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Purification and characterization of Alkaline phosphatase from patients women breast cancer

Mohammed A. Al-Dolaymi [a], Khalid F. Al-Rawi [a],
Firas T. AL-Shamary [b], Mohammad M. Al-Halbosiy [c],

a) Department of Chemistry, College of Science, University of Anbar , Iraq
b) Department of Chemistry, College of Science, University of Tikrit, Iraq
c) Biotechnology Research Center , Al-Nahrain University, Iraq

Abstract

our study includ determination of the activity of alkaline phosphatase followed by isolation and purification of that enzyme. The process also includ the estimation of molecular weight, effecting of some factors on enzyme activity and studying the chemical kinetics of alkaline phosphatase.

Optimization of the extraction process for purify the alkaline phosphatase (ALP) from the serum was achieved by purification steps included:

Firstly, the precipitation; Using ammonium sulphate in saturated degree (60 %) showed increased in specific activity of partial purification ALP enzyme from (0.0021U/mg) in crude sample to (0.0031U/mg) after first step of purification .

Secondly, utilize the ion-exchange chromatography; Using DEAE-cellulose to isolate three types of isoenzymes. The partial purification of the tissue - nonspecific (TNSALP) isoenzyme(I) was carried out by using Sephadex G100 as gel - filtration technique , and purified by sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE) which only one single band appeared. On the other hand, the molecular weight of isoenzyme (I) purity detection by PAGE (Polyacrylamide-gel electrophoresis) which revealed single dense bands along the gel was 97 KD.

In addition to the previous processes,the kinetic studies of the purified isoenzyme (I) was carried out, and it was found that the optimum concentration for the substrate disodium phenyl phosphate (DPP) was (5mM) and the Km for the purified isoenzyme was (1.4)mM , While its pH optimum was 10 , while optimum temperature was 37Co , and study the effect of the reaction time on the enzymatic activity and we found that the maximum activity is after 15 minute of incubation period.

Keywords: breast cancer, alkaline phosphatase, purification.

تنقية وتوصيف الفوسفاتيز القاعدي من النساء المصابات بسرطان الثدي

المستخلص :

اشتملت هذه الدراسة تقدير نشاط انزيم الفوسفاتيز القاعدي كخطوة اولية ومن ثم عزله وتنقيته وتحديد الوزن الجزيئي له وكذلك دراسة بعض العوامل الموثرة على نشاطه و الدراسة الحركية له

لقد تم استخدام طرق مختلفة لعزل وتنقية انزيم الفوسفاتيز القاعدي في المصل بواسطة عدة خطوات تضمنت:

الترسيب باستخدام كبريتات الامونيوم بنسبة اشباع 60 ٪ . حيث اظهرت زيادة واضحة في التنقية الجزيئية للانزيم من0.0021 وحدة/ ملغم في النموذج الاصلي الى 0.0031 وحدة/ ملغم بعد الخطوة الاولى من التنقية

كروموتوغرافيا التبادل الايوني باستخدام DEAE- Cellulose في هذه الطريقة تم فصل ثلاثة متناظرات لانزيم الفوسفاتيز القاعدي من مصل النساء المصابة بسرطان الثدي، المتناظر المنقى جزئياً غير المتخصص بالانسجة (TNSALP) حمل على جل السيفادكس بتقنية الترشيح الهلامي، وفصل بواسطة SDS-PAGE والذي اظهر حزمة مفردة واحدة في حين ان الوزن الجزيئي للمتناظر المنقى بواسطة PAGE الذي اظهر حزمة كثيفة واحدة نقية على امتداد الهلام كان (97 KD).

الدراسات الحركية للمتناظر المنقى شملت ظروف المثلى للتفاعل والتي تضمنت تركيز 5 mM لثنائي صوديوم فنيل فوسفاتيز ، Km = 1. 4 mM ، pH = 10 عند درجة حرارة 37 م^{O} . وكذلك تضمنت الدراسة تأثير زمن التفاعل على فعالية الانزيم حيث وصل الى اقصى فعالية بعد مرور 15 ثانية من مدة الحضانة.

1.Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer-related mortality among women, accounting for 23% of all new cancer cases and 14% of cancer deaths [1]. Bone is one of the most preferential target sites of metastasis for breast cancer and up to 70% of women with advanced disease develop bone metastases [2]. Such lesions have devastating effects, including pain, pathologic fractures, spinal compression and hypercalcemia, all of which greatly compromise the quality of life and outcome [3].

Alkaline phosphatase (ALP) (Ec: 3.1.3.1)is ahydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. The process of removing the phosphate group is called dephasphorylation [4], in (pH=95) (10-).

Alkaline phosphatase is considered one of non-specified enzyme which interacts with a number of substrates, and it is a very stable enzyme [6] . As he remains active for six months for six months at 40 C with the existence of the regulating solution 5mM Tri-Hcl and when it reserves at 25oC then it Keeps his activity for ten days [7] , and the alkaline phosphates is different in molecular weights [8]. the alkaline phosphates existed in high concentrations in liver, bones, intestine, placenta, kidney, spleen and leukocytes, but its main source is liver and bone [9]

2. Experimental

2.1. Partial Purification of ALP from serum of Breast cancer patient

2.1.1 Precipitation of enzyme by ammonium sulphate

Crud enzyme was precipitated with 60% saturation of $(NH_4)_2SO_4$ by adding 36.1 g from salt to 6 ml crude enzyme under cooled conditions, with continuous stirring for 45 minutes, and centrifugated at (6000 rpm for 30 minutes at 4°C) . Precipitates were dissolved in small amount of Tris-HCl (0.1M) buffer then activity of enzyme and protein concentration and specific activity were calculated.

The solution obtained from the precipitation step was dialyzed against 0.1M Tris-HCl buffer pH 7.2 for 24 hr at 4°C with stirring for desalting .The ALP activity, protein concentration were estimated and the specific activity

was determined .

2.1.2.Dialysis

It is one of the oldest methods used in the purification of proteins and the aim is to remove the remaining ammonium sulphate added to the deposition of proteins by placing the protein in the bag dialysis and immersing the sachet in buffer solution 0.1M Tris-HCl pH 7.2, the buffer solution was changed from time to time for 24 hours. This step was performed at 4 ° C. After the end of the period, the enzyme activity was measured and the protein concentration was determined .

2.1.3.Ion-exchange

Chromatography (DEAE-Cellulose) (10 -11)

A-Solutions preparation:

- Sodium hydroxide (0.25 N)

It was prepared by dissolving 5g of NaOH in 500 ml of distilled water.

