for the activities of these extracts. The extracts were obtained from mats grown for the same period (4 days) on modified Czapek Dox's medium consisting of mineral salts plus glucose (Materials and Methods). From Table 1, it appears that, with the exception of the *Fusarium oxysporium* strain, the three *Aspergilli* and the *Penicillium politans* NRC 510 strain contained a constitutively expressed L-serine and L-threonine deaminating (dehydrating) enzyme(s). Highest deaminating activity with both amino acids was almost that of the *A. terreus* extracts. Based on this result, the *A. terreus* DSM 826 was chosen for the following study.

**Relationship between specific activity of L-serine deaminating enzyme and growth phase of *Asphergillus terreus*:** Figure 1 shows that the highest specific activity of L-serine deaminating enzyme of *A. terreus* extracts was at the 5th day of growth. This day seems to represent the

Table 1: Deamination of L-serine and L-threonine by extracts of some filamentous fungi

| | Products (μmol) from | | | |
|---|---|---|---|---|
| | L-serine | | L-threonine | |
| Organism | Pymvate | Ammonia | α-oxobutyrate | Ammonia |
| *Aspergillus terreus* DSM 826 | 8.16 | 8.00 | 4.89 | 5.00 |
| *Aspergillus niger* NRRL3 | 5.53 | 5.60 | 3.20 | 3.16 |
| *Aspergillus terricola* strain | 3.27 | 3.31 | 2.00 | 2.11 |
| *Penicillium politans* NRC 510 | 7.38 | 7.25 | 4.50 | 4.51 |
| *Fusarium oxysporium* strain | 0.00 | 0.00 | 0.00 | 0.00 |

Reaction mixture contained (in 1 mL vol): L-serine or L-threonine, 50 μmol; extract protein, 2.5 mg; Tris-HCl buffer at pH 8, 80 μmol; temperature, 40°C and reaction time, 30 min

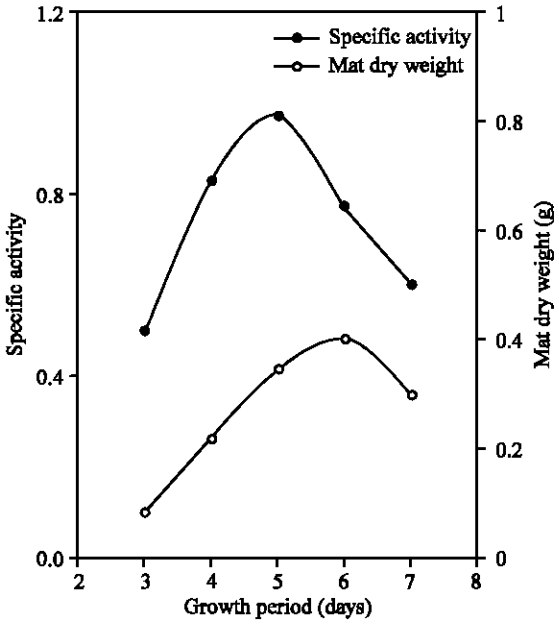


Fig. 1: Dependence of serine dehydratase specific activity on the growth phase. Reaction mixture contained (in 1 mL vol.): substrate, 20 μmol; extract protein, 2.5 mg; Tris-HCl buffer at pH 8, 80 μmol; temperature, 40°C; time of the reaction, 30 min

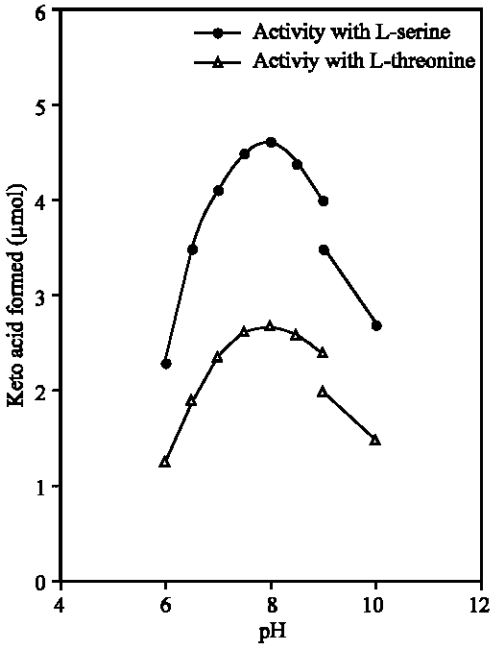


Fig. 2: *A. terreus* L-serine and L-threonine deaminating activities as function of the pH of the reaction. Reaction mixture contained (in 1 mL vol.): substrate, 20 μmol; extract protein, 2.5 mg; buffer, 80 μmol (Tris-HCl pH 6-9, carbonate bicarbonate pH 9-10); temperature, 40°C; time of the reaction, 30 min

