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L-alanine-glycine and L-alanine-glutamate - Transaminases of *Rhizoctonia solani* AG 4

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Abstract. Cell free extracts of *Rhizoctonia solani* AG 4 grown on L-alanine as the sole source of nitrogen were found to contain two L -alanine transaminases activities namely L-alanine- glycine and L-alanine-glutamate transaminase. The reversibility of L-alanine-glutamate transaminase catalyzed reaction was demonstrated. Alternatively, the transamination reaction catalyzed by L-alanine-glycine transaminase was found to be irreversible . The optimum activity of the first enzyme obtained at pH 8 and 40 ^{o}C , while that of the second enzyme obtained at pH 7.5 and 20 ^{o}C. K_m values for all substrate was calculated. The activity of second enzyme was stimulated by addition of pyridoxal phosphate, whereas hydroxylamine inhibited it. The inhibitory effect of hydroxylamine was overcome by pyridoxal phosphate, while pyridoxal phosphate did not stimulate the activity of the first enzyme. Dialysis for 24 h against 0.05 M phosphate buffer at pH 8 completely inhibited the activity of the first enzyme, while the second enzyme was stable. From these result , it may be concluded that the two transamination reaction of L – alanine in *R . solani* AG 4 are catalyzed by two different transaminases .

Keywords : *Rhizoctonia solani* AG4 , L-alanine-glycine transaminase and L-alanine-glutamate transaminase.

Introduction

L-alanine participates in number of transamination reactions in fungi. There are three major transaminases involvoing L-alanine known in different fungal extract, L- alanine-glycine transaminase (L-alanine-glyoxylate transaminase EC 2.6.1.44); L-alanine-glutamate transaminase (L-alanine α-ketoglutarate transaminase EC 2.6.1.2) and L-alanine-aspartate transaminase (L-alanine-oxalacetate transaminase EC 2.6.1. 1).

Some literatures reported the presence of two active enzymes in fungal extract, while others reported the presence of only one , the investigation which demonstrated the presence of an active L-alanine-glycine transaminase and L-alanine-glutamate transaminase in fungal extract was reported by El-Awamry *et al* . ,1990 while others demonstrated the presence of an active L- alanine-glutamate and L- alanine-aspartate

transaminase in fungal extract (Quoreshi *et al*.,1995). (Aurich, 1961; Chalot *et al*., 1995; Elzainy *et al*., 1975; Ishihara *et al*., 1980 and (Piskorz-Binczycka *et al*., 1995) reported the existence of only L-alanine-glutamate transaminase in fungal extract, while (McCurdy and Cantino, 1960; Galbraith and Smith, 1969; Takada and Noguchi, 1985 and El-Awamry and El-Rahmany, 1990) reported the existence of only L-alanine--glycine tranaminase in fungual extract.

The present work aims at investigating the ability of extracts from *R . solani* to catalyze the transamination reactions of L – alanine and to study the properties of L-alanine-glycine transaminase and L-alanine-glutamate transaminase in *R. solani.*

Materials and Methods

Organism

Rhizoctonia solani AG 4 studied was obtained from Plant Prevenient Department, College of Agriculture, King Saud University, and maintained in potato dextrose agar medium.

Growth conditions

The organism was grown on glucose – Czapek – Dox liquid medium with L-alanine replacing $NaNO_3$ on nitrogen equivalent basis to induce the formation of L-alanine transaminases (El-Awamry and EL-Rahmany, 1990). Five ml aliquots of spore suspension of *R. solani* were used to inoculate 250 ml Erlenmeyer flasks, each containing 50 ml sterile medium. The inoculated flasks were incubated at 25 ^{o}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 g for10 min and the supernatant was used as the crude enzyme preparation .The crude extracts were dialysed against 200 volumes of distilled water or (0.05 M pH 7.5 phosphate buffer) for 24 hr at 4 ^{o}C.

Chemical analysis methods

The amounts of L-alanine, glutamate or glycine were 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 Bradford (1976) , with bovine serium albumin as the standard .

