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Purification Of Alt Enzyme From Rabbit Liver Intoxicated With Acrylamide With Special Reference To The Protective Role To Garlic

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ABSTRACT

Alanine aminotransferase (ALT) is one of special liver enzymes and used as indicator of liver damage. ALT was purified from the liver of rabbits treated with Acrylamide (ACR) with or without garlic. The results revealed that, Acrylamide is a non competitive inhibitor of ALT. In the contrary, garlic increases the affinity of ALT to its substrate. The results showed also that, the optimal temperature, pH and incubation time was 37 ºC, 7.5 and 30 minutes respectively.

Key words:

Introduction

Alanine aminotransferase (ALT) is a pyridoxal enzyme found mainly in the liver and kidney, but also in small amounts in the heart, muscle, fat, and brain. Serum aminotransferase activities have been used broadly as surrogate markers for tissue injury and disease in human and veterinary clinical settings and in safety assessment of chemicals and pharmaceuticals. Because of its relative abundance in liver, increased serum ALT activity is generally considered indicative of liver damage (Francis *et al*., 2006). Alanine aminotransferase (ALT) also known as glutamate–pyruvate transaminase (EC 2.6., 1.2 GPT), is a pyridoxal enzyme that reversibly catalyzes transamination between alanine and 2-oxoglutarate to form pyruvate and glutamate (Welch,, 1972). In human, ALT is found primarily in the liver and kidney, with lesser amounts in the heart and in skeletal muscle, fat, and brain (Jung *et al*., 1981). This enzyme is localized in both cytosol and mitochondria as isoenzymes (Kamoda *et al*., 1980). In the liver, ALT participates in the synthesis of glucose from pyruvate in a reverse ALT reaction, the so-called alanine–glucose cycle (Felig, 1973). The precursor, pyruvate, is produced during intensive exercise when skeletal muscles operate anaerobically, producing pyruvate from glycolysis (De Rosa and Swick, 1975). ALT is also believed to contribute to the synthesis of glutamate, which is an important neurotransmitter in the brain (Peng, *et al*., 1991). Acrylamide (ACR) is a water soluble vinyl monomer used extensively in the production of polyacrylamide which have several uses (e.g. water purification, cosmetics and as a soil stabilizer) and in the molecular laboratories for the production of polyacrylamide gel electrophoresis (PAGE) (Gold and Schaumburg, 2000). Recently, ACR has been found as a contaminant in certain potato and grain based food that cooked at high temperature (Tareke *et al*., 2002 and Tareke etal., 2000).

Garlic (Allium sativum L) is one of the most herbal medicines. It has been used since ancient time by many cultures as a medical folk remedy for a variety of illness. It used as antibacterial, antithrombotic, antineoplastic, antipyretic, antifungal, larvacidal, antiviral, antispasmodic, diuretic and against chest pains and intestinal disorders (Dubick, 1986; Guyonnet *et al*., 2000 and Lohani *et al*., 2003).

Material And Methods

Chemicals:

Acrylamide was obtained from Sigma Chemical Co. It was given in drinking water at a dose rate of 0.05% (Hedaya *et al*., 1999). Garlic was purchased from commercial sources. The outer husks of garlic were peeled off before slicing the cloves by a knife. The sliced garlic was dried in an oven at 60 °C up to dryness and moisture content of fresh garlic was determined to be around 72%. The dried garlic was ground and kept in dry glass package until used (EL-Afify, 1997). Garlic given in diet at a dose rate of 1% (Myung-Jakwan *et al*., 2003).

Animals:

The present study was carried out on 40 male rabbits (3 months old, weighted from 1000-1500g). They were given diet and water *adlibitum*. All rabbits were housed in metal cages and received diet for two weeks

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before the start of experiment for acclimatization and to ensure the normal growth and behaviour. All animals remained healthy throughout the experimental period (one month).

The animals were divided into 4 groups.

Group I : 10 rabbits were fed basal diet and served as control.

Group II: 10 rabbits were kept on basal diet and water contains acrylamide at concentration of 0.05%.

Group III: 10 rabbits were given basal diet containing 10 g of garlic powder per kg diet (1%).

Group IV: 10 rabbets were fed on basal diet containing 10 g of garlic powder per kg diet (1%) and water contains acrylamide at concentration of 0.05%.