beginning of the stationary phase of growth. This result may indicate that the physiological role of the enzyme is providing the cell with pyruvate and ammonia. Pyruvate can then directly feed into the central metabolism and ammonia can be used as a nitrogen source for biosynthetic reactions.

**Constitutivity of the *A. terreus* L-serine deaminating enzyme:** From data of Table 1, it appears that only about 16% of the amount of L-serine and about 10% of the amount of L-threonine were converted into products under the used experimental conditions. This result led to investigating whether or not this low percentage was due to inducibility of the deaminating enzyme(s). Consequently, L-serine was used as the sole nitrogen source, for the growth of the organism, instead of sodium nitrate. This replacement was made on nitrogen equivalent bases. Specific activity of the obtained extracts, with L-serine as substrate, was then compared with the analogous specific activity obtained on using extracts of nitrate grown mats. Data obtained showed that the ratio of activity of extracts of L-serine grown mats represented about 85% of that of extracts of nitrate-grown mats. This result indicates that the L-serine deaminating enzyme was not induced in mats grown with L-serine as the sole source of nitrogen.

**Pattern of dependence of L-serine and L-threonine deamination on pH of the reaction as well as on nature and molarity of the buffer:** From Fig. 2, it appears that L-serine and L-threonine deaminating activities were optimum at pH 8 and with almost similar profiles. However, the activity with L-threonine represented only about 60% of that obtained with L-serine. The data also show that, this ratio was more or less constant over the whole range of the tested pH values. Table 2 shows that Tris-HCl buffer was some what more suitable for both activities than phosphate and borate buffers. The table also shows almost similar responses of the two activities to changes of these buffer systems.

Figure 3 demonstrates almost the same profiles when the activity with L-serine and that with L-threonine were assayed at different molarities of Tris-HCl buffer at pH 8.

It is worth mentioning here that, in these experiments, formation of pyruvate from L-serine was always accompanied with formation of equimolar amounts of ammonia and formation of the keto compound (which is presumably α-oxobutyrate) from L-threonine was always accompanied with formation of equimolar amounts of ammonia. This result with L-threonine substantiates its subjection to the action of only a deaminase and not to

Table 2: Effects of the nature of the buffer on L-serine and L-threonine deamination by *A. terreus* extracts

| | Products (μmol) from L-serine | | | Products (μmol) from L-threonine | | |
|---|---|---|---|---|---|---|
| Buffer system | Pymvate | Ammonia | Relative activity (%) | α-oxobutyrate | Ammonia | Relative activity (%) |
| Tris-HCl | 3.80 | 3.65 | 100 | 2.50 | 2.55 | 100 |
| Phosphate | 2.95 | 2.92 | 78 | 1.87 | 1.82 | 75 |
| Borate | 2.70 | 2.26 | 72 | 1.71 | 1.72 | 68 |

Reaction mixture contained (in 1 mL vol): substrate, 20 μmol; extract protein, 2.5 mg; buffer, at pH 8, 80 μmol; temperature, 40°C and time, 30 min

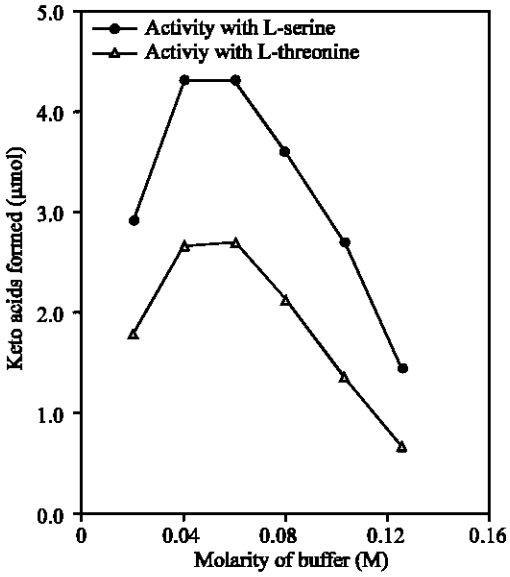


Fig. 3: Effect of different molarities of the buffer on L-serine and L-threonine deaminating activities. Reaction mixture contained (in 1 mL vol.): substrate, 20 μmol; extract protein, 2.5 mg; Tris-HCl buffer at pH 8, molarity, as indicated; temperature, 40°C and time of the reaction, 30 min