Identification of products

L-alaline, glutamate and glycine were identifed by paper chromatography using Whatman No.1 filter paper and two solvent systems. Solvent I consisted of n- butanol-aceton-acetic acid-water (70 : 70 : 20 : 40) (Erasser and Smith, 1976) and solvent II was water - saturated phenol (Kay *et al.*,1956). The R_f values of identified spots were identical to those of the standard samples. The keto acids, pyruvate , α - ketoglutarate and glyoxylate were identified by paper chromatography of their 2,4-dinitrophenylhydrazine derivatives (Friedemann and Haugen ,1943) , using whatman No.1 filter paper and two solvent systems. Solvent I consisted of n - butanol - ethanol - water (40 : 10 : 20) (Germano and Anderson, 1968) and solvent II consisted of n - butanol-ethanol-0.5 N NH_4OH (70:10 : 20) (Seakins *et al.*, 1976).The identification spots had the same R_f values as standard samples .

Assay of transaminase activity

L-alanine-glycine transaminase activity was assayed by following the formation of glycine, when glyoxylate and alanine were incubated with dialyzed cell-free extracts . L-alanine-glutamate transaminase activity was assayed by following the formation of glutamate. When the same extracts were incubated with L-alanine and α-ketoglutarate .The reverse reaction of L-alanine-glutamate transaminase enzyme was assayed by following L-alanine formation from pyruvate and glutamate .

All data were statistically analyzed using Person coefficient (Marija , J. and Norusis / spss Inc. , 1990) .

Results and Discussion

Coversion of L-alanine and glyoxylate into pyruvate and glycine by dialyzed cell-free extracts of *R. solani* AG4

Figure 1 demonstrates the formation of glycine when the dialyzed extracts were incubated with L-alanine and glyoxylate. It shows that the increase in the amount of glycine was accompained with a decrease in the added amount of L-alanine. An equilibrium was reached after 90 min where the amount of L-alanine disappeared was almost equivalent to that of the formed glycine .

Formation of glutamate from α-ketoglutarate and L-alanine by transamination

Figure 2 shows the formation of glutamate after incubation L-alanine and α-ketoglutarate with dialyzed cell-free extracts. It can be seen that the increase in the amount of glutamate was accompained by decrease in the mount of L-alanine. The equilibrium of reaction was reached after about 60 min at which the amount of L-alanine disappeared was almost equivalent to that of glutamate. It assumed that reaction did not proceed to completion because of the reverse reaction.

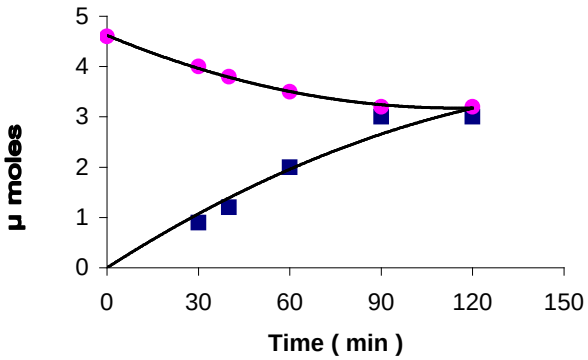

Fig. 1. Formation of glycine from L-alanine and glyoxylate by *R. solani.*

Reaction mixture contained : L-alanine, 4.6 µmoles; glyoxylaye, 6 µmoles; phosphate buffer at pH 8.0 , 80 µmoles ; dialyzed extract protein, 1.1 mg ; time, as indicated; temp., 40 °C; total volume, 1ml.

(●) Remaining alanine. (■) Formed glycine.

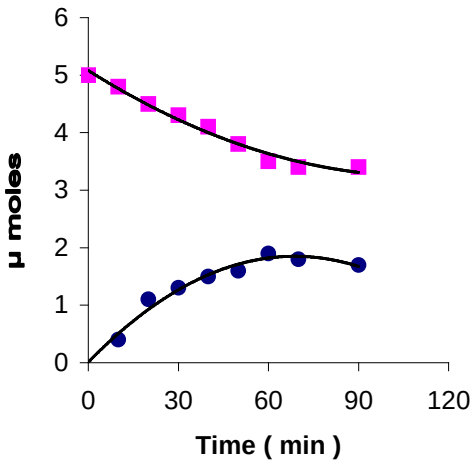

Fig. 2. Formation of glutamate from L-alanine and α-ketoglutarate by *R. Solani*.

Reaction mixture contained : L-alanine, 4.6 µmoles; α-ketoglutarate, 6 µmoles; phosphate buffer at pH 7.5, 80 µmoles; dialyzed extract protein, 1.1 mg; time, as indicated; temp., 20 °C; total volume, 1ml.