- Hydrochloric acid (0.25 N)

It was prepared by the addition of 10.4 ml of concentrated (12N) HCl to 400 ml of distilled water, and then the volume was completed to 500 ml by distilled water.

- Sodium chloride (200 mM)

This solution was prepared by dissolving 11.7g of sodium chloride in 1000 ml of 0.1M Tris-HCl pH 7.2, from which attended a series of solutions containing graduated concentrations of NaCl starting (50 - 200) mM.

- Tris- HCl buffer (0.1 M, pH 7.2)

This solution was prepared by dissolving 15.76g of Tris-HCl in 900 ml distilled water and pH adjusted to 7.2, then the volume was completed to 1000 ml by distilled water.

B-Preparation of Ion-exchange column (DEAE-Cellulose)

According to Whitaker and Bernhard, [10] this method was prepared by using 20g of diethyl amino ethyl-cellulose (DEAE-Cellulose) powder was suspended in 1000 ml distilled water and left to settle, the supernatant was discarded, and this step was repeated several times until supernatant became clear. DEAE-Cellulose was activated with 0.25 N HCl for 30min, then filtrated by Buchner funnel containing Whatman No.1 filter paper and washed with distilled water twice.

After that DEAE-Cellulose was activated with 0.25N NaOH, the filtration and washing processes were repeated twice. The activated DEAE-Cellulose was equilibrated with (0.1M

Tris buffer pH 7.2), and packaged in a column with dimension (3 × 18) cm.

The concentrated enzyme from previous steps was added to the ion-exchanger column. Flow rate was organized to be 1ml / min for washed and eluted samples, which collected was 3 ml per each fraction. Tris buffer (0.1M, pH 7.2) was prepared for washing ALP and the enzyme was eluted by the same buffer with NaCl (50 - 200) M. The absorbance for each fraction was measured at 280 nm. Active fractions were collected for measuring enzyme activity, protein concentration and specific activity [11] .

After these measurements, the enzyme was concentrated by filling semipermipul membrane with it, and covered by sucrose in dialysis process for next purification step.

2.1.4. Gel Filtration Chromatography (12).

A-Solutions preparation:

- Tris- HCl buffer (0.1 M, pH 7.2)

This solution was prepared by dissolving 15.76g of Tris-HCl in 900 ml distilled water and pH adjusted to 7.2, then the volume was completed to 1000 ml by distilled water.

- Sodium chloride (500 mM)

This solution was prepared by dissolving 29.25g of sodium chloride in 1000 ml of 0.1M Tris-HCl pH 7.2 .

B-Column preparation:

The Sepharose 6B-CL gel was prepared according to the instructions of the Pharmacia Fine Chemical Company. Gel was suspended in 0.1M Tris-HCl buffer pH 7.2 as in and degassing under vacuum, then the gel was poured into the glass column with dimensions of 2×40 cm.

The equilibrium of the column was done by adding enough amount of 0.1M Tris-HCl buffer pH 7.2 until the pH of the leaching fractions becomes 7.2 with a flow rate of (5ml/3min).

After column preparation, the concentrated enzyme solution obtained from the elution fractions of ion exchange chromatograph was added gradually on column surface and eluted using 0.1M Tris-HCl buffer pH 7.2 with flow rate of (5ml/3min). (5ml for each fraction). The absorbance of each fraction was measured at 280 nm. The peak fractions with enzyme activity were collected and their volumes were determined. Protein concentrations in peak fractions were also measured to determine the specific activity of the

enzyme. The collected fractions of the peak were re-concentrated by filling semipermipul membrane with it, and covered by sucrose in dialysis process and passed on the same column at the same conditions for further purification.

The enzyme activity and protein concentration were determined in the purified fraction; the collected fraction (purified enzyme) was stored under freezing conditions for characterization processes.

2.1.5.Electrophoresis

Electrophoresis is the movement of charged molecules in an electric field. It is a rapid and often employed technique for the determination of the molecular weight of protein and for the separation of biological molecules such as nucleic acid, nucleotides, amino acids and protein [12].

2.2.1.Effect of substrate concentration Disodium Phenyl Phosphate (DPP):

This effect has studied different concentrated of Disodium Phenyl Phosphate (DPP) on ALP enzyme activity if the concentration used (0.5,0.75,1.5,2,3,4,5,6) mM to find the optimal substrate concentration for the action of ALP enzyme , whereas interaction velocity measurement . Then draw the relation between interaction velocity and concentration of Disodium Phenyl Phosphate (DPP) to identify the optimal substrate concentration where the velocity of the interaction its maximum value (Vmax) .

2.2.2.Specifies the value of Michaelis-Menten constant (Km).

To identify constant value (Km) of Disodium Phenyl Phosphate (DPP) specified in ALP enzyme , in which we got Km value by using diagram as in Line Weaver-Burk plot linked with reverse values for both velocity and concentration of DPP (1/v vs.1/[s]) .

2.2.3.Determination of optimum pH

Studied the effect of buffer solution (Sodium Carbonate –Bicarbonate) on the velocity of ALP , used solutions pH different (7,8,9,10,11,12) by existence of Disodium Phenyl Phosphate (DPP)with concentration of 5mM and 37°C. that determination of optimum pH by drawing the relation between interaction velocity and pH .

2.2.4. Determination of optimum Temperature

Measure ALP enzyme activity by conducting interaction in different temperature (7 ,17 ,27 ,37,47 ,57) °C with buffer solution (sodium carbonate –Bicarbonate) with PH 10 concentration of Disodium Phenyl Phosphate (DPP) 5mM , then the relation was drawn between interaction velocity and temperature to identify the optimum temperature of interaction .

2.2.5. Determination of optimum Incubation time

studied the effect of Incubation time interaction mixture on ALP enzyme activity by using 5mM concentration from Disodium Phenyl Phosphate (DPP) with accurate time period (0 , 5 , 10 , 15 , 20, 25) minute, and under 37°C temperature with buffer solution (sodium carbonate –Bicarbonate) of pH 10 , then drawing the relation between enzyme activity and time and identifying the effect of Incubation time on enzyme interaction velocity .

2.2.6. Effect of Metallic Ions and EDTA on ALP

The effect of some ions have been studied upon the activity of ALP specially which has stimulation effect or inhibition effect as cobalt CO+2 and Magnesium Mg+2, Nickel Ni+2 ,Zinc Zn+2, Copper Cu+2 and EDTA if the following concentrations are used (1 , 3 , 5 ,10) mM and interaction was made with the buffer solution(sodium carbonate –Bicarbonate) pH10 .Enzyme activity was measured and calculation of percentage of inhibition or stimulation for those ions and EDTA.

