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PARTIAL PURIFICATION OF ALANINE AMINOTRANSAMINASE (ALT) ENZYME IN THE SERA OF GALLSTONE PATIENTS

Manar Mohammed Jassim

Noor Qasim Ahmed

Arwa Saud Alwan

Abstract:

Alanine Amino Transaminase (ALT, GPT) is the plasma enzyme most frequently tested as a sign of damaged liver cells. Even though there is no discernible failure of function, an increase in plasma transaminases activity is a crucial sign of damage to cytoplasmic or mitochondrial membranes. Hepatic ability to synthesize and secrete substances is vast; only severe and typically chronic liver disease can clearly impair prothrombin and albumin synthesis.

This study included fifty-five patients with gallstones, their ages ranged between (71-17) years, and forty apparently healthy persons aged between (71-17) years were used as a control group. For the period from the beginning of September to January 2022 in Salah El-Din General Hospital

We estimated the activity of the enzyme alanine aminotransferase in the first step, where it was isolated, purified and determined its molecular weight, as well as a kinetic study was conducted for the enzyme, where different methods were used to work and purify this now in the blood by several steps that included precipitation using ammonium sulfate for 50% saturation, which showed a clear increase in purification Molecular enzyme (3.228IU/mg) to (6.664IU/mg) after the first purification step.

In the second stage, ion exchange chromatography using DEAE-Cellulose was used in this method to separate the purified enzyme from the sera of people with gallstones, which showed one band, while the molecular weight of the enzyme purified by PAGE, which showed one dense band along the length of the gel was 35 kilo dalton

As for the kinetic study of the purified enzyme, it included the optimum conditions for the reaction, which included the concentration of the enzyme 2 mM, and Km= 0.89, pH = 8, and the temperature of 37 °C. incubation time 30 min

INTRODICTION:

The gallbladder contains one or more stones, which can range in size and shape. Depending on the amount of cholesterol in the gallstone, which can range in size from a pinhead to a golf ball, we can categorize them. Types of gallstones (a) cholesterol monohydrate, (b) cholesterol gallstone, (c) large cholesterol gallstone (d) Mixed cholesterol gallstones, and (e) Pigment stones[1,2]. Calcium, bile pigments, and cholesterol make up the mixed stone. These mixed stones generated from compounds rely on the solubility level.

Gallstone complications.The bile ducts that carry bile from the gallbladder to the liver may become blocked as a result of gallstones. The flow of digestive enzymes from the pancreas can potentially

be obstructed by gallstones. Clinical symptoms such as nausea, vomiting, and stomach pain are brought on by blocked bile ducts. There is still a blockage in the bile duct, and jaundice may be developing. If there are any gallbladder stones[3-5]

Aspartate aminotransferase and alanine aminotransferase levels rise in response to acute or chronic liver inflammation.

glutamic pyruvic transaminase (GPT) is other names for alanine amino transferase (ALT), [E.C.2.6.1.2].Patients with liver and biliary tract diseases typically get a liver function test to measure their condition. Instead of examining a particular function, the increased serum enzyme activity is used to identify the abnormal release of specific enzymes from injured liver cells[6,8]. increased blood levels of aminotransferases signify harm to cells that contain large amounts of these enzymes; A gallstone that becomes stuck in the bile duct and produces bile congestion can also cause damage. These conditions include chronic diseases like cancer, sudden trauma like a heart attack, and chronic diseases like cancer. The most likely explanation for the early and brief increase in ALT is transient ampullary obstruction, which causes a quick rise in bile duct pressure and subsequent liver cell damage[7,9].

Applications of Alanine Amino Transaminase: Aspartate Amino Transaminase (AST, GOT) and Alanine Amino Transaminase (ALT, GPT) are plasma enzymes that are frequently tested as markers of liver cell damage. The hepatic synthetic and secretory abilities are enormous; only severe and typically prolonged liver disease demonstrably impairs prothrombin and albumin synthesis[10,11]. However, the rise in plasma transaminases activities is an important indicator of damage to cytoplasmic or mitochondrial membranes even if there is no detectable failure of function. The level of liver damage is thought to be caused by the effect of radiation on the activity of the enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT)[12,13]. When there is an infection, Similar to viral hepatitis, the main area of damage is to the cytoplasmic membrane, which allows cytoplasmic contents to seep out and elevate plasma ALT rather than AST activities. The type of cell injury may be identified using the relative plasma activity of ALT and AST. The former is more sensitive than ALT and more specific for hepatic illness; AST may be detected in skeletal muscle. A ratio of <1 indicates chronic viral hepatitis or hepatic steatosis, while a ratio of >2 is suggestive but not definitive of alcoholic liver disease[14,15].

Materials and methods:

Sample collection:

The study involved 95 blood serum samples, of which 55 contained gallstones. The subjects' ages ranged from 71 to 17 years, and the samples were obtained from Salah El-Din General Hospital. The control group included (40) healthy samples, whose ages ranged from (71 to 17). In the current investigation, blood samples were obtained by taking 5 cm3 of venous blood with a single-use syringe, depositing it in tubes, centrifuging it to separate the serum, and then draining the serum through a micropipette. To avoid the procedure of repeatedly thawing and freezing the sample, it was acquired in three parts and stored in a (Eppendorf) container. It was held at a deep freeze temperature of (-20°C) until used.