(■) Remainig alanine. (●) Formed glutamate.

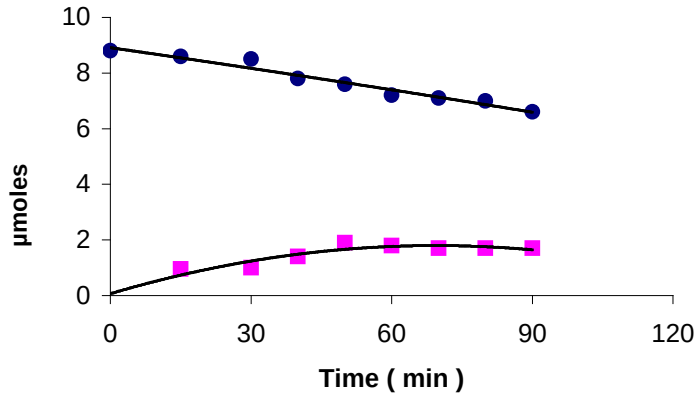

Fig. 3. Formation of L-alanine from L-glutamate and sodium pyruvate by *R. solani*.

Reaction mixture cotained: L-glutamate, 8.8 µmoles; sodium pyruvate, 5 µmoles; phosphate buffer at pH 7.5, 80 µmoles; dialyzed extract protein, 2.5 mg; time, as indicated; temp., 20 °C; total volume, 1ml.

(●) Remaining glutamate . (■) Formed L- alanine.

Demonstration of the reverse reaction of L-alanine transaminase of *R .solani* AG4

The reverse reaction of L-alanine- glutamate transaminase was demonstrated by determining the amount of L-alanine formed when the dialyzed extracts were incubated with pyruvate and glutamate. Figure 3 shows that the increase in the amount of L-alanine was associated with a decrease in the added amount of glutamate. The equilibrium of reaction was reached after 60 min. Similar results were reported for the enzyme isolated from *Neurospora crassa* (Aurich and Ngo Ke Suong, 1974), *Aspergillus awamori* IF0422 (Ishihara *et al.*, 1980), *Aspergillus niger* (Elzaniy *et al.*, 1975), *Penicillium martensii* (El-Awamry, *et al.*, 1990) and *Streptomyces nitrosporeus* (El-Rahmany *et al.*, 1995). Alternatively, the transamination reaction catalyzed by L-alanine-glyoxylate transaminase was found to be irreversible. Similar result was reported for the enzyme isolated from *Saccharomyces cerevisiae* (Takada and Noguchi, 1985). On the other hand, the reaction catalyzed by L-alanine-glycine transaminase of *Blastocladiella emersonii* (McCurdy and Cantino, 1960), *Cunninghamella elegans* (El-Awamry and El-Rahmany, 1990) and *S . nitrosporeus* (El-Rahmany *et al.*, 1995) was reported to be reversible.

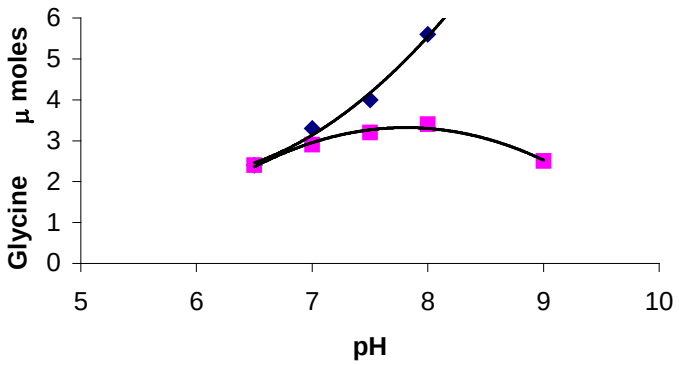

Fig . 4. Effect of pH on the formation of glycine from L-alanine and glyoxylate by *R . solani* .

Reaction mixture contained: L-alanine, 6 µmoles; glyoxylate, 6 µmoles; phosphatebuffer, pH as indicated, 80 µmoles; dialyzed extract protein 2.75 mg; time 90 min; temp., 40 °C; total volume, 1ml.

(◆) Phosphate buffer . (■) Pyrophosphate buffer .