2.2.7.Some Amino acid effect on ALP

studied the effect that was made by different concentration of some amino-acids (Trp ,Cys ,Phe ,Leu) on ALP activity by using concentration (1 , 3 ,5) mM than inhibition rate of amino acid was calculated on ALP activity and measuring enzyme activity.

3. Results and discussion

3.1.Partial purification of alkaline phosphatase from serum of breast cancer patients

purification of biological molecules (protein, carbohydrate, nucleic acid and lipid) can be done under special laboratory techniques of cell lysis , tissue homogenization, filtration, centrifugation, chromatography, salt or organic solvent precipitation,

and concentration [13]. As with many other biological materials, extreme conditions must be avoided when attempting to isolate proteins, and physical rather than chemical methods are employed. Generally speaking a high protein concentration, low temperature and pH close to neutrality are best; otherwise denaturation occurs .Enzyme purification involves the isolation of specific enzyme protein from crude extract of whole cells containing many other components. Small molecules may be removed by dialysis. The problem is to separate the desired enzyme from a mixture of hundreds of chemically and physically similar proteins [14].

Precipitation with varying salt concentration (generally ammonium sulfate), gel filtration and electrophoresis. Selective adsorption and elution of proteins from the polydextran derivatives anion exchange Diethylaminoethyl (DEAE-Sephadex) and the cations exchange carboxymethyl (CM-Sephadex) have also been extremely successful for extensive and rapid purification which used in many other studies [15] . In addition to exhaustive physical, chemical and biological tests must still be applied as criteria of purity for many proteins, the isolation and purification procedure is an exacting task requiring days of effect to obtain only few milligrams or less of the desired product [16].

3.1.1 Concentration of ALP by ammonium sulphate precipitation

Protein precipitation by salting out technique using of inorganic salts such as ammonium sulphate [NH4(SO4)2] was carried out with constant gentle stirring [13] .This occurs by using varying saturating ratio to concentrate and precipitate the sample by dissolving calculating amount from solid ammonium sulphate, gradually, added slowly over a period of time on ice with a constant stirring up to a final concentration [17] .

Our Results in table (3 -1) show that most of proteins in our sample were precipitating at partial purification in saturated degree (60 %) and this result caused elevated in specific activity for partial purification ALP from (0.0021U/mg)in crude sample to (0.0031U/mg) after first step in purification ,and the purification fold also increases after precipitating from (0 to 1.48). All these results are obtained because

ammonium sulphate has no adverse effects upon enzyme activity. It is used successfully to reduce the complexity and to remove interference of small molecules from protein/enzyme solutions , the immune portions were precipitated. Protein solubility is dependent on the ionic strength of the solution, as the salt concentration (ionic strength) increases; the solubility of the protein begins to decrease. At certain ionic strength, the protein will almost be completely precipitated from the solution. Salting-out is a very useful procedure to assist in the purification of a given proteins.

Ammonium sulfate has been widely used in salting out for protein purification [18], as it is very water soluble, forms two ions, and has no adverse effects upon enzyme activity as the ammonium sulfate dissolves, a large amount of water is bond to each ammonium sulfate molecule. Therefore, as the number of ammonium sulfate molecules in solution increases, less water is available to interact with proteins that may be present [19] .

3.1.2 Dialysis

Low molecular weight materials, such as salts, and some biological materials, such as amino acids, coenzyme and low molecular weight carbohydrate, can be removed by dialysis from macromolecular materials such as proteins, nucleic acid and polysaccharides. Dialysis is a process that separates molecules according to size through the use of semipermeable membranes containing pores of less than macromolecular dimensions. These pores allow small molecules, such as those of solvent, salts and small metabolites, to diffuse across the membrane but block the passage of larger molecules (13,16).

A loss of ALP activity on dialysis may be found. This may be due to instability of the enzyme, or to removal of some cofactor, but a common cause, unless very pure water is used, is the dialysis of traces of inhibitory metals from the water. The enzyme usually has a very high affinity for these, and even though their concentration in the external water is very small. It may take up all the traces present in a very large volume of water. This is not usually as important with crude preparations as in the later stages [20].

Step	Elute (ml)	Activity (I U/L)	Total activity (I U)	Protein .conc (mg/ ml)	Total protein (mg)	Specific activity (I U/mg)	Purifica-tion (Fold)	Yield %
Crud serum	5.5	109.912	0.605	51.14	281.25	0.0021	-	100
Ammonium sulphate	5	73.53	0.368	23.64	118.24	0.0031	1.48	60.8
Dialysis	4	57.653	0.231	13.77	55.102	0.0041	1.95	38.1
(Ion exchange) DEAE-Cellulose A50	5	26.023	0.130	4.17	20.85	0.0062	2.95	21.5
Isoenzyme - I	5	19.822	0.099	3.46	17.3	0.0057	2.71	16.4
Isoenzyme - II	5	24.313	0.121	3.98	19.9	0.0060	2.85	20
Isoenzyme - III								
(Gel filtration) Sephadex G100	5	25.013	0.125	3.00	15.00	0.0083	4	20.7

Table (3 - 1): Partial purification of ALP from serum of BC patients

3.1.3. Ion - Exchange Chromatography

Ion exchange chromatography occurs due to electrostatic attraction between buffer-dissolved charged proteins and oppositely charged binding sites on a solid ion exchange adsorbent. An ion exchange adsorbent (also called media, resin, gel, or matrix) usually consists of spherical porous inert beads with charged groups (functional groups) densely grafted onto the beads' surfaces; the charges of functional groups are neutralized by free counter - ions

$$R^{+}A + B \rightleftharpoons R^{+}B + A$$

Here, R+A- are an anion exchanger in the A- form and B- which represent anions in solution. These principles are used for a great advantage in isolating biological molecules by ion exchange chromatography. In purifying a given protein, pH, salt concentration, and ion exchange are chosen so that the isolated protein is immobilized on the ion exchange [16].

In our study, the partial purified enzyme solution (supernatant) obtained from Ammonium sulfate precipitation was applied directly to a (DEAE-cellulose) column.

Figure (31-) showed the presence of three peaks for purified ALP after eluted by increasing concentration from NaCl solution, the first peak (elution peak) that appeared between (2 -7) tubes ,refers to elimination of disassociated proteins , the second active peak that appeared between (9-13) tubes, and the third active peak that appeared between (16 -19) tubes where after ALP got eluted by NaCl .This peak indicated that the ALP has been purified till utmost homogeneity .These results are accordance with study AL-TAII [21] which uses the same column, After second purification steps, the ALP activity was still higher than normal level ,but there was decreasing as compared with ALP activity in crud enzyme ,the decreasing was from (73.53 IU/l) after ammonium sulphate concentration step to (26.023, 19.822, 24.313 IU/l)after ion exchange chromatography as show in table (3 -1).