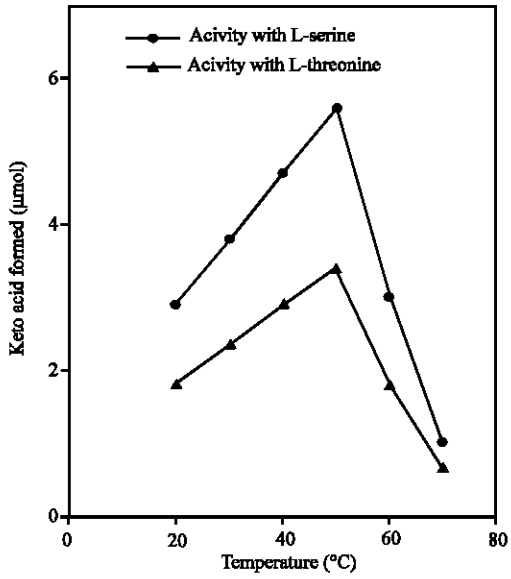


Fig. 4: Temperature-activity relationships with L-serine and L-threonine. Reaction mixture contained (in 1 mL vol.): substrate, 20 μmol; extract protein, 2.5 mg; buffer, 80 μmol Tris-HCl pH 8; temperature, as indicated and time of the reaction, 30 min

the action of threonine aldolase. The activity of threonine aldolase leads to formation of glycine plus acetaldehyde with no ammonia formation.

Data of Fig. 2, Table 2 and Fig. 3 demonstrate almost similar behaviors of the deaminating enzyme(s) with L-serine and L-threonine. These data indicate that the two amino acids were either deaminated by one enzyme or each of them was deaminated by a distinct enzyme and these two enzymes behaved similarly under the different conditions used.

**Temperature-activity relationship with L-serine and L-threonine:** Figure 4 shows that deamination of L-serine and L-threonine by *A. terreus* extracts were optimally achieved, with almost similar profiles, at 50°C. It also shows almost constant ratios of activity of L-threonine to that of L-serine (about 0.6:1.0) over the range of temperature used.

Figure 5 demonstrates heat inactivation kinetics of the two activities due to exposure of the extracts to different temperatures (in absence of the substrate), before assaying enzyme(s) activities. This figure also demonstrates that the activity with L-threonine was affected in almost similar manner to that obtained with L-serine due to heating the extracts.

From Fig. 4 and 5, it can be seen that, inspite of having its optimum activity at 50°C (Fig. 4), the deaminase (s) lost about 30% of its activity when the extracts were exposed to this same temperature in absence of the substrate (Fig. 5). This indicates that presence of substrate in the reaction mixture protected the catalytic site of the enzyme(s) from partial (30%) inactivation at 50°C.

**Incompletion of L-serine and L-threonine deaminating reactions:** When each of L-serine and L-threonine were incubated with the *A. terreus* extracts at the optimum conditions reached above, only about 30% of L-serine and about 20% of L-threonine were converted into

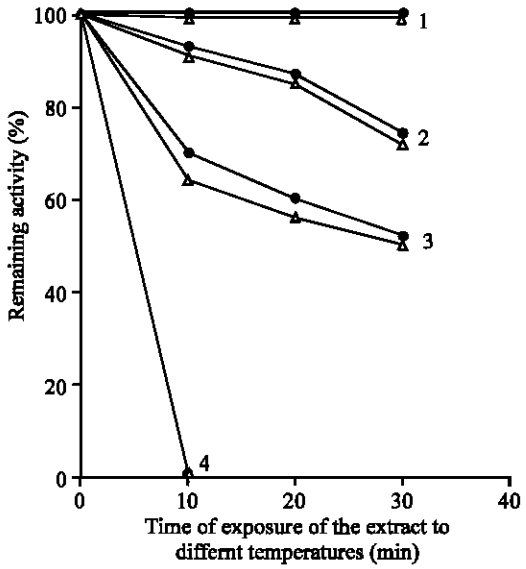


Fig. 5: Heat inactivation kinetics of the extract activities with L-serine and L-threonine. Reaction mixture contained (in 1 mL vol.): substrate, 20 μmol; extract protein, 2.5 mg; buffer, 60 μmol Tris-HCl pH 8; temperature 50°C and time of the reaction, 30 min, 1-activities of the extracts after it's exposure to 40°C in absence of the substrate for the indicated period, 2-activities after exposure to 50°C, 3-activities after exposure to 60°C, 4- activities after exposure to 70°C (solid circle represents activity with L-serine and the triangle represents activity with L-threonine)