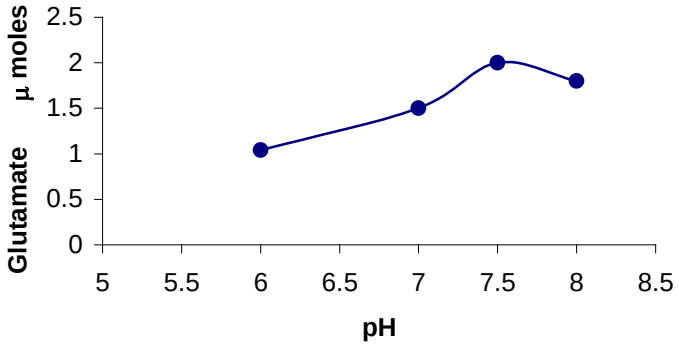

Fig . 5. Effect of pH on the formation of glutamate from L-alanine and α- ketoglutarate by *R. Solani.*

Reaction mixture contained: L-alanine, 5 µmoles; α-ketoglutarate, 5 µmoles; phosphate buffer, pH as indicated, 80 µmoles; dialyzed extract protein, 2.64 mg; time 60 min; temp., 20 °C; total volume, 1ml.

Efect of pH on L-alanine-glycine and L-alanine-glutamate transaminases

Figure 4 demonstrated that maximal enzyme activity for glycine formation from L-alanine and glyoxlate to take place at pH 8. These results were in close agreement with

those reported for L-alanine-glycine transaminase *C. elegans* (El-Awamry and El-Rahmany, 1990) and *S. nitrosporeus* (El-Rahmany *et al.*, 1995).

Figure 5 shows the effect of pH on glutamate formation from α-ketoglutarate and L-alanine. It is clear that maximal activity of L-alanine-glutamate transaminase was at pH 7.5.

These result of pH optima indicate that transamination reactions between L-alanine and either glyoxylate or α-ketoglutarate may be catalyzed by two different enzymes, since the pH profiles for the two transaminases are somewhat different. In order to clarify this point, studies were carried out to compare the temperature activity relationship and the heat inactivation kinetics of the two activities.

Temperature - activity relationship

As shown in Fig. 6 maximal enzyme activity for glycine formation from L-alanine and glyoxylate occurs at 40 ^{o}C. Similar results were demonstrated for L-alanine-glycine transaminase of *P. martensii* (El-Awamry *et al.*, 1990), *C. elegans* (El-Awamry and El-Rahmany, 1990) and *S .nitrosporeus* (El-Rahmany *et al.*, 1995). Results shown in Figure 6 indicate the ratio for the activity of glycine formation at 60 ^{o}C to that at 40 ^{o}C (optimum) is 50 %. The data indicated the relative sensitivity of enzyme to higher degree.

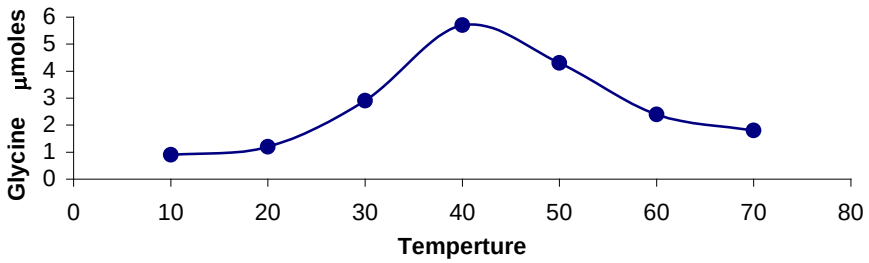

Fig. 6. Effect of temperture on the formation of glycine from L-alanine and glyoxylate by *R . solani* .

Reaction mixture contained: L-alanine, 6 µmoles; glyoxylate, 6 µmoles phosphate buffer, pH 8.0, 80 µmoles; dialyzed extract protein, 2.3 mg; time 90 min; temp., as indicated; total volume, 1ml.

Figure 7 demonstrates that the maximal activity of L-glutamate formation from α-ketoglutarate and L-alanine at 20 ^{o}C. The ratio of enzyme activity at 50 ^{o}C to that at 20 ^{o}C is 61 %. This data also suggest, that the enzyme was thermolable.