Estemation the Alanine Amino Transaminase Activity in Blood Serum.

A- The principle of the method used:

α- oxoglutarate +L-alanine → pyruvate + L-glutamate

By estimating the amount of pyruvate hydrazine that 2,4-dinitrophenylhydrazine creates, it is estimated.

Reagent's chemical composition:

Contents	Initial Concentration of solution
R1Buffer Phosphate buffer L-alanine α-oxoglutarate	**100mmol /l, pH 7.4 200mmol/l 2.0 mmol/l**
R2 2,4-dinitrophenylhydrazine	**2.0 mmol/l**
CAL Pyruvate standard	**See lot specific insert**
R3 Sodium Hydroxide	**4.0mmol/l**

Procedure

Wavelength	**Hg 546nm(530-550)**
Cuvette	**1 cm light path**
Incubation Temperature	**37 °C**

Comparison to the Reagent Blank

Pipette in to test tubes --	Reagent Blank	Sample
Sample	------	0.1ml
Buffer(R1)	0.5ml	0.5ml
Distilled water	0.1ml	----

30 minutes of mixing and incubation at 37 degrees

2,4-DNP(R2)	0.5ml	0.5ml

mixing and incubating for 20 minutes at (20–25) °C

Sodium Hydroxide (R3)	5.0ml	5.0ml

After mixing for five minutes, check the absorbance[16-18].

Partial purification of ALT from serum of Gallstone patient:

The alanine aminotransferase was isolated from gallbladder patients using serum as:

Precipitation of enzyme by ammonium sulphate

Ammonium sulfate was added to 50% of the cell extract while stirring it at 4 °C, and the resulting solution was centrifuged at 20000 for 50–60 minutes to precipitate the enzyme. The supernatant layer is created by ammonium sulfate. The supernatant was removed and dissolved in buffer Tris-HCl

Dialysis:

It is one of the most often used techniques for isolating enzymes, and it also involves removing too much ammonium sulfate. The bag should then be dipped into the 100 mM HCl solution. After the conclusion, the answer changed occasionally. of the separation process measure concentration of ALT and Protein concentration.

Solution and Buffers:

Diethyl amino ethyl cellulose (DEAE-Cellulose) is a packing material. Potassium hydroxide and potassium chloride in a 0.25M B-solution 100 mM solution of C At pH7.3, tris HCl includes 10% glycerol and 1mM dithiothreitol (DTT).

Ion Exchange Column Preparation:

According to the method outlined by Whitaker, anion exchanger (DEAE-Cellulose) is manufactured as follows: 500 ml of distilled water are used to dissolve 15 g of DEAE-Cellulose before waiting for the granules to settle. The gel is then reconstituted with distilled water once the supernatant has been decanted. Until the supernatant is clear, these processes are repeated. The gel is then activated by washing it twice with solution C, followed by filtering and rinsing it with distilled water, before being suspended in a buffer solution. The gel is then placed in a glass column of 1.6 x 40 cm, and the equilibrium procedure for the column is run using the same buffer that is used for the gel and is similar in pH to the buffer that is placed in the column.emerging from the column at a flow rate of 0.5 ml/min to make sure the pH of the column's environment is stable.

Sample Injection:

A DEAE-Cellulose column is injected with 23 ml of partly pure enzyme precipitated by 50% ammonium sulfate and dialyzed.0.1M Tris buffer pH 7.3 is used to wash unbound proteins, and the absorbance of fractions that have been separated is measured at 280 nm. Up until the absorbance drops to less than 0.1, the buffer solution is kept running over the column.After that, the bounded protein, or target protein, is eluted from the resin using a linear gradient of KCl concentration that ranges from 0.07 to 0.15M. Five milliliters of each fraction are collected in test tubes, with a flow rate of 0.5 milliliters per minute.The active fractions are then gathered, and their volume, activity, and protein content are assessed.

Gel Filtration:

Simple methods are employed in biochemistry to isolate chemicals based on the size and molecular mass of the molecules. Wherever the high molecular weight particle is first filtered, the high molecular weight proteins are migrating through the gel. The separation column estimates the volume of the solution or distal water, and the isolated proteins are read using the absorbance at 280 nm.

1) Buffer Solution (100mM Tris HCl pH8.0): This buffer has 100 mM Tris/HCl pH 7.3 and was made by dissolving 15.8 g of Tris HCl in 1 L of distill water that also contains 10% (by volume) glycerol, 1 mM dithiothreitol, and 10 mM pyridoxal phosphate.
2) The gel filtration suspension method: Prepared by combining 2.5 g of Sephadex G150 filler with 200 ml of 100 mM Tris-HCl pH 7.3 and allowing the mixture to sit for 20 to 24 hours at 4°C.
3) Potassium chloride solution with a 500 mM concentration 23.77 grams were dissolved in 1 L of buffer solution (0.01 M Tris HCl, pH 8.0) to make the preparation.