Purification of alanine aminotransferase (ALT):

Alanine aminotransferase was purified according to (Vedavathi *et al*., 2006)

Tissue preparation:

About 100 g of the liver tissues were collected and placed immediately in ice-cold physiological saline. The liver tissues were perfused with ice-cold saline and homogenized in a sorvall-omni mixer at full speed for 30 sec in five volumes of 0.25 M sucrose containing 0.01 M β-mercaptoethanol. The homogenate after words was centrifuged at 10,000 xg for 30 min and the resulting supernatant was taken. Afterwards, the supernatant was heated at 55°C for 5 min. Then the pH was lowered to 5.0 by the addition of 1 M acetic acid with constant stirring for denaturation of other unrequired protein. The denatured protein was removed by centrifugation at 10,000 xg for 30 min. In addition, the clear supernatant was subjected to 48% ammonium sulfate precipitation (275 mg of ammonium sulfate per ml of supernatant were added slowly with gentle stirring). After a period (overnight) the precipitate was collected by centrifugation at 10,000 xg for 30 min and dissolved in a small volume of 0.01 M potassium phosphate buffer, pH 5.7, containing 0.01 M β-mercaptoethanol. The solution was dialyzed against the same buffer (0.01 M potassium phosphate buffer, pH 5.7, containing 0.01 M β-mercaptoethanol) overnight. Buffer is changed several times and the end point of complete dialysis was reached when the dialyzate failed to give precipitate with an acid solution of barium chloride.

Determination of protein content and enzyme activity:

The protein content was determined by modified lowry´s method according to (Ohnishi and Bar, 1978). Also, the enzyme activity was determined according to the method described by (Reitman and Frankel, 1957) in each step of purification.

Gel filtration by sephadex G120:

Preparation of sephadex G120:

-Ten gram of sephadex G 120 were swelled in excess of 0.01 M potassium phosphate buffer, pH 7.4, containing 0.01 M β-mercaptoethanol.

- After degassing, the swollen sephadex G120 was allowed to settle and the excess buffer was removed.
- The gel was poured into the column (1.5 x 80 cm) down the glass rod to avoid air bubbles formation.
- The column was equilibrated with 0.01 M potassium phosphate buffer, pH 7.4, containing 0.01 M β-mercaptoethanol at a flow rate of 25 ml / hr.

Sample application:

- The eluent (buffer) above the gel surface was allowed to drain and the dialyzed ammonium sulfate fraction was carefully layered on top of the bed. The column outlet was then opened till the sample was drained into the bed and the protein was eluted with 0.01 M potassium phosphate buffer, pH 7.4, containing 0.01 M β-mercaptoethanol at flow rate of 3 ml / fraction using fraction collector LKB-ultorase. Each fraction was monitored for protein by reading the absorbance at 280 nm and also for alanine aminotransferase activity. The most active fractions were collected, concentrated and stored at 4°C.

-The protein content and the enzyme activity were determined in each step of purification for check on the efficiency of purification technique.

- All purification steps were repeated with group II, III and IV.

Estimation of protein content in different stages of purification:

The protein content was determined by the modified lowry´s method, which can be summarized as follows (Ohnishi and Bar, 1978).

Reagent:

1- Biuret reagent: 9 g Na-K tartrate in 500 ml 0.2 N NaOH + 3 g $CuSO_4$. $5H_2O$+ 5 g KI, the volume was made up to 1000 ml with 0.2 N Na OH.
2- 2.3% $NaCO_3$.
3- 2 N folin-Ciocalteu phenol reagent.
4-Standard: protein standard (6 g/dl).

Procedure:

Onto 1 ml of the solution (crude; supernatant; heated supernatant; ammonium sulfate fraction and concentrated fractions) 4 ml diluted Biuret reagent were added. After standing for 10 min at room temperature 0.125ml of 2 N folin- Ciocalteu phenol reagent was added. Thereafter the developed blue colour was measured at 650 nm and a standard curve was prepared with crystalline bovine albumin.