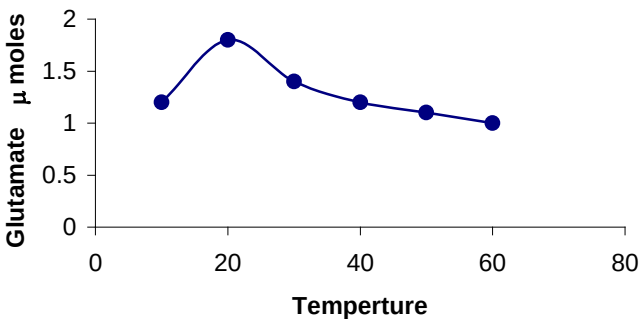

Fig. 7. Effect of temperture on the formation of glutamate from L-alanine and α -ketoglutarate by *R . solani.*

Reaction mixture contained : L-alanine , 5 µmoles ; α-ketoglutarate, 5 µmoles; phosphate buffer, at pH 7.5, 80 µmoles; dialyzed extract protein, 2.64 mg; time 60 min; temp., as indicated; total volume, 1ml.

Comparative heat inactivation kinetics of L-alanine-glycine and L-alanine-glutamate transaminases of *R.solani* AG4

Activity of L-alanine-glycine transaminase was studied as a function of incubating the dialyzed extracts at 50^{o}C for different time intervals in presence of phosphate buffer at pH 8.0, while activity of L-alanine-glutamate transaminase was studied as a function of incubating the dialyzed extracts at 30 ^{o}C for different time intervals in presence of phosphate buffer at pH 7.5. Figure 8 indicates the activity of L-alanine-glycine transaminase decreassed gradually by time during incubation at 50 ^{o}C. The data show that about 50 % and 60 % of the enzyme activity lossed after 3-20 min incubation . Where as Fig. 9 indicates the activity of L-alanine-glutamate transaminase was decreassed gradually by time during incubation at 30 ^{o}C.The data show that about 13 % and 34 % of the enzyme activity after 20-70 min incubation .

These results collectively with the preceding ones indicate that extracts of *R.solani* cotain two different alanine transaminase.

Determination of the apparent K_m values of L-alanine-glycine and L-alanine-glutamate transaminases of *R . solani* AG4

Since L-alanine-glycine transaminase of *R.solani* catalyzed this reaction :

L-alanine + glyoxylate $\longrightarrow$ pyruvate + glycine

Whereas L-alanine-glutamate trasaminase catalyzed the reversible reaction :

L-alanine + α – ketoglutarate $\rightleftharpoons$ pyruvate + glutamate

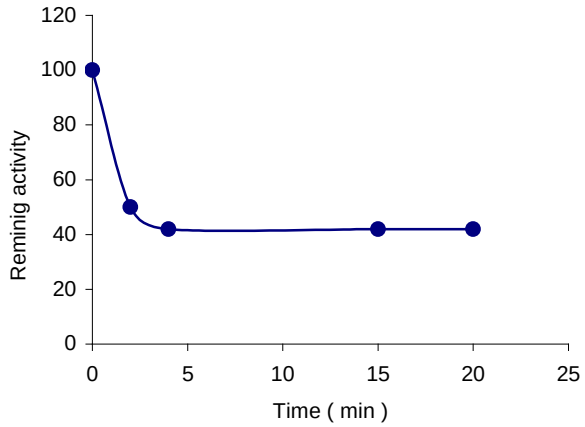

Fig. 8. Thermal stability of L-alanine glycine transaminase of *R . solani.*

Dialyzed extract (24.8) was incubated at 50 °C with an equal volume of 0. 2 M phosphate buffer at pH 8.0. Samples were withdrawn at different time intervals and assayed as usual for L-alanine-glycine transaminase.

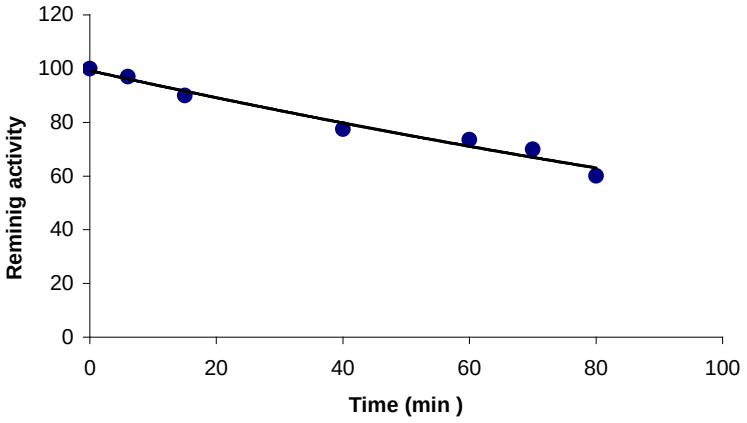

Fig . 9. Thermal stability of L- alanine - α – ketoglutarate transaminase of *R . solani* .