The purified ALP peak revealed specific activity (0.0062 IU/mg) with 2.95folds of purification indicating that this peak represents a good source of enzyme for further purification.

The using of (DEAE - cellulose) column after ammonium sulphate concentration step is effective tool used in the purification step of enzymes Because of the hydrophilic nature of cellulose, these exchangers have little tendency to denature proteins which are used as the first ion exchangers designed for use with biological substances like (Protein, carbohydrates, Nucleic acid) [22] .

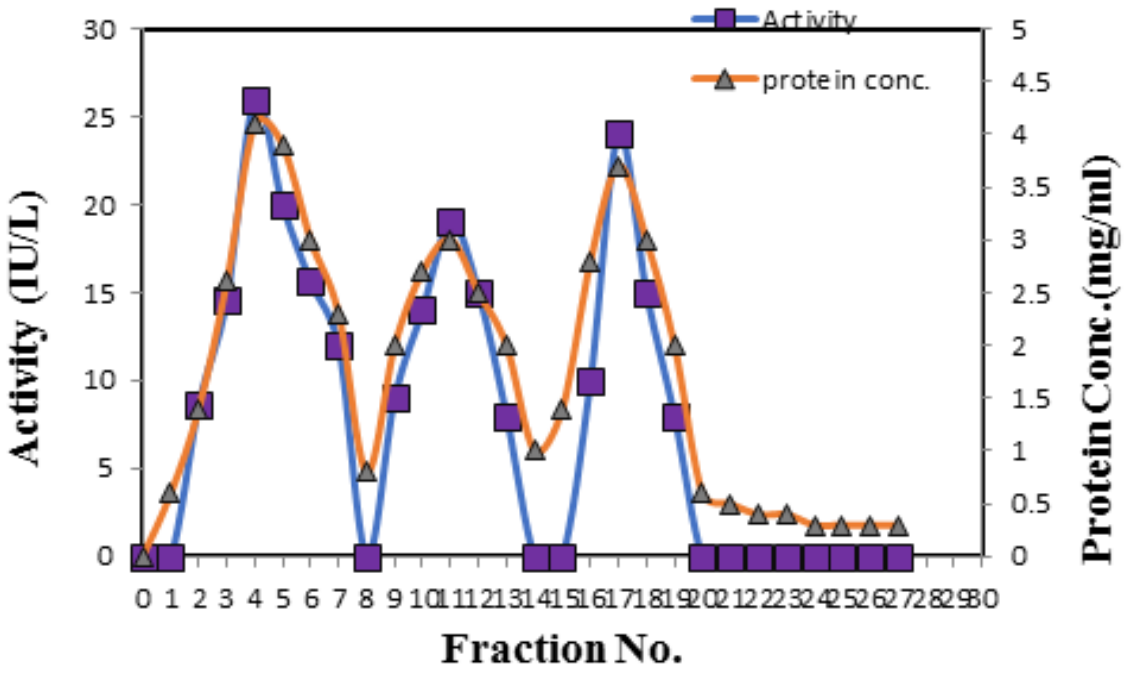

Figure (3 -1): DEAE-cellulose ion exchange chromatography for ALP.

3.1.4 Gel Filtration or Size-exclusion chromatography (SEC)

It is a chromatographic method in which molecules in solution have fractionation, isolation and purification of protein, enzymes, hormones, antibiotics and nucleic acids. It is usually applied to large molecules or macromolecular complexes which separated by their size, and in some cases molecular weight. as both are analytical and preparative tool. Typically, when an aqueous solution is used to transport the sample through the column, the technique is known as gel-filtration chromatograph. The main application of gel-filtration chromatography is the fractionation of proteins and other water-soluble polymers, while gel permeation chromatography is used to analyze the molecular weight distribution of organic-soluble polymers [23] .The advantages of this method include good separation of large molecules from the small molecules with a minimal volume of elute, and that various solutions can be applied without interfering with the filtration process. With size exclusion chromatography, there are short and well-defined separation times and narrow bands, which lead to good

sensitivity. There is also no sample loss because solutes do not interact with the stationary phase.

Finally, minor disadvantages of gel filtration are the dilution of the solute that occurs during the separation and the period of separation is long, and oxidizing agents cannot be used with Sephadex as well as only a limited number of bands can be accommodated because the time scale of the chromatogram is short [24] .This technique was applied to separate and purified ALP in this study. This was obtained by ammonium sulfate precipitation and DEAE-cellulose Ion exchange methods using column containing Sephadex G100. Our results are shown below in Figure (3 -2).

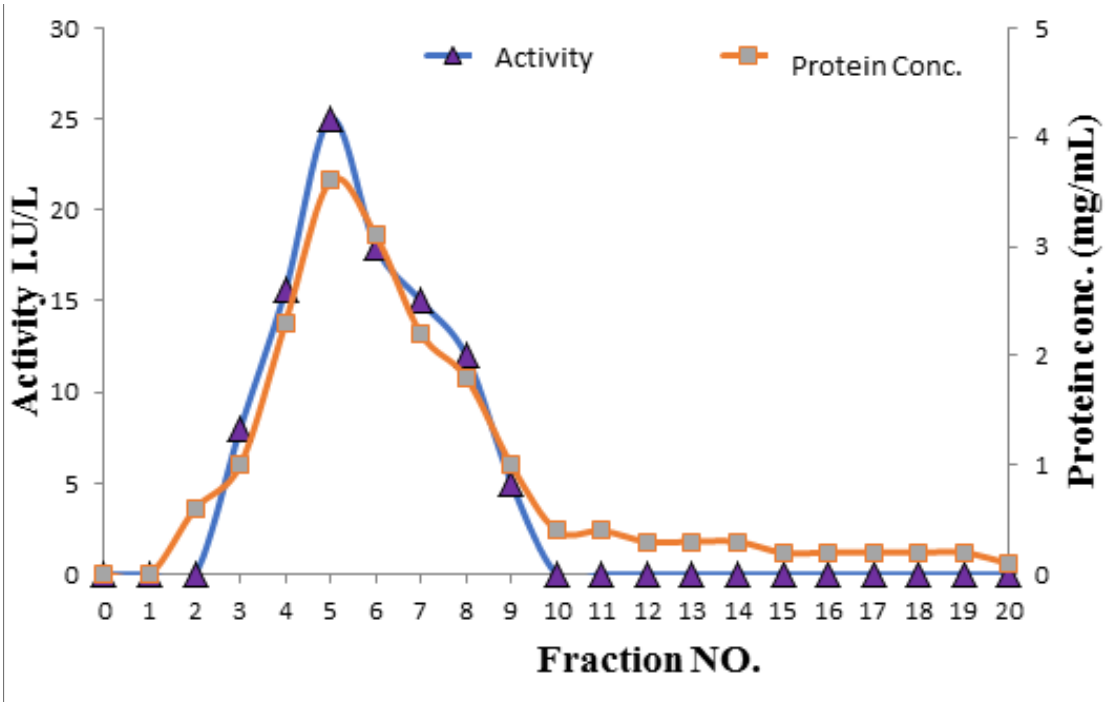

Figure (3 -2): Gel filtration chromatography of ALP by Sephadex G100.