Determination of enzyme kinetics from group (I):

The effect of enzyme concentration:

Different enzyme concentration 5, 10, 15, 20, 25, 30, 40 and 50 µl of sample were added to 100 µl of solution 1 (phosphate buffer, pH 7.4, DL-alanine and oxoglutarate), mixed and incubated in water bath at 37°C for exactly 30 min then 100 µl of solution 2 (2,4-dinitrophenyl hydrazine) were added and mixed, let to stand at 20 to 25°C for exactly 20 min. then added 1 ml of sodium hydroxide solution 0.4 N. was added and mixed, Poured into cuvettes and after 5 minutes, read the absorbance of the sample against the reagent blank at 546 nm.

The effect of substrate concentration:

Different substrate concentration (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mmol of DL-alanine in phosphate buffer pH 7.4) were added to 20 µl of sample, mixed and incubated in water bath at 37°C for exactly 30 min then added 100 µl of solution 2 (2,4-dinitrophenyl hydrazine) were added and mixed, let to stand at 20 to 25°C for exactly 20 min. then 1 ml of sodium hydroxide solution 0.4 N. was added and mixed, Pour into cuvettes and after 5 minutes, read the absorbance of the sample against the reagent blank at 546 nm.

The effect of temperature on enzyme activity:

The enzyme assay was carried out using 20µl of sample were added to 100 µl of solution 1 (phosphate buffer, pH 7.4, DL-alanine and oxoglutarate), mixed and incubated in water bath at different temperature values (15, 25, 30, 37, 45, 50, 55, and 60°C) for exactly 30 min then added 100 µl of solution 2 (2,4-dinitrophenyl hydrazine) mixed, let to stand at 20 to 25°C for exactly 20 min. then added 1 ml of sodium hydroxide solution 0.4 N. mixed, Pour into cuvettes and after 5 minutes, read the absorbance of the sample against the reagent blank at 546 nm.

The effect of pH on enzyme activity:

The enzyme assay was carried out using 20µl of sample were added to 100 µl of solution 1 (phosphate buffer, pH of a wider range of (4 to11), DL-alanine and oxoglutarate), mixed and incubated in water bath at 37°C for exactly 30 min then added 100 µl of solution 2 (2,4-dinitrophenyl hydrazine) mixed, let to stand at 20 °C to 25°C for exactly 20 min. then added 1 ml of sodium hydroxide solution 0.4 N. mix. Pour into cuvettes and after 5 minutes, read the absorbance of the sample against the reagent blank at 546 nm.

Time course on ALT activity:

The enzyme assay was carried out using 20µl of sample were added to 100 µl of solution 1 (phosphate buffer, pH 7.4, DL-alanine and oxoglutarate), mixed and incubated in water bath at 37°C for a different times (10, 15, 20, 25, 30, 40, 50 and 60 min) then added 100 µl of solution 2 (2,4-dinitrophenyl hydrazine) mixed, let

to stand at 20 to 25°C for exactly 20 min. then added 1 ml of sodium hydroxide solution 0.4 N. mix. Pour into cuvettes and after 5 minutes, read the absorbance of the sample against the reagent blank at 546 nm.

Similarly, the method of purification of ALT enzyme and factors affecting on the enzyme activity (enzyme conc, substrate conc, temperature, pH and time) were done in other groups (II, III and IV).

Results:

ALT was purified from rabbits liver in the four groups used in the experiment (I, II, III and IV). The purification was performed firstly by addition of 48% ammonium sulfate to the crude enzyme preparation followed by sephadex G_{120} gel filtration column chromatography. The purification profile of ALT enzyme in group I indicated that, the specific activity was increased to $24x10^{-5}$ U/mg protein after ammonium sulfate precipitation. The dialyzate after ammonium sulfate precipitation was loaded on sephadex G_{120} gel filtration column chromatography and eluted with 0.01 M potassium phosphate buffer pH 7.4 containing 0.01 M mercaptoethanol. The total protein decreased to 70 mg while specific activity increased to$15x10^{-4}$ with15.31 purification factor fold and 25.3 yield.

The purification profile of ALT enzyme in group II showed that, different purification steps decreased the total protein and increased the specific activity, purification fold and yield. Similarly in Table 1, 2 and 3. The total protein was decreased and specific activity, purification fold and yield % was increased from purification step to another.

The results indicated in Table 3. Showed that in sephadex G_{120} column chromatography, the specific activity, purification factor fold and yield % were (15×10^{-4}, 15.31, 25.3), (13×10^{-4}, 15.1, 23.66), (14×10^{-4}, 15.53, 24.04) and (14×10^{-4}, 15.5, 24.3) in group I, II, III and IV respectively.