Table 3: Effects of addition of products of the reaction on L-serine deamination

| Additions | Pyruvate formed (μmol) | Activity (%) |
|---|---|---|
| 0.0 | 3.50 | 100 |
| Pyruvate | 2.60 | 74 |
| Ammonia | 2.35 | 67 |

Reaction mixture contained (in 1 mL vol): L-serine, 20 μmol; pyruvate or ammonia, 10 μmol; extract protein, 2.5 mg; Tris-HCl buffer, at pH 8, 60 μmol; temperature, 50°C and reaction time, 30 min

Table 4: Relative activity in presence of both L-serine and L-threonine

| | Product (μmol) | |
|---|---|---|
| Substrate (μmol) | Keto acid | Ammonia |
| L-serine 20 | 3.50 | 3.45 |
| L-threonine 20 | 2.10 | 2.20 |
| L-serine+L-threonine 20+20 | 2.25 | 2.30 |

Reaction mixture contained (in 1 mL vol.): substrate, as indicated; extract protein, 2.5 mg; buffer, Tris-HCl pH 8, 60 μmol; temperature, 50°C and reaction time, 30 min

products (Fig. 6). This low percentage of conversion may represent some sort of metabolic regulation.

**Inhibition of L-serine deaminating activity by the products of the reaction:** Incompletion of the deamination

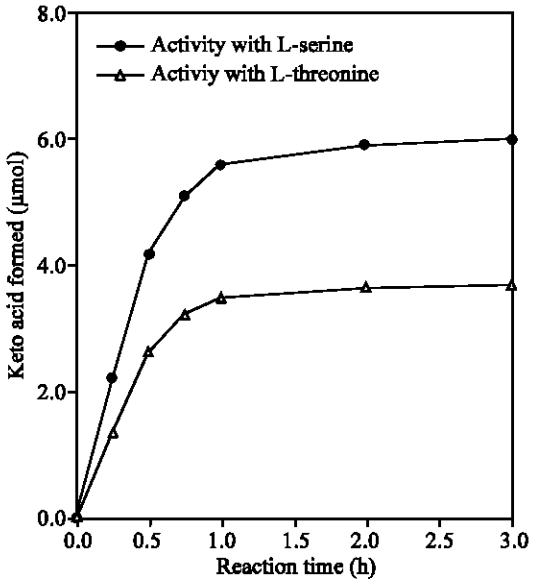


Fig. 6: Incompletion of L-serine and L-threonine deaminating reaction. Reaction mixture contained (in 1 mL vol.): substrate, 20 μmol; extract protein, 2.5 mg; buffer, 80 μmol Tris-HCl, pH 8; temperature, 50°C; time of the reaction, as indicated

reaction observed in Fig. 6 led to the suggestion that this could be probably due to product inhibition. Substantiation of this assumption can be observed from results of Table 3 which show that, addition of pyruvate or ammonia to the reaction mixture containing L-serine as substrate, at the beginning of the reaction, revealed about 30 and 34% inhibition, respectively.

**Evaluation of the sum of the two deaminating activities in presence of both L-serine and L-threonine in the same reaction mixture:** Data of Table 4 show that when both L-serine and L-threonine were used as substrates in the same reaction mixture (mixed substrate), the activity obtained was much less than additive. It represented only about 40% of the sum of the two separate activities. This indicates that both amino acids may compete for the same catalytic site.