This figure indicates that the enzyme was eluted as a single symmetrical peak with the fraction Figure (32-) Activity for purified ALP reached to (25.013) IU/l and the specific activity of ALP enzyme was increased to (0.0083) IU/mg by (4) folds than the specific activity in last purification step (0.0062 IU/mg)by (2.95) folds as shown in Table (3 -1) .

3.1.5 Molecular Weight Determination by SDS-PAGE

Electrophoresis" refers to the electromotive force (EMF) that is used to move the molecules through the gel matrix. By placing the molecules in wells in the gel and applying an electric field, the molecules will move through the matrix at different rates, species that are positively charged (cations) will migrate towards the cathode which is negatively charged. If the species are negatively charged (anions) they will migrate towards the positively charged anode [21] . Polyacrylamide gel electrophoresis (PAGE) is used for separating proteins due to the uniform pore size provided Which is controlled by modulating the concentrations of acrylamide and bis-acrylamide powder used in creating a gel. Care must be used when creating this type of gel, as acrylamide is a potent neurotoxin in its liquid and powdered forms Buffers in gel electrophoresis are used to provide ions that carry a current and to maintain the pH at a relatively constant value [25]

study and in order to investigate the purity of ALP, which was purified from serum, Polyacrylamide gel electrophoresis involve two samples, the first sample was normal serum and the second sample was the purified ALP ,when the gel is immersed in Comassie brilliant blue G-250, several protein bands seemed with different molecular weights along the gel ,while the purified enzyme sample demonstrated one clear band only,as shown in figure (3 -4).

The appearance of many protein bands along the gel is imputed to that crude extract which contains a large number of different proteins with different molecular weights, the purified enzyme sample gave only one band, this means that there is no contamination from other proteins, as referred by (26,27) , since the analysis of purified ALP using polyacrylamide gel electrophoresis with SDS revealed that there was no detectable contamination, as it represented just one distinct band and the molecular weight of the subunit about (97 KD).

Previous studies showed agreement with our result and detected that purified ALP gives at (95 KD) [28] .

Table (3 -2) . Standard proteins used in determination of molecular weightof ALP by SDS-PAGE.

Molecular weight	Log	RF
250	2.4	0.06
150	2.18	0.1
100	2	0.21
75	1.88	0.31
50	1.7	0.39
37	1.57	0.45
25	1.4	0.58
15	1.18	0.8
10	1	0.91

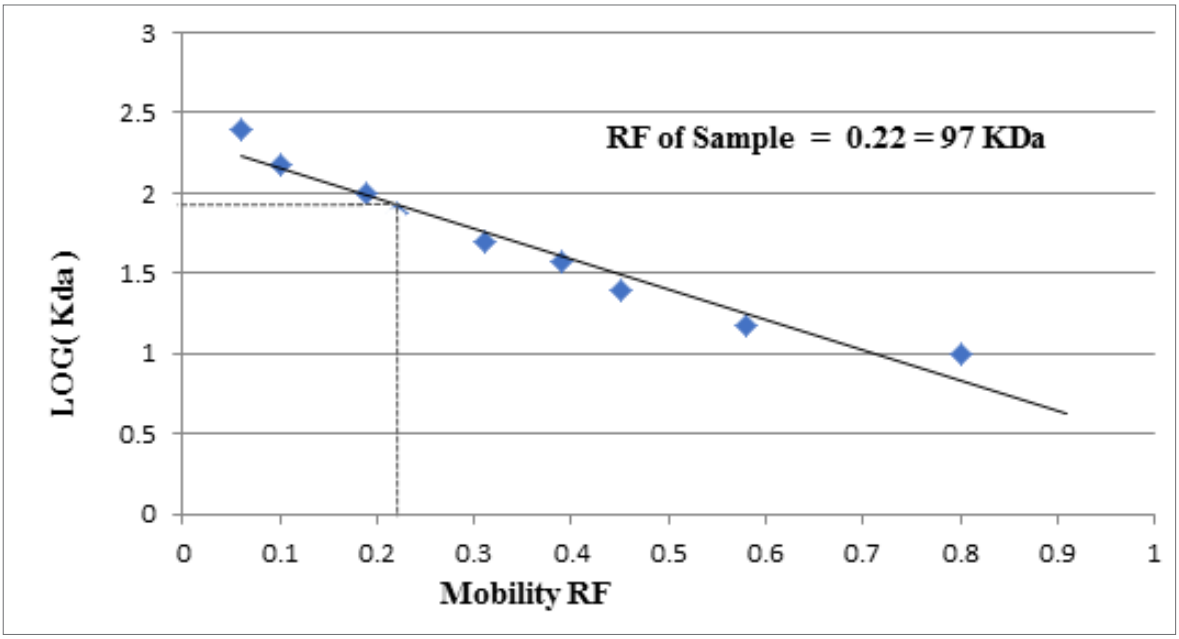

Figure (3 -3): Determination of molecular weight of the purified ALP by SDS-PAGE.