The yield percentage indicated that the purification steps preserved most of the enzyme activity and the purification factor indicating the efficiency of sephadex G_{120} in purifying ALT enzyme.

The study was extended to examine the factors affecting ALT activity in the four groups. The result showed that, the ALT enzyme was progressively increased as the enzyme concentration increased in all groups. As substrate concentration increased, the initial reaction rate of activities increased in four groups. In addition, the ALT reaction was subjected to Lineweaver-Burk analysis to determine the Km and Vmax of the four groups. The data illustrated in Fig. 2 showed that, the acrylamide (group II) is a non competitive inhibitor for ALT where Vmax was decreased to 0.0033 u/ml/min and the Km value was constant at 45 mM. In contrast, Fig. 2 revealed that garlic powder (group III) decreased the Km value to 30 mM and increased the Vmax to 0.0038 u/ml/min. Therepy garlic increased the affinity of ALT for its substrate. On testing the effect of garlic powder and acrylamide together at a concentrations of 1% and 0.05% respectively (group IV). It was found that the addition of garlic powder to acrylamide caused no effect on Vmax value (0.0033 u/ml/min) while the Km decreased (33 mM) in compared with group II. Fig. 2.

The effect of temperature on the pure ALT in four groups were tested. The results illustrated in Fig. 3 and Table 4 showed that the maximum ALT activity was noticed at 37 ºC for the four groups. The preparation of purified ALT enzyme in four groups permitted a study of the effect of pH on ALT activity over a wider pH range (pH 4 to pH11). The results illustrated in Fig. 4and Table 4 proved that the purified enzyme from the four groups had a maximum activity at pH (7.5).

On testing the effect of different incubation times (5, 10, 15, 20, 25, 30, 40, 50 and 60 minutes) on the activity of ALT. The results revealed that, the ALT activity from four groups increased as the incubation time increased reaching a maximum activity at 30 minutes after which the enzyme activity decrease. The decrease in ALT activity with time may be due to drop in substrate concentration, increasing potentiality of the reverse reactions and even progressive inactivation of the enzyme protein as showed in Table 4.

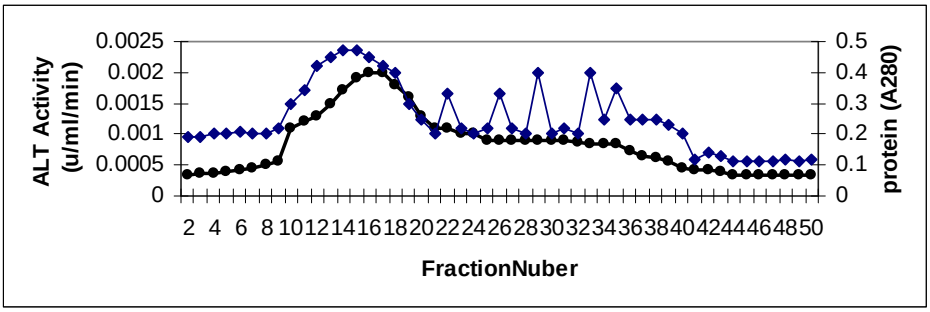

Fig. 1: Gel filtration sephadex G_{120}, chromatography of alanine aminotransferase in group I. the dialyzed ammonium sulfate was chromatographed on sephadex G_{120} column. The total protein was monitored at 280 nm. The fractions were assayed for ALT activity.