**Protein concentration-activity relationship and substrate saturation kinetics of the deaminating enzyme(s) with L-serine and L-threonine:** Figure 7 shows proportionality of protein concentration-activity relationships and Fig. 8 demonstrates hyperbolic saturation kinetics of the deaminase(s) with the two substrates. From the data of both figures, it appears that the enzyme(s) has no cooperative multiple sites for binding each of the two

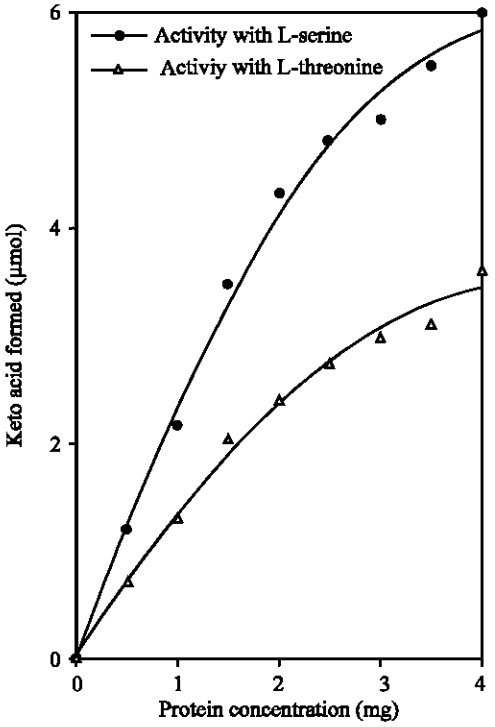


Fig. 7: Protein concentration-activity relationships. Reaction mixture contained (in 1 mL vol.): substrate, 20 µmol; extract protein, as indicated; buffer, 60 µmol Tris-HCl pH 8; temperature, 50°C and time of the reaction, 30 min

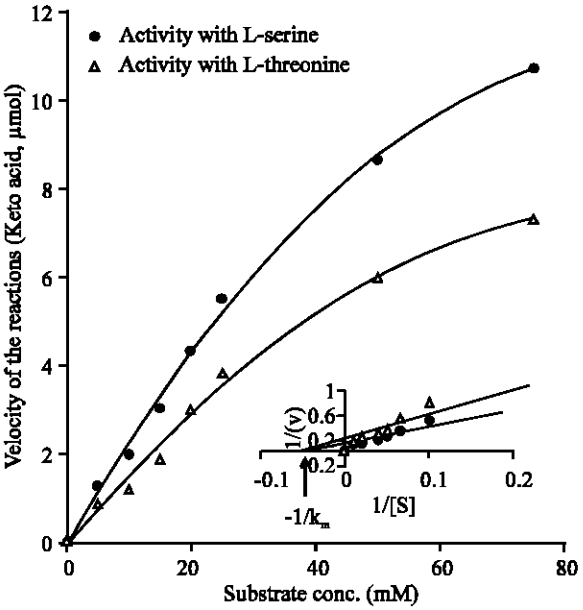


Fig. 8: Saturation kinetics of the deaminase(s) with each of the two substrates. Reaction mixture contained (in 1 mL vol.): substrates indicated, extract protein, 2.5 mg; buffer, Tris-HCl pH 8, 60 µmol; temperature, 50°C and time of the reaction, 30 min

substrates. The apparent $K_m$ values of the enzyme(s) for L-serine and L-threonine were calculated from the Lineweavers-Burk plot (Fig. 8) and were found to be $2.7\times10^{-4}$ and $5\times10^{-4}$ M, respectively.

**Elution of L-serine and L-threonine deaminating activities in the same protein fractions during fractionation of the extract proteins using DEAE Sephadex A-25 column chromatography:** Figure 9 shows elution of both L-serine and L-threonine deaminating activities in the same protein fractions. In addition, the peak of activity with L-threonine coincided with the peak of activity with L-serine. Also, almost constant ratios of L-threonine activity to that of L-serine was observed in the different fractions. These data collectively indicate that both L-serine and L-threonine deaminating activities could be assigned to one enzyme.

In conclusion, the present research provides evidence for detection of L-serine (L-threonine) deaminase (dehydratase) activities in extracts of nitrate-grown mats of *Aspergillus terreus* DSM 826. The constitutive level of this enzyme did not increase when the growth medium contained L-serine as sole source of nitrogen. Constitutivity of the analogous enzyme from the thermophile *Chloroflexus aurantiacus* has been reported (Laakmann and Klemme, 1986). However, L-serine (L-threonine) deaminase of *Sccharomyces cerevisiae* was inducible and its synthesis was induced by L-serine and L-threonine (Ramos and Wiame, 1982).