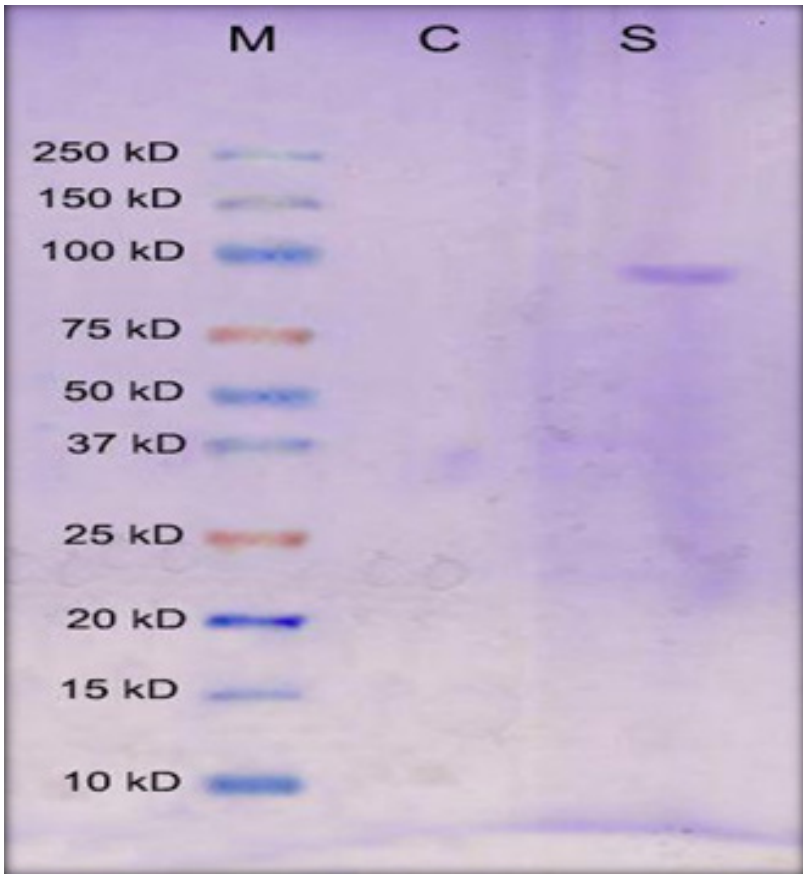

Figure (3 - 4) : SDS-PAGE stained with Comassie brilliant blue G250 for purified ALP and healthy sample .

3.2 Kinetic properties for ALP partially purified from breast cancer patients

3.2.1 Effect of substrate concentration on enzyme activity

The activity of the ALP was measured in the presence of different concentration of DPP as a substrate. It was found that the maximum activity of the ALP was obtained by using (5) mM of DPP as show in Table (3 -3).

Table (3 -3) The effect of substrate conc. on ALP activity

Conc.[S]mM	Activity IU/L
0.5	**13.7**
0.75	**18.6**
1.5	**27**
2	**32**
3	**37.4**
4	**41**
5	**42.8**
6	**40**

figure (3 -5) shows increasing enzyme interaction velocity with increasing concentration of DPP, also the same figure shows that purified ALP enzyme from serum subjected to Michaelis-Menten equation where the resulted figure is of hyperbola and these results agree with Ul-Qader et al., [29] and Mahesh et al(30), who used Paranitrophenol phosphate(PNPP) also agree with what Al-Taii [21] .

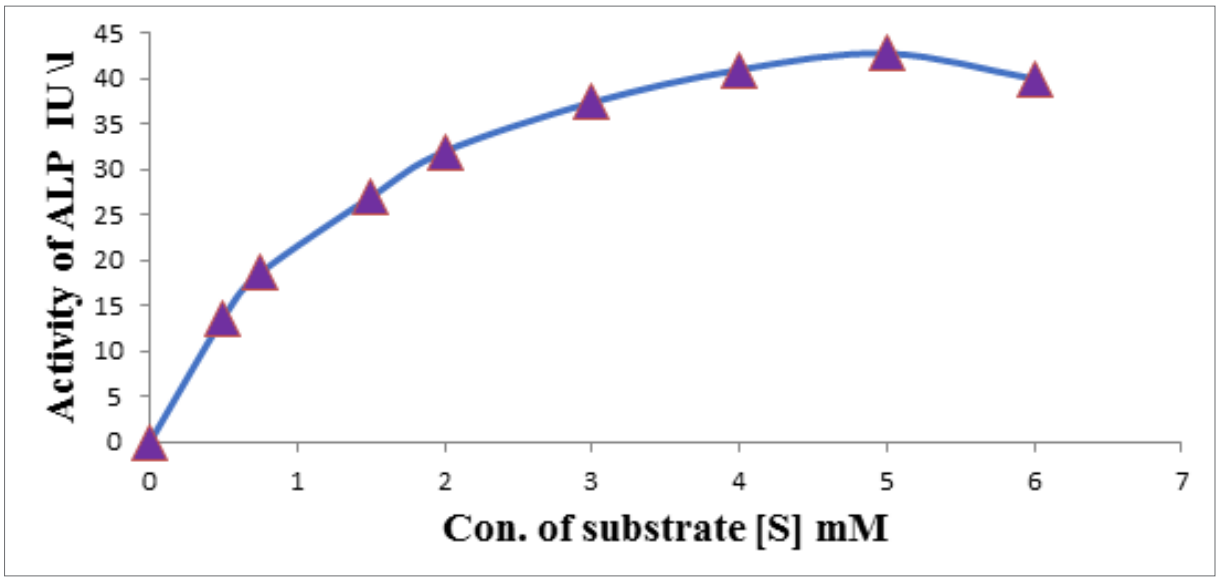

Figure (3 -5) : (Michaelis–Menten) plot show effect of substrate concentration on ALP activity.

3.2.2 Calculation of Km and Vmax

Results in our study showed from Line weaver–Burk plot by plotting the reciprocal of the initial velocity versus the reciprocal of the substrate concentration. A linear relationship was obtained with a Km and Vmax value of the enzyme were 1.4 mMol and 54.3 mMol/L/min ,respectively.

While Cho-Ngwa et al,. has pointed out to study the purified Alkaline phosphatase from Onchocerca Km value 0.56 Mm with the substrate (PNPP)(31) and Zhang et al., study,has pointed out the purified Alkaline phosphatase ALP from Geobacillus thermodenitrificans that Michaelis–Menten constant reached 31.5 mM [32] and the value of Michaelis–Menten constant for ALP in KIBGe-HAS equals to 2.74 mM [29], while Al-Taii [21] has reached that Michaelis–Menten constant value for Alkaline phosphatase from diabetic saliva equals to 5.88mM.