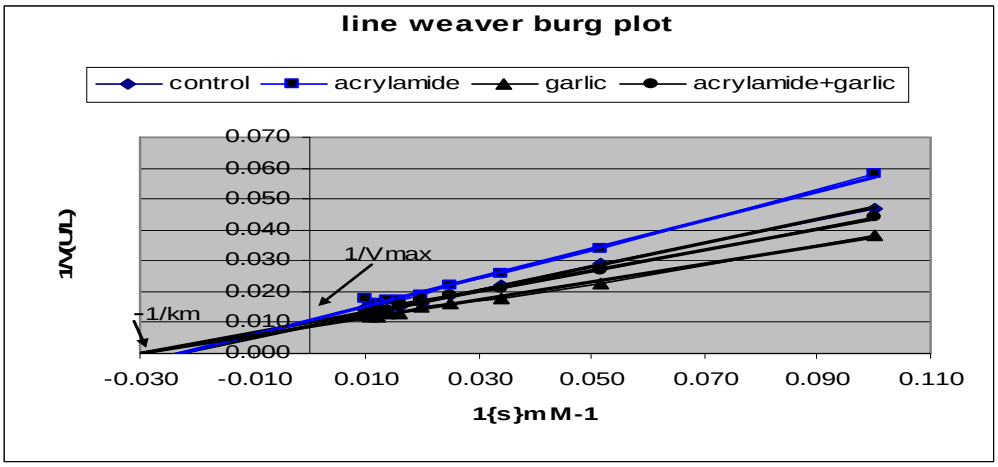

Fig. 2: Line weaver-Burk plot of ALT activity versus L-alanine (four groups).

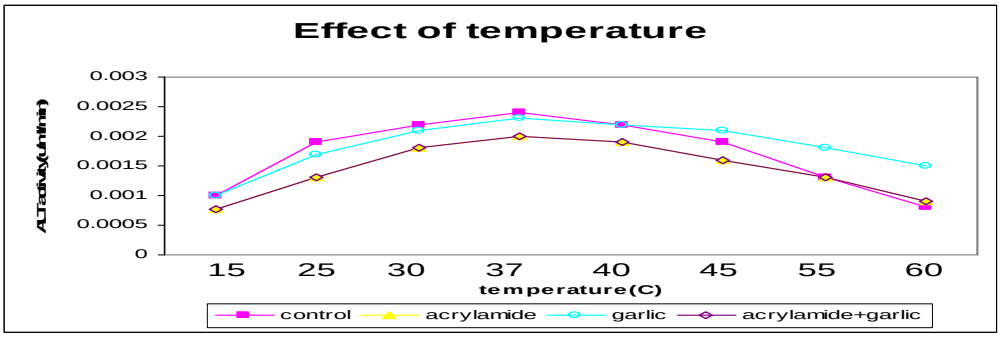

Fig. 3: Effect of incubation temperature on ALT activity (four groups).

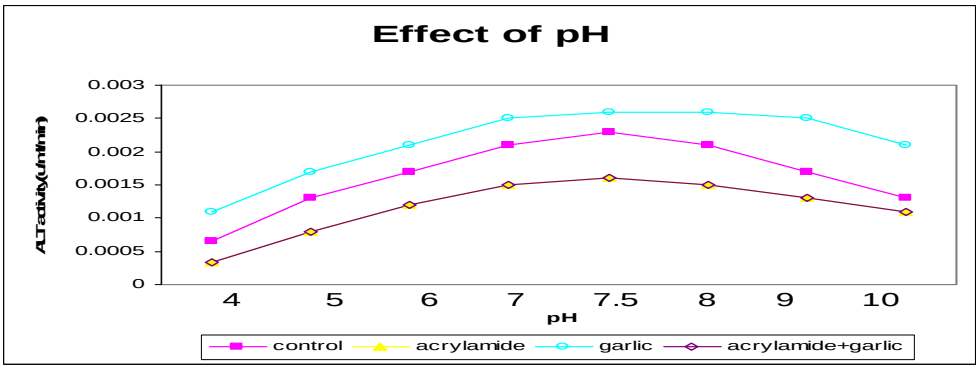

Fig. 4: Effect of pH on ALT activity (four groups).

Table 1: Crude extract of ALT enzyme in different groups

Groups	Total activity (u/ml/min)	Total protein(mg)	Specific activity (u/ml/min/mg protein)x 10^{-4}	Purification fold	Yield (%)
Group I	0.442	4498	0.98	0.00	100
Group II	0.338	3926	0.86	0.00	100
Group III	0.416	4498	0.92	0.00	100
Group IV	0.390	4316	0.90	0.00	100

Table 2: Partial purification of ALT enzyme by ammonium sulfate in different groups

Groups	Total activity (u/ml/min)	Total protein(mg)	Specific activity(u/ml/min/mg protein)x 10^{-4}	Purification fold	Yield (%)
Group I	0.169	689	2.4	2.50	38.2
Group II	0.136	435.5	3.1	3.60	40.4
Group III	0.143	604.5	2.4	2.61	34.4
Group IV	0.143	500.5	2.8	3.17	36.7