The suggested dual specificity of the *A. terreus* enzyme towards both L-serine and L-threonine was based on the given lines of evidence which all indicate that one enzyme was involved in partial conversion of each of the two amino acids to the corresponding α-keto acid plus ammonia. These lines of evidence included: similar responses of the two activities to changes in the conditions of the reactions such as, the pH, nature and molarity of the buffer, temperature, using preheated extracts, protein concentration as well as substrate concentration. Further more, the sum of the two activities in the mixed substrate experiment was much less than additive. Also, elution of the two activities in the same protein fractions, with the same peak and with almost constant ratio. All these criteria are in favor of classification of the deaminating enzyme as an L-serine (L-threonine) deaminase (dehydratase).

As for the studied kinetic properties of the *A. terreus* enzyme, this enzyme showed optimum activity at pH 8 Tris-HCL buffer and hyperbolic substrate saturation

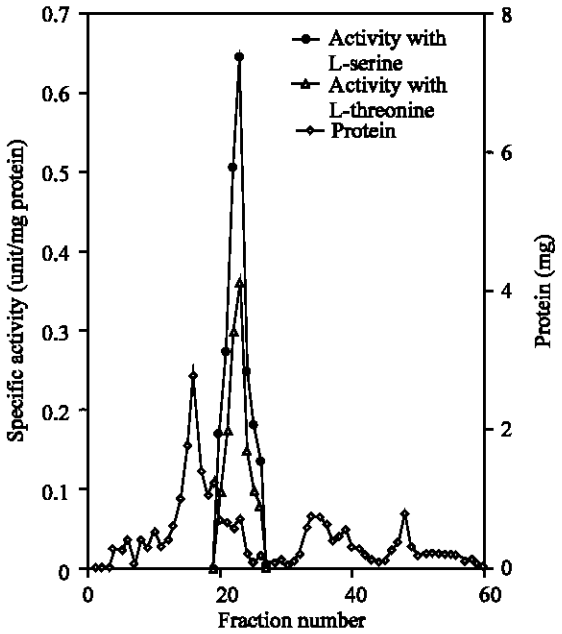


Fig. 9: Elution diagram of L-serine and L-threonine deaminating activities using DEAE-Sephadex A-25 column chromatography

kinetics as well as proportionality of the enzyme concentration-activity relationships. In these properties it resembles the enzyme from *Saccharomyces cerevisiae* (Ramos and Wiame, 1982) as well as the enzyme from *Chloroflexus aurantiacus* (Laakmann and Klemme, 1986). However, *Saccharomyces cerevisiae* enzyme had better activity in phosphate buffer than in Tris-HCl buffer. Being a typical Michaelian enzyme, indicates that this enzyme has no cooperative substrate binding sites for each of the two amino acids.

Incompletion of the two deamination reactions observed during the present work and which was suggested to be due to product inhibition, may indicate that the involved enzyme is under metabolic control and its physiological role can be regulation of the amounts of these amphibolic intermediates during dynamics of cell metabolism. Thus, whenever the amounts of L-serine and L-threonine are less needed than the amounts of their corresponding α-keto acids, deamination reaction proceeds. These reactions then are terminated whenever the required amounts of pyruvate and α-oxobutyrate are formed.

Obtaining higher activity of the deaminating enzyme with L-serine than with L-threonine, in all experiments carried out, may suggest that the cell always requires pyruvate more than α-oxobutyrate. This higher requirement for pyruvate can be attributed to its central role in cell metabolism and to its faster integration in it. Thus, pyruvate can enter the Krebs cycle via one step-reaction which is its conversion to acetyl Co-A. It can also be converted to alanine via also one (transamination) reaction. However, α-oxobutyrate has to undergo three reactions in order to be converted to succinyl Co-A which enters the Krebs cycle; and four reactions to be converted to isoleucine. Thus, for its conversion to succinyl CoA, α-oxobutyrate has to be converted to propionyl CoA which then is converted to methyl malony CoA and this in turn forms succinyl Co-A which enters the Krebs cycle (Yudkin and Offord, 1980). In case of its conversion to isoleucine, α-oxobutyrate is first converted to α-aceto-α-hydroxybutyrate, this then is converted to α, β-dihydroxy β-methyl valerate which is transformed to α-keto β-methyl valerate and this in turn gives isoleucine (Stanier *et al*., 1990).

Regarding the fate of ammonia formed as a result of these two deamination reactions, it can also be assimilated via any of three fixation reactions, one forming the amino group of glutamic acid and two others forming the amido groups of asparagine and glutamine.

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