Dialyzed extract (26. 8) was incubated at 30°C with an equal volume of 0. 2 M phosphate buffer at pH 7.5. Samples were withdrawn at different time intervals and assayed as usual for L-alanine glutamate transaminase activity.

Therefore it was of interest to know the relative affinity of each enzyme for its substrate. This was achieved by determined the K_m values for these enzymes with each

substrate. The apparent K_m values of L-alanine-glyoxylate transaminase for L-alanine and glyoxylate and of L-alanine-α-ketoglutarate for L-alanine, α-ketoglutarate, glutamate and pyruvate were determined from Linweaver-Burk plots of the reciprocal of intial velocities and substrate concentrations. Table 1 shows , the K_m values which demonstrated that L-alanine-glycine transaminase has the highest affinity for glyoxylate than L-alanine, while the affinity of L-alanine-glutamate transaminase to its substrates was L-alanine < α-ketoglutarate < glutamate < pyruvate.

Table 1. Apparent K_m values of L-alanine-glycine and L-alanine-glutamate transaminase of *R . solani.*

Enzyme	Substrate		Km
L-alanine-glycine		L-alanine	7.7
transaminase	Glyoxylate		5.25
*L-alanine-glutamate		L-alanine	3.03
transaminase	α –ketoglutarate		2.77
	glutamate		2.5
	pyruvate		2.38

Reaction mixture contained: L-alanine (1.25-10 µmoles); ketoacid (1.25-10 µmoles); phosphate buffer at pH 8.0, 80 µmoles; dialyzed extract protein 2.7 mg; time, 45 min; temp., 20 °C; total volume 1 ml. When the concentration of one substrate was changed the other substrate was added at the saturation level.

*Assay of L-alanine-glutamate transaminase activity was carried out at pH 7.5; time, 60 min and temp., 20 °C.

Table 2. Effect of pyridoxal phosphate and hydroxylamine on L-alanine glycine and L-alanine-glutamate transaminases activity

Additions	Relative activity (%)	
	L-alanine-glycine transaminase	*L-alanine-glutamate transaminase
Control	100	100
Pyridoxal phosphate (0.1 mM)		
Pyridoxal phosphate (0.5 mM)		
Hydroxylamine (0.1 mM)		
Hydroxylamine (0.5 mM)		
Pyridoxal phosphate (0.5 mM) And Hydroxylamine (0.5 mM)		

Reaction mixture contained: L-alanine, 6 µ moles; ketoacid, 5 µ moles; phosphate buffer at pH 8. 0, 80 µ moles; dialyzed extract protein 2.7 mg; time, 90 min; temp., 40 °C; total volume 1 ml.

*Assay of L-alanine-glutamate transaminase activity was carried out at pH 7.5; time, 60 min and temp., 20 °C.

Effect of pyridoxal phosphate and hydroxylamine on L-alanine-glycine and L-alanine-glutamate transaminases activity

Table 2 shows that addition of pyridoxal phosphate at a concentration of 0.1 and 0.5 mM to the reaction mixture containing L-alanine and α-ketoglutarate resulted in stimulatory effect, while addition of pyridoxal phosphate at the same concentration to the reaction mixture containing L-alanine and glyoxylate had no effect. These data indicate that L-alanine-glutamate trasaminase require pyridoxal phosphate as a coenzyme.Comparable results were reported for L-alanine-glutamate transaminase of *N. crassa* (Aurich ,1961), *A. niger* (El-Zainy *et al.,* 1975), *P. martensii* (El-Awamry, e*t al.,* 1990) and *S. nitrosporeus* (El-Rahmany *et al.,* 1995).While pyridoxal phosphate did not stimulate the activity of L-alanine-glycine transaminase.