Table 3: Purification by sephadex G_{120} gel filtration column in different groups

Groups	Total activity (u/ml/min)	Total protein(mg)	Specific activity(u/ml/min/mg protein)x 10^{-4}	Purification fold	Yield (%)
Group I	0.112	73.5	15	15.31	25.3
Group II	0.080	59.5	13	15.10	23.66
Group III	0.100	70	14	15.53	24.04
Group IV	0.095	68.25	14	15.50	24.3

Table 4: Kinetic parameters of ALT enzyme.

Groups	K_m (mM)	V_{max} (u/ml/min) x 10^{-4}	Temperature (ºC)	pH optimum	Time (min)
Group I	45	38	37 ºC	7.5	30
Group II	45	33	37 ºC	7.5	30
Group III	30	38	37 ºC	7.5	30
Group IV	33	33	37 ºC	7.5	30

Discussion:

The purification profile of ALT enzyme showed that, different purification steps decreased the total protein and increased the specific activity, purification fold and yield. Similarly in Table (1, 2 and 3). The total protein was decreased and specific activity, purification fold and yield % was increased from purification step to another. Also, the results indicated in Table (3). Showed that in sephadex G_{120} column chromatography, the specific activity, purification factor fold and yield % were (15 x 10^{-4}, 15.31, 25.3), (13 x 10^{-4}, 15.1, 23.66), (14 x 10^{-4}, 15.53, 24.04) and (14 x 10^{-4}, 15.5, 24.3) in group I, II, III and IV respectively.

The yield percentage indicated that the purification steps preserved most of the enzyme activity and the purification factor indicating the efficiency of sephadex G_{120} in purifying ALT enzyme.

These results are in agreement with that obtained previously (Anand *et al*., 2004) who purified the ALT enzyme from the liver of two fresh water fish (clarias batrachus and labeo rahita) by heat extraction, ammonium sulfate, DEAE Sephacel column, $Ca(PO_4)_2$ Gel column and Acetone precipitation they found that the ALT was purified 202, 120 fold with 38, 27% yield in C.batrachus and L.rohita, respectively. Moreover, the ALT was purified from B. subtilis by ammonium sulfate, DEAE sephadex and hydroxy apatite with 90 fold and 13% yield (Martinez-Carrion and Terry, 1965). Furthermore ALT purified from Vibrio fluvialis by ammonium sulfate, Q-Sepharose FF and Butyl-Sepharose FF, the ALT was purified 49 fold with 42% yield (Shin *et al*., 2003). Also, ALT from Anaerobic Barley Root Tissue was purified using Gel filtration, Q-Sepharose, Mono-P and Mono-Q. the enzyme was obtained with 349 fold and 3% yield (Allen and Douglas, 1992). The present findings are also in agreement with those obtained by others (Blanca *et al*., 1991) who purified ALT from

Chlamydomonas reinhardtii by using heat extraction, ammonium sulfate, Anion-exchange chromatography and hydroxy apatite. They found that the ALT was purified 157 fold with 12% yield. In addition, ALT was purified from canine liver by ammonium sulfate, Gel filtration and the enzyme was purified with 229.81 fold and 60.4% yield (Francis *et al*., 2006). The present results also agree with previous (Vedavathi *et al*., 2006) who purified ALT from fasted rat liver using heat and acid treatment, ammonium sulfate, Sephadex G-150, DEAE-Sephadex, hydroxyapatite and Electroelution. They found that the ALT enzyme was purified 275.14 fold with 13.7% yield. Simillary, the ALT enzyme purified from Trypanosoma cruzi using Phenyl-Sepharose CL, Red-120 Sepharose and Sephacryl S-200 column, the enzyme was purified with 180 fold and 8% yield (Cecilia *et al*., 1996).