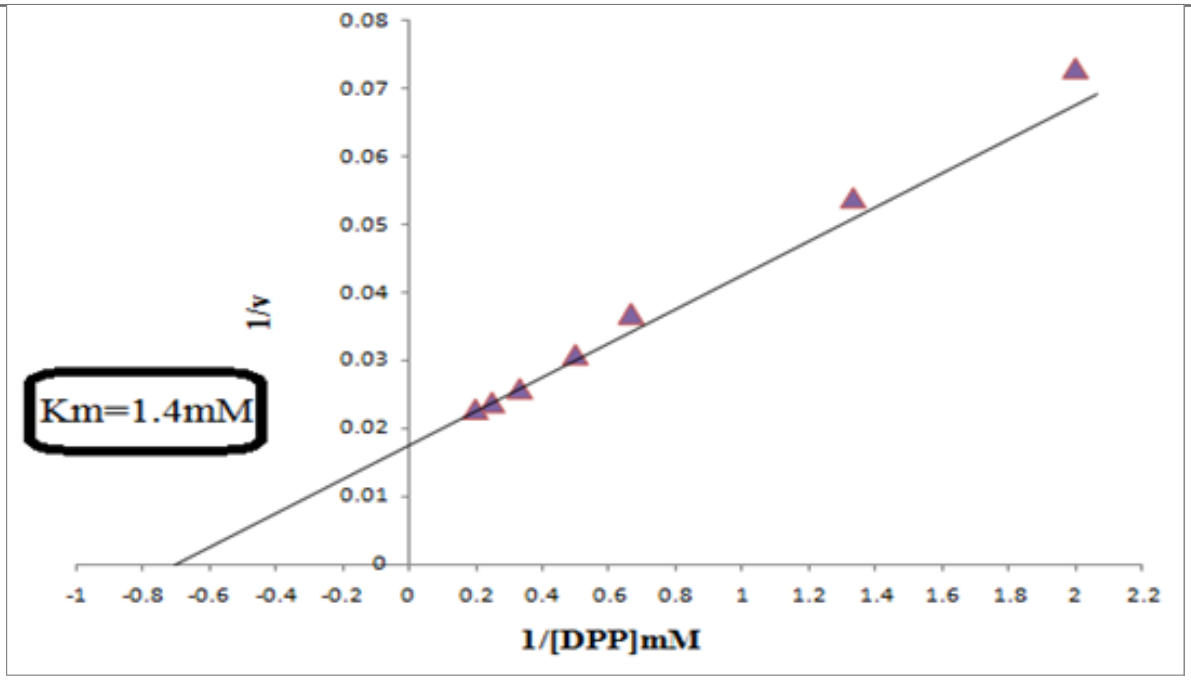

Figure (3 -6): Parameters of (Km and Vmax) by Line weaver–Burk plot for ALP purified from serum of breast cancer patients.

3.2.3 Effect of pH on enzyme activity

The different effect of pH on the ALP enzyme activity was studied. as show in figure (3 -7).The results showed that maximum enzyme activity was at (pH 10) for purified ALP.

The velocity of enzyme-catalyzed reactions depends on pH, it's also act as a factor in the stability of enzymes. Enzymes have optimum pH and frequently give bell-shaped curves of velocity against pH, even though other shapes have been observed [13].

Extremely high or low pH values generally result in complete loss of activity for most enzymes ,decreasing in enzyme activity at low pH may be due to consequence of pH environment of reaction in ionic groups of active site, or changing in ionic state for substrate, or complex enzyme-substrate at the concentration of substrate above than Km, if the substrate concentration is small , it will depend on enzyme [33].

The pH has also an effect on the tertiary structure of the enzyme, therefore , on its activity, so that the enzyme may be irreversibly denaturized at extreme pH value [34].

There are many studies in the effect of pH on ALP activity, The results of

showed that the optimum pH for ALP was 10 these results agree with Al-Taii et al (21) for ALP purified from diabetic saliva ,and Raimi et al., (35). Other study found that the optimum (pH =9) for ALP in Geobacillus thermodenitrificans(32) , and (pH =8.8) in Bacillus spp. (30).While in the study was carried on beans roots optimum pH value (8) [36].

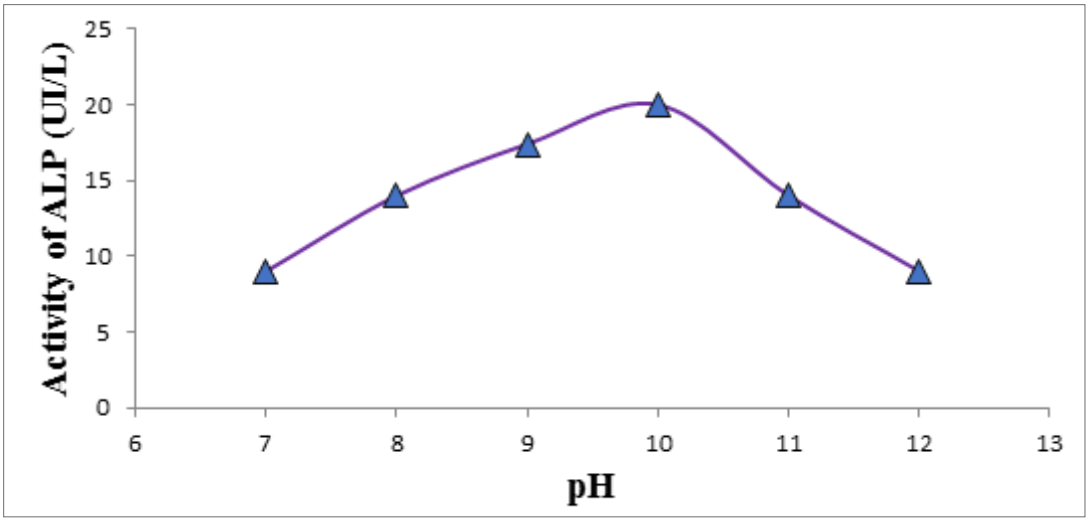

Figure (3 - 7) : Effect of pH on ALP activity .

3.2.4 Effect of temperature on enzyme activity

Different temperatures were tested to determine optimal temperature for ALP activity (from 7 to 57)ºC, the optimal temperature was (37ºC) and the results showed that there was elevate in ALP activity with increasing the temperature followed by reducing in ALP activity as shown in figure (3 - 8).