Use a glass column with a 1.5 cm diameter and a 30 cm length for the first step. To stop the gel granules from escaping the column, add a little piece of glass wool. When the gel reaches 12 cm, the mobile phase is then slowly and uniformly poured into the column to prevent the formation of air bubbles that would obstruct the separation. The column is then washed with enough of the

solution controlled at 100 mM Tris -HCl 8.0 to achieve a flow velocity of 1 ml/min. After membrane separation, add 5 mL of the enzyme slowly over the Sephadex G150 gel's surface and let it soak for 15 minutes. A 100 mL regulator solution of 500 mL KCL solution was used for the separation process, with 5 ml of solution being collected for each portion.

Kinetic Study of Alanine Aminotransferase (ALT):

After being separated and meticulously purified from people with gallstones, including, the kinetic properties of the Alanine Aminotransferase were examined.

1) **Effect of substrate concentration:** Different substrate concentrations (0.5, 1, 2, 3, 4, and 5) were used to study the impact of the substrate on the activity of ALT. If the ALT enzyme's rate of reaction is measured in accordance with paragraph (5), and the relationship between the rate of reaction and the concentration of the base material is plotted, it will be possible to determine the impact of substrate concentration on the enzyme's work. This will reveal whether the enzyme is subject to the Mikals-Menten equation.The Lenover-Burke graphical approach, which links the inverse values of both velocity and substrate concentration (1 / [s] vs. 1 / v), was used to get the Km values.
2) **Effect of Optimum pH:** Using various pH solutions, the impact of the controlled solution's pH on the rate of the ALT response was investigated (4,5, 6, 7, 8, 9) The enzyme activity was assessed as previously described using the substrate at a concentration of 2 mM and a temperature of 37 ° C.
3) **Effect of Temperature:** As the reaction was carried out at various temperatures (7,17,27,37,47,57) °C with a buffer solution with a pH of 7.3 and a substrate concentration of 2 mM, the temperature and reaction velocity were measured to determine the reaction temperature**.**
4) **Effect of incubation time on activity ALT:** With a substrate concentration of 2mM and time periods of 10, 20, 30, 40, 50, and 60 minutes at a temperature of 37°C and a buffer solution with a pH =8.0, the impact of the reaction mixture's duration on the activity of ALT was investigated. The link between enzyme activity and time was esterified to ascertain how incubation time affected the rate of an enzymatic process.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis to Estimation molecular weight of Purified Enzyme (SDSPAGE).

To determine the purity of an enzyme, polyacrylamide electrophoresis under the previously described non-denatured conditions is used.

Solutions and Buffers

1) Stock Solution for Acrylamide: The solution is made up of pure water (100 milliliter), 30 grams of acrylamide, and 8 grams of bisacrylamide. It is then filtered and kept at 4 degrees Celsius.
2) Resolving Gel Buffer is made by dissolving 36.3g of tris base (tris [hydroxyl methyl] aminomethane) in 48 ml of 1 M hydrochloric acid, followed by the addition of water to make 100 ml. The mixture is filtered, titrated to a pH of 8.8, and then kept at 4°C.

3) Stacking Gel Buffer: Six grams of tris-base are dissolved in 40 ml of water, the pH is adjusted to 6.8 with 1 M HCl, and then 100 ml of distilled water is added to make the final volume.
4) Reservoir Buffer: One liter of water is dissolved in three grams of tris-base and 14.4 grams of glycine, and the combination is titrated to pH 8.3.
5) Ammonium persulfate $(NH2)_2S_2O_8$) is freshly made by dissolving 0.15g of it in 10ml of water. Its weight percentage is 1.5% (w/v).
6) TEMED: (NNN'N' Tetra methylene ethylene di amine) is catalyst.
7) Staining Solution: The staining solution is made up of 0.25g of Commasie Brilliant blue R-250 that has been dissolved in equal parts of acetic acid, methanol, and distilled water (4:5:1).
8) Destaining solution: The solution is made up of 100 ml of distilled water and 40 ml of ethanol and 10 ml of acetic acid.
9) Fixing Solution: The fixing solution is made up of 10% tri-chloroacetic acid and 40% methanol.
10) Solution for Preserving: The solution is made by combining 500 ml of distilled water with 150 ml each of ethanol, glycerol, and acetic acid.
11) Enzymatic Solution: It contains 50 ml of 0.25% bromophenol blue, 1 ml of resolving buffer, and 2 ml of purified alanine amino transferase. The addition of seven drops of glycerol increases the solution's density.
12) Standard proteins in solution: Bovine Serum Albumin (BSA), trypsin inhibitor, Ovalbumin, Glycogen phosphorylase, Carbonic anhydrase, Alanine amino transferase (GPT), and α-lactalbumin are the standard proteins that have been dispersed in the sample solution.

where the relationship between the relative movement (Rm or RF) with the logarithm of the molecular weight of standard proteins was drawn, then the molecular weight of the ALT enzyme was estimated. After extracting the Rf of the enzyme and dropping it on the standard curve where the length of the gel was (10.4 cm).