The study was extended to examine the factors affecting ALT activity in the four groups. The result showed that, the ALT enzyme was progressively increased as the enzyme concentration increased in all groups. These results come in agreement with others (Puppo and Blassco, 1995) who purified ALT from Ruditapes philippinarum and reported that, the alanine conversion rate increased linearly with the amount of enzyme added to the reaction mixture. As substrate concentration increased, the initial reaction rate of activities increased in four groups. In addition, the ALT reaction was subjected to Lineweaver-Burk analysis to determine the Km and Vmax of the four groups. The data illustrated in Fig. (2) showed that, the acrylamide (group II) is a non competitive inhibitor for ALT where Vmax was decreased to 0.0033 u/ml/min and the Km value was constant at 45 mM.

In contrast, Fig. (2) revealed that garlic powder (group III) decreased the Km value to 30 mM and increased the Vmax to 0.0038 u/ml/min. Therepy garlic increased the affinity of ALT for its substrate.

On testing the effect of garlic powder and acrylamide together at a concentrations of 1% and 0.05% respectively (group IV). It was found that the addition of garlic powder to acrylamide caused no effect on Vmax value (0.0033 u/ml/min) while the Km decreased (33 mM) in compared with group II. Fig. (2).

The effect of temperature on the pure ALT in four groups were tested. The results illustrated in Fig. (3) and Table (4) showed that the maximum ALT activity was noticed at 37 ºC for the four groups.

These results are in accordance with that obtained by previous (Anand *et al*., 2004) who purified the ALT enzyme from the liver of two fresh water fish (clarias batrachus and labeo rahita) and found that the enzyme show optimum activity at 37 ºC. Simillary, ALT purified from Vibrio fluvialis the optimal temperature for ALT was 37 ºC (Shin *et al*., 2003). Furthermore, ALT purified from Ruditapes philippinarum the enzyme has a maximal activity at 40 °C (Puppo and Blassco, (1995). On the other hand, these results disagree with those obtained by others (Donald *et al*., 2000) who showed that, ALT purified from pyrococcus furiosus had a maximal activity at 95 ºC. Also, the ALT enzyme purified from fasted rat liver had maximal activity at 55 °C (Vedavathi *et al*., 2006). Moreover, the ALT purified from Chlamydomonas reinhardtii had optimal activity at 50 °C (Blanca *et al*., 1991). The preparation of purified ALT enzyme in four groups permitted a study of the effect of pH on ALT activity over a wider pH range (pH 4 to pH11).

The results illustrated in Fig. (4) and Table (4) proved that the purified enzyme from the four groups had a maximum activity at pH (7.5).

These results are come in accordance with previous (Blanca *et al*., 1991) who revealed that, the ALT purified from Chlamydomonas reinhardtii had optimal activity at pH (7.3). Simillary, the present findings agree with those obtained by others (Puppo and Blassco, (1995) who purified ALT from Ruditapes philippinarum and reported that, the enzyme has a maximal activity at pH (7.0). Moreover, the ALT enzyme purified from fasted rat liver had maximal activity at pH (8.0). (Vedavathi *et al*., 2006), Furthermore, the ALT purified from Trypanosoma cruzi had a maximal activity at pH (7.0) (Cecilia *et al*., 1996). Moreover, ALT purified from pyrococcus furiosus had a maximal activity at pH (6.5 to 7.8) (Donald *et al*., 2000). Also, ALT purified from Anaerobic Barley Root Tissue, the enzyme had a high activity at pH (8) (Allen and Douglas, 1992). The ALT enzyme purified from the liver of two fresh water fish (clarias batrachus and labeo rahita) show optimum activity at pH (7.5) (Anand *et al*., 2004). On the other hand, the present findings disagree with those obtained by others (Shin *et al*., 2003) who purified ALT from Vibrio fluvialis and reported that, the optimal pH was (9.2).

On testing the effect of different incubation times (5, 10, 15, 20, 25, 30, 40, 50 and 60 minutes) on the activity of ALT. The results revealed that, the ALT activity from four groups increased as the incubation time increased reaching a maximum activity at 30 minutes after which the enzyme activity decrease. The decrease in ALT activity with time may be due to drop in substrate concentration, increasing potentiality of the reverse reactions and even progressive inactivation of the enzyme protein as showed in Table (4).

From these obtained results, we can concluded that ACR is a non competitive inhibitor for ALT, while the garlic alone was found to increase the affinity of alt to its substrate. Furthermore, it evident from the present work, that the optimal temperature of the enzyme, PH and incubation time was 37°C,7.5 and 30 minutes respectively.

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