Addition of hydroxylamine at concentration of 0.1and concentration of 0.5 mM caused inhibition in activity of L-alanine-glutamate transaminase, while there was no effect on the enzymatic activity of L-alanine-glycine transaminase. On carrying the L-alanine-glutamate transaminase reaction in presence of hydroxylamine and pyridoxal phosphate, the inhibitory effect of hydroxylamine on enzyme was overcome by pyridoxal phosphate.

Enzyme stability

The results demonstrated that L-alanine-glutamate transaminase was relatively stable , while L-alanine-glycine transaminase was unstable when extracts of *R. solani* kept at 4 ^{o}C or – 15 ^{o}C, the first enzyme was active for more than one month while the second lost 51 % of activity after 48 hr, then the enzyme activity decreased gradually. Frequent freezing and thawing of the cell extracts had no appreciable effect on L-alanine-glutamate transaminase.

Dialysis of *R . solani* extracts for 24 hr against 0.05 M phosphate buffer at pH 7.5 or pH 8 resulted in complete loss in activity of L-alanine-glycine transaminase, while L-alanine-glutamate transaminase was stable. Alternatively dialysis of *R. solani* extracts for 24 hr against distilled water resulted in complete loss in L-alanine-glutamate transaminase, while L-alanine-glycine transaminase was stable.

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النشاط الإنزيمي ل ل – ألانين – جليسين ترانسأمينيز و ل– ألانين – جلوتامات ترانسأمينيز في فطرة الرايزوكتونيا سولاني أج ٤

سهام عبد المحسن القضيب و دلال حسين الخليفة
قسم البنات، الأقسام العلمية،كلية التربية للبنات،الرياض، المملكة العربية السعودية

(قدم للنشر في ١٤٢٢/٦/٢٣هـ ؛ قبل للنشر في ١٤٢٣/٨/٢٢هـ)

ملخص البحث.أوضحت النتائج التي تم التوصل إليها أن خلاصات خلايا فطرة الرايزوكتونيا سولاني أج٤ حفزت النقل الأميني بين ل - ألانين وكل من الجلايوكسيلات وألفا - كيتوجلوتارات لتكوين كل من الجليسين والجلوتامات وبرهنت النتائج أن التفاعل الثاني والمحفّز بإنزيم ل - ألانين - جلوتامات ترانسأمينيز قابل للإنعكاس. أما التفاعل الأول والمحفّز بإنزيم ل - ألانين - جليسين ترانسأمينيز فيعتبر تفاعلاً غير قابل للإنعكاس. وأتضح أن أعلى نشاط للإنزيم الأول كان عند الرقم الهيدروجيني ٧,٥ ، بينما كان أعلى معدل لنشاط الإنزيم الثاني عند الرقم الهيدروجيني ٨.

أما فيما يختص بدرجة الحرارة المثلى ،برهنت النتائج على أن النشاط الأمثل للإنزيم الأول قد تم الحصول عليه عند درجة حرارة ٤٠ْم وكانت الدرجة ٢٠ْم هي المثلى بالنسبة للإنزيم الثاني. وتم حساب قيمة ثابت ميكالس لكلا الإنزيمين. كما أثبتت التجارب أن نشاط أنزيم ل - ألانين - جلوتامات ترانسأمينيز يتم تحفيزه بإضافة فوسفات البيرودوكسال، بينما كان للهيدروكسيل أمين تأثير مثبط على هذا النشاط. وتم معادلة هذا التأثير المثبط للهيدروكسيل أمين بإضافة فوسفات البيرودوكسال، ويدل هذا على أن الإنزيم يتطلب فوسفات البيرودوكسال كمرافق إنزيمي ، ومن ناحية أخرى، أثبتت التجارب أن إضافة فوسفات البيرودوكسال لاتزيد حافزية إنزيم ل - ألانين - جليسين ترانسأمينيز .

وتم دراسة مدى ثبات نشاط كلا الإنزيمين حيث لوحظ أن الفصل الغشائي في المحلول المنظم فوسفات الصوديوم ٠,٠٥ مولارعند الرقم الهيدروجيني ٨ قد تسبب في فقد الإنزيم الأول لنشاطه كاملا بينما بقي نشاط الإنزيم الثاني ثابتا.

من خلال النتائج السابقة ،يمكن استنتاج أن تفاعلي النقل الأميني لـ ـ ألانين في فطرة الرايزوكتونيا
سولاني أج ٤ ـ يتم حفزهما بإنزيمي نقل أميني مختلفين.