Statical Analysis:

Statistical analysis was carried out using SPSS (version 20). Graphs were drawn using the Excel (2010), arithmetic mean and standard deviation were used. The minimum probability factor ($p< 0.05$) was statistically significant

Results and Discussion:

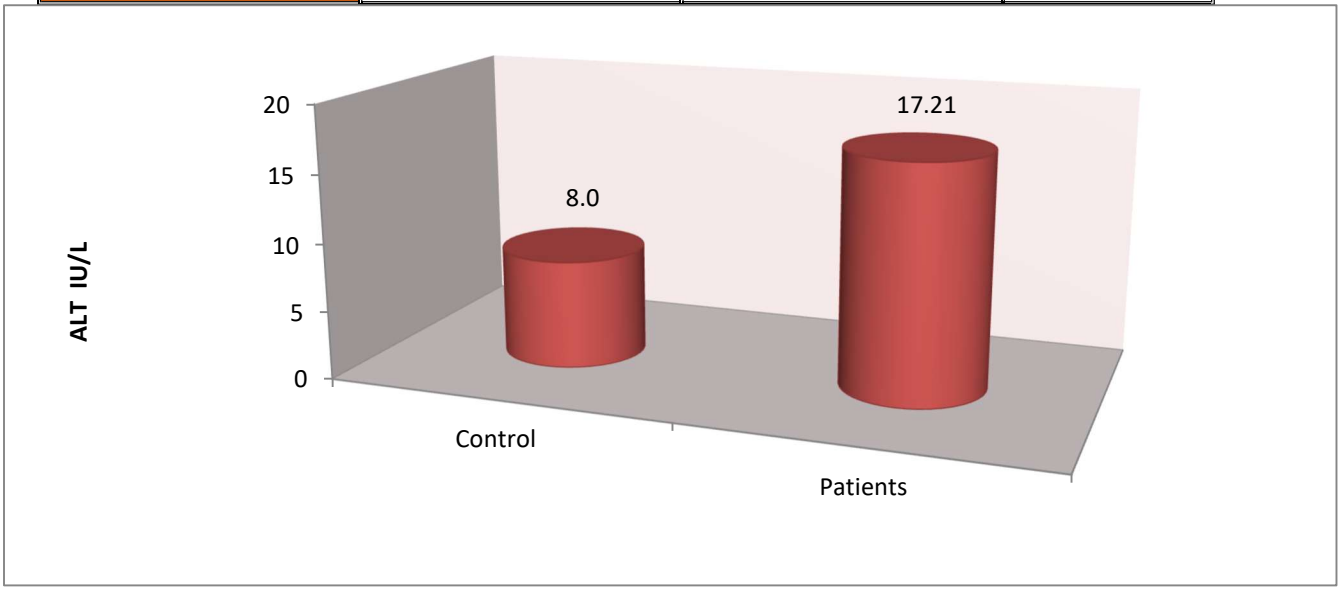
The study included (55) sample Gallstone. The study also included (40) samples of healthy (control) males, as comparison groups, and the range the age for patients and healthy between (71-17) years

Measurement of alanine aminotransferase Activity in Blood Serum:

Gallstone patients and a healthy group had their ALT activity measured. The study's findings included statistical information about gallstone patients as well as biochemical characteristics determined in blood serum samples taken from both the study's control group and the patients.

According to the findings, patients with gallstones had higher levels of ALT activity in their serum. The study of the ALT activity in the patients revealed that they had significantly higher ALT activity than the healthy individuals, with a P value of (P< 0.001).

Parameters	Mean ± S.D. Patients (55)	Mean ± S.D. Control (40)	P value
ALT (IU/L)	17.21±5.85	8.00±2.79	0.001



Fig(1): Activity of ALT in sera of control and gallstone patients.

Separation and Purification of alanine aminotransferase from Serum Patients of gallstone: alanine aminotransferase was separated and purified in several steps as shown in the Table(1).

Table 1. Separation and purification of the alanine aminotransferase enzyme from serum patients gallstone

Total protein (mg)	Flods	Yield %	Specific activity (IU/g)	Protein con. (mg/mL)	Total activity (IU)	Activity (IU/L)	Eluteml	purification stages
185.21	1	100	3.228	8.053	598	26	23	Cruds serum
42.913	2.064	47.82	6.664	3.301	286	22	13	ammonium sulphate 70%

15.505	2.797	23.41	9.029	2.215	140	20	7	Dialysis
10.806	3.096	18.06	9.994	1.801	108	18	6	Gel filtration sephadex G150
6.881	3.219	11.95	10.391	1.251	71.5	13	5.5	Ion exchange DEAE

The first stage was employing ammonium sulphate salt at a concentration of (0-50%)% to precipitate and separate the enzyme from the serum. To achieve a level of cleanliness and desalting, dialysis was carried out in the second phase. The final stage involved separating the ALT from the proteins and other salts that were linked to the enzyme using the size-exclusion chromatography technology. In this phase, a sephadex G-150 resin filtration column was utilized, and as shown in Fig(2), a single peak was obtained at yield (18.06)% and (3.09) Number of purification. The ALT isoenzyme was separated using ion exchange chromatography in the last step based on the difference in charge. Diverse levels of DEAE-Cellulose A50 resin purity were used in this experiment, a single peak was obtained at yield (11.95)% and (3.21) Number of purification as shown in fig (3).

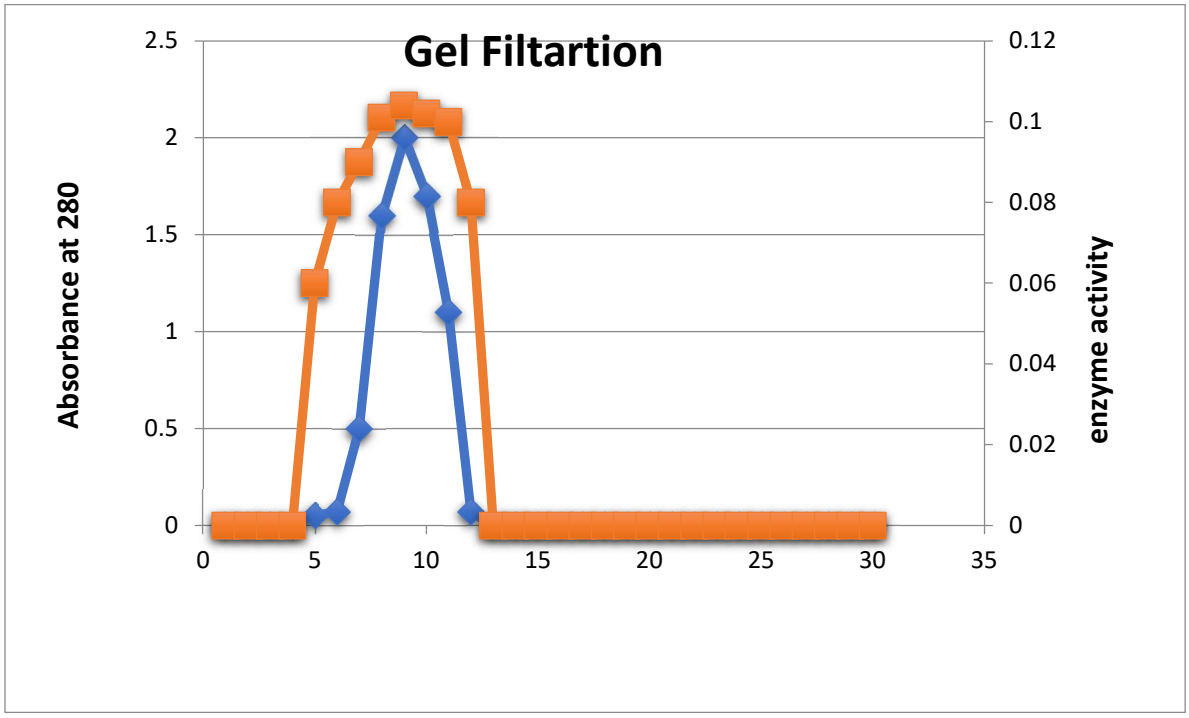


Fig (2) Purification of Alanine aminotransferase enzyme using gel filtration

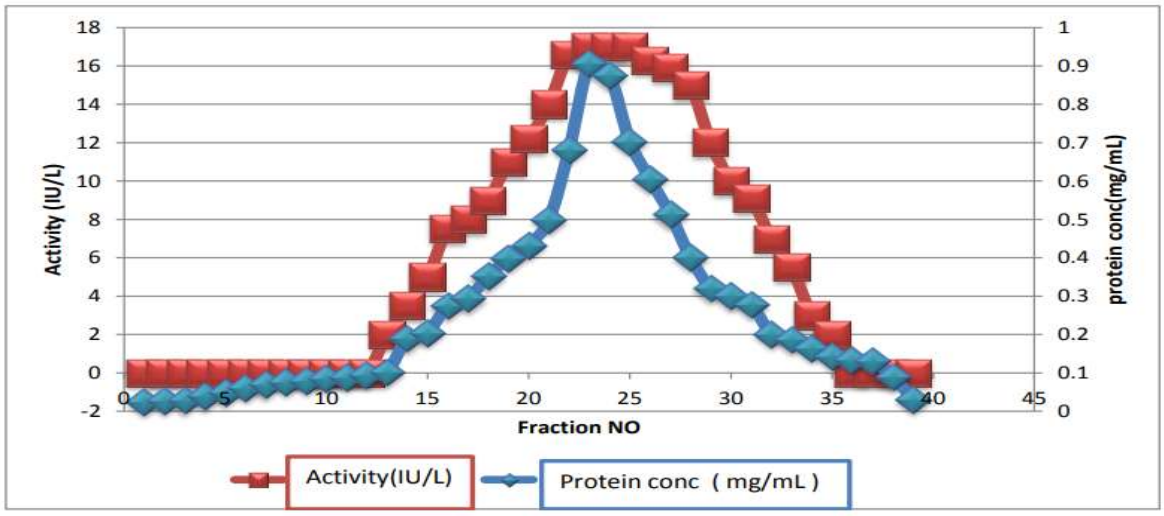
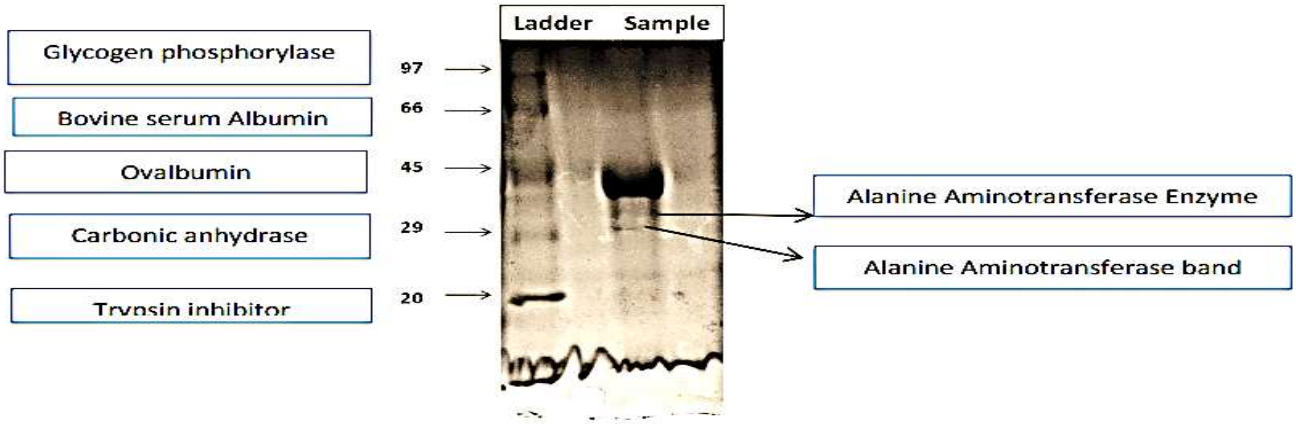


Fig (3) DEAE-cellulose ion exchange chromatography for Alanine Aminotransferase.

Estimation Molecular weight for enzyme by electrophoresis

The electro-migration technique is a method that can be used to determine the molecular weight through the linear relationship between the movement of the protein within the gel and the logarithm of the molecular weight. The gel can be titrated with proteins of known molecular weight to be used later to determine the unknown molecular weights. The standard curve (logarithm relationship of molecular weight and RM) for standard proteins in a polyacrylamide gel in the absence of teratogenic factors was used to calculate the molecular weight of the enzyme. (35KD)

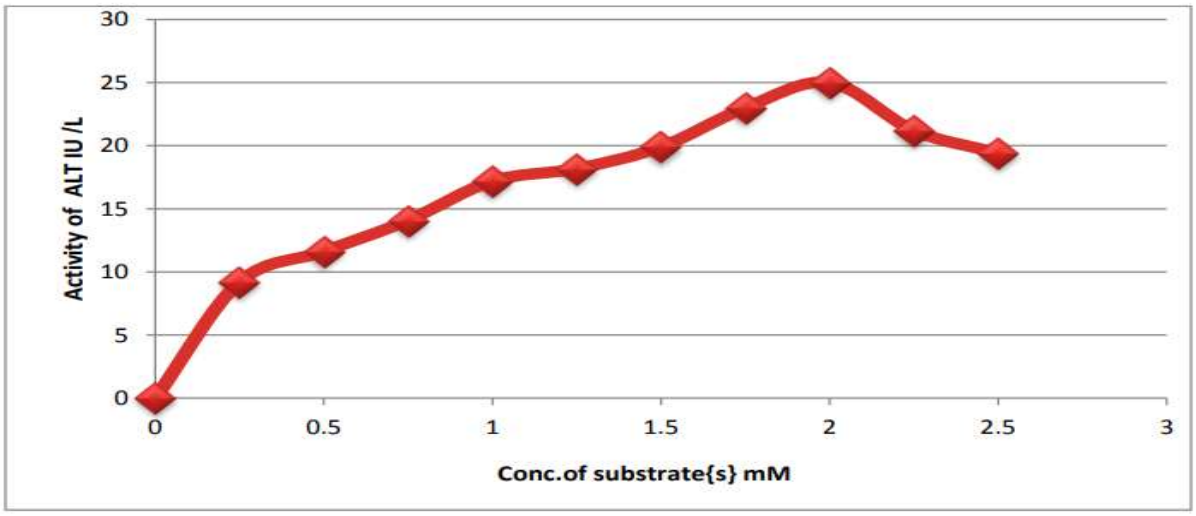


Fig(4) Purified ALT from patient serum electrophoresed on polyacrylamide gel in the absence of teratogenic substances.

ALT Kinetic Properties partially purified from patients with gallstones.

Effect of Substrate Concentration on Enzyme Activity.

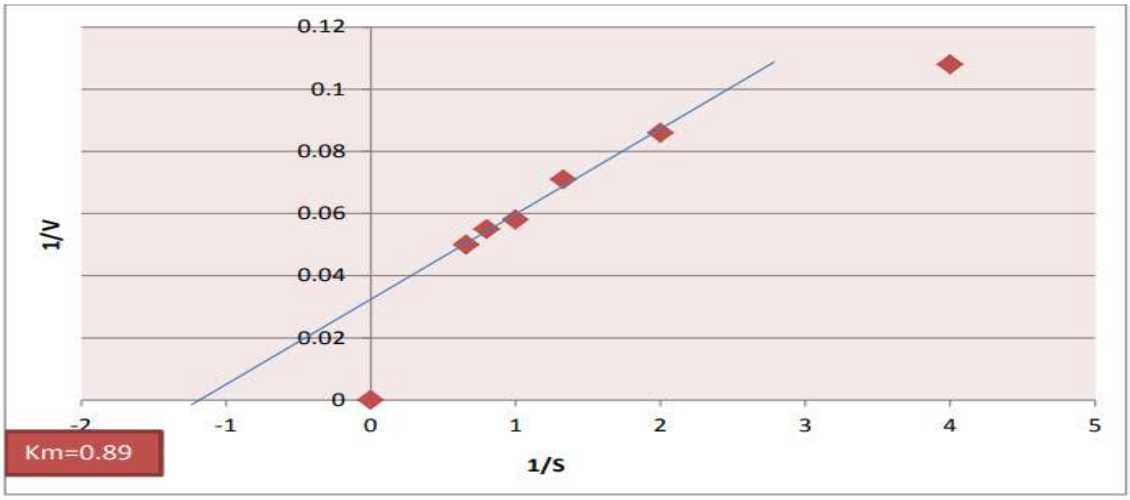
With varied substrate concentrations present, the ALT's activity was assessed. The greatest ALT activity was discovered to be attained by employing (2 mM) of the substrate.



Fig(5) Alanine Aminotranferase activity was influenced by substrate concentration according to a Michaelis-Menten plot.

Calculation of Km and Vmax

The Km value of partially purified prolidase from the serum of patients with acute renal failure was calculated using Lineweaver Burk's drawing. This value represents the reciprocal of the enzyme's activity 1/v. vs. reciprocal matrix concentration 1/[s][21].

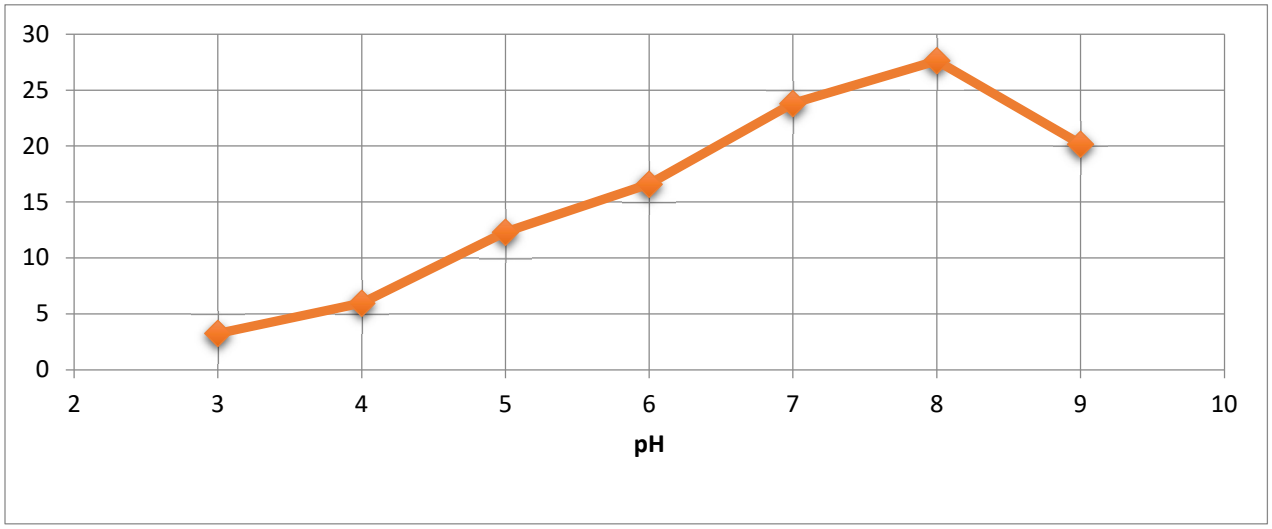


Fig(6) Linover Burke's drawing of ALT for the samples under study

Effect of pH on Enzyme Activity:

Enzymes' efficiency is influenced by pH because they operate most effectively in an environment with a balanced acid/base ratio. As a result, an increase or decrease in pH causes an enzyme's activity to decline. Since altering the pH results in the removal of the enzyme's natural form, or denaturation, it was discovered by examining the effects of different pH levels on the reaction speed of partially purified ALT that a discernible increase in the reaction speed occurred with the

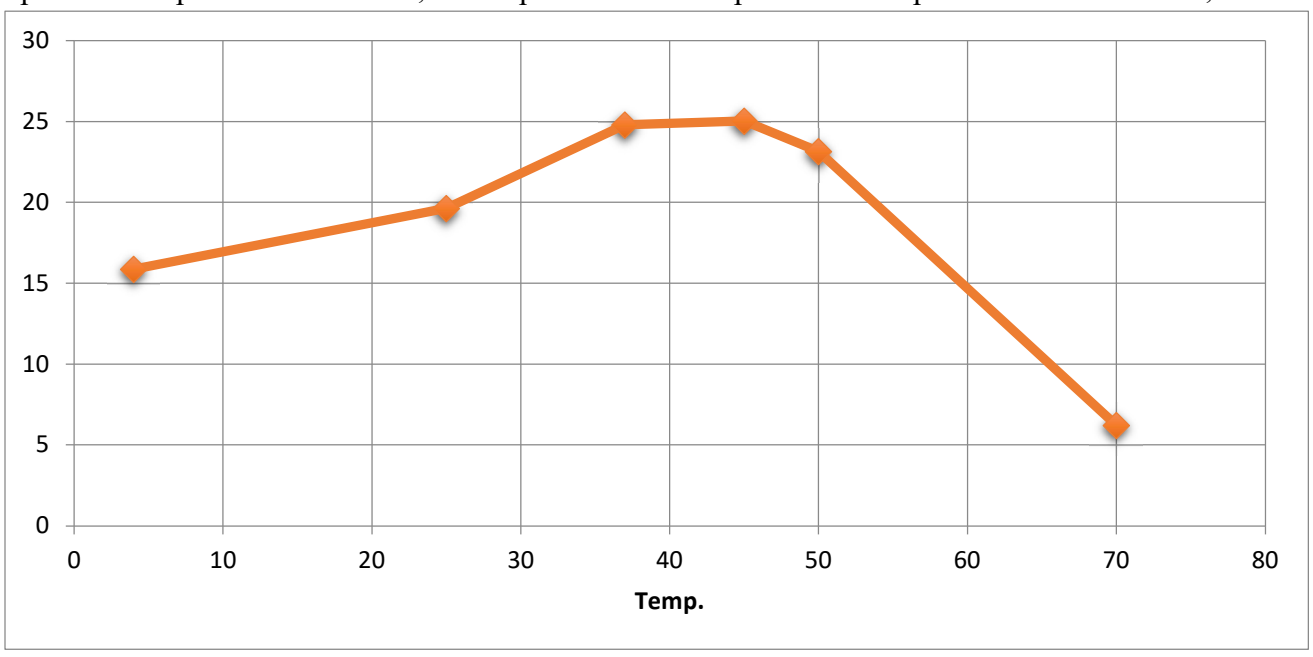
pH degree rising to the point of reaching The highest reactional speed occurs at pH = 8, the ideal pH[19].



Fig(7) Effect of pH on the activity of Alanine aminotransferase

Effect of Temperature on Enzyme Activity.

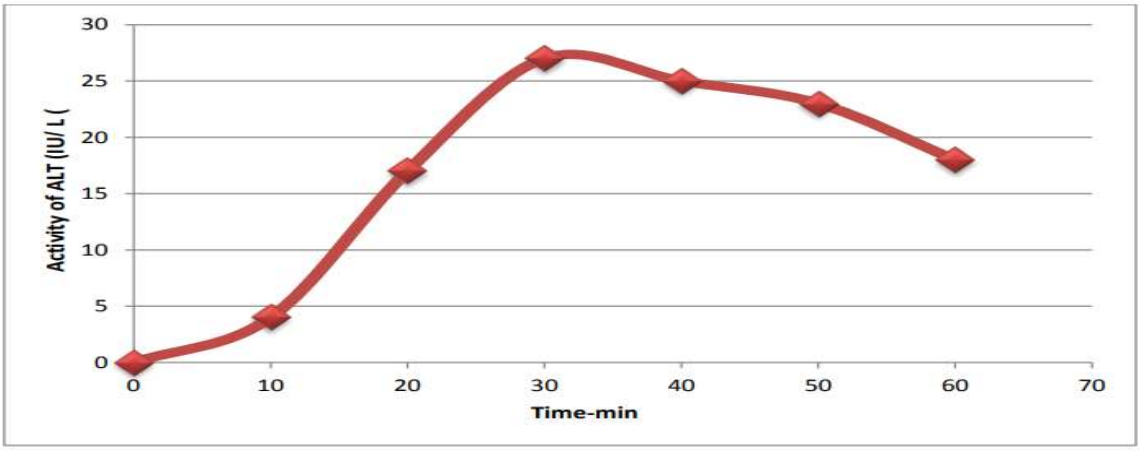
Temperature has a significant impact on the effectiveness and activity of enzymes because reaction speed increases with rising temperatures until the reaction reaches its ideal temperature, at which point it starts to gradually fall in order to denature the enzyme, alter its three-dimensional structure, and thereby lose its efficacy and activity. It was discovered through a study of how temperature affected the partial purification of ALT from the blood serum of individuals with renal failure and how quickly it reacted. As we observe an increase in reaction speed with temperature up until the optimum temperature is reached, which peaked at 37o C purified from patient blood serum[20],



Fig(8) Effect of Tempurature on the activity of Alanine aminotransferase

Effect of Enzyme Activity on Incubation Period:

The dependency of enzyme activity on incubation period (0,10,20,30,40,50,and 60) minutes was depicted in figure (9). Because it pertains to a linear area of the curve and takes into account trustworthy absorbance readings, thirty minutes (30)min was chosen as the ideal incubation time throughout the task. This might be related to the enzyme's assigned temperature, where the heat has more time to disrupt the bonds between two amino acids[22].



Fig(9) Alanine aminotransferase incubation time optimization

Conclusions:

After doing this study, the following considerations may be made:

1. According to patients with gallstones, high ALT levels are associated with the disease.
2. The level of Alanine Aminotransferase purification (ALT) Ionic exchange column partial purification (2.92), yield (15.63%), and a particular activity was (0.00943).
3. The isolated enzyme's molecular weight from the serum of patients with gallstones was (35KD).
4. The enzyme-purified WAS Km values (Km =0.89) Mm

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