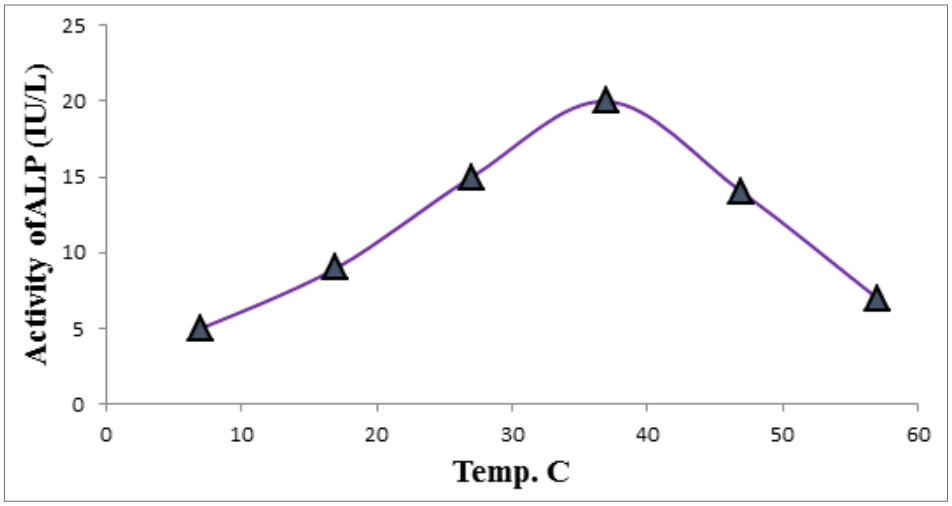

Figure (3 -8): Effect of temperature on ALP activity.

The result of the present study showed that the change in activity of serum ALP above and below optimum temperature, This might be due to the alterations in the ratio of isoenzymes in the serum of patient for the reason that isoenzymes have not the same stability to temperature. The tertiary structure of an enzyme is kept principally by amounts of non-covalent links, When molecule absorbs much energy the tertiary structure will broke, and enzyme will be denatured, which loses enzymes activity [37] while below 37oC. ALP activity decreases due to insufficient energy that required to perform enzyme substrate complex.

this result agree with Al-Taii [21] for ALP purified from diabetic saliva and Morales et al., that optimum temperature for ALP purified from beans roots is 37oC [36], and Njoku et al., results have shown that the optimum temperature for the enzyme ALP work in Rabbit liver is 45OC [38] .

3.2.5 Effect of incubation time on enzyme activity

The figure (3 - 9) shows enzyme activity dependence on incubation time (0 – 25 minutes). thirty minutes (15 min) was adopted as the standard incubation time throughout the work because it relates to a linear region of the curve and presents reliable absorbance values.

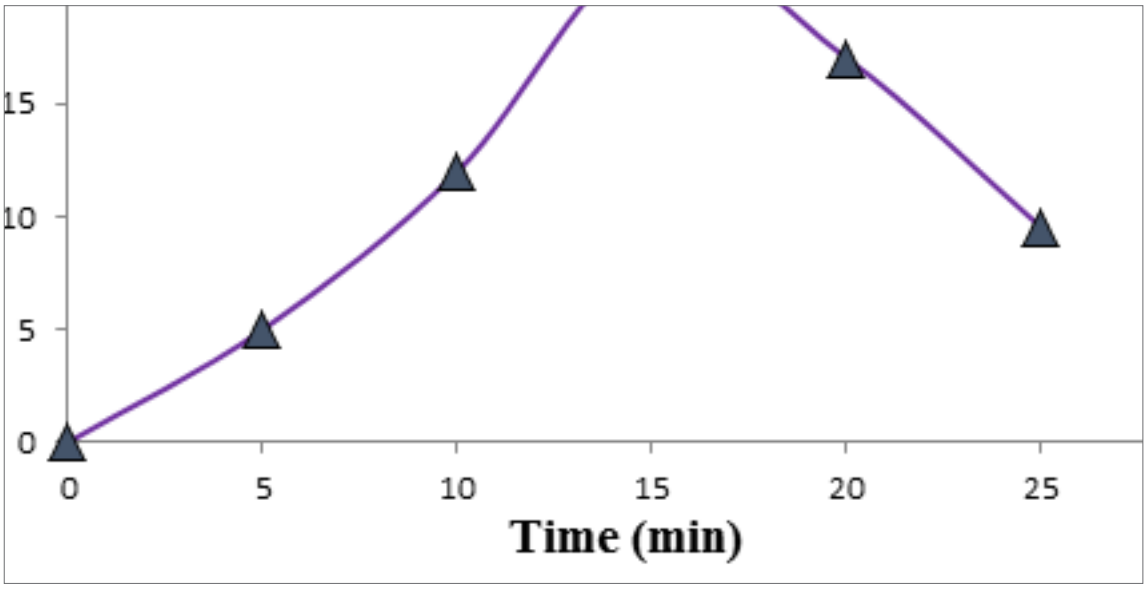

Figure (3 - 9) : Optimization Incubation time of ALP

This may related to the temperature ascribed to the enzyme where the more time the heat break the bonds between two Amino acids, the time stability results are agree with results of Raimi et al., [35] and Al-Taii [21].

3.3. The Effect of Metallic Ions , Amino Acids and complex on Purified ALP from Serum Breast Cancer Patients

3.3.1 The Effect of Metallic Ions and EDTA on Purified ALP

Inhibition and activation studies by ions are regarded a model in used methods in Enzymology to identify active centers upon enzyme surface . Mechanics illustrate the effect of these metallic ions on enzyme's activity through making bridges between the enzyme and substrate or occurring changes in enzyme structure which helps in activation process or inhibition enzyme [39] .

Accordingly a study was made on the effect of some Ions (Co^{+2}, Mg^{+2}, Ni^{+2}, Zn^{+2}, Cu^{+2}). Also chelating factor (EDTA) upon the activity of enzyme ALP purified from serum breast cancer patients . Several concentrations for this ion (1 ,3,5,10) mM added to the mixture of enzyme interaction containing 5mM from substrate (DPP) and buffer solution (carbonate-Bicarbonate) pH 10.

Ions (Co^{+2}, Mg^{+2}, Ni^{+2}) showed an active effect on enzyme when above concentration was used . The highest percentage of the activation was at concentration(10mM) (62. 67% , 52.35% , 64.83%) of ions (Co^{+2}, Mg^{+2}, Ni^{+2}) respectively as illustrated in the table (3 -4) .

Results have agreed with Qiao et al.,[40] and Ying et al.,[41] ,and Al-Taii [21] . Proposal was made for strong interaction occurring between metallic ions and the enzyme because of having ALP some mineral related with active site when studying the effect of metallic ions upon ALP activity in Rhizobium sp [42] .

ion (Cu^{+2}, Zn^{+2}) have showed inhibition effect for the enzyme .highest rate inhibition effect at concentration (10mM), (Zn^{+2}) inhibition rate (53.86%) and (Cu^{+2}) inhibition rate (71.65%) as illustrated in the table (3 -5)

Results have agreed with Yan et al., attributed occurring of inhibition to complicated formation between (Zn^{+2}) and substrate [43]. also what we have reached was similar to what was reached by Al-Taii [21]. Studies of

Marhova and Kostadinova agreed with inhibition of the enzyme by (Cu^{+2}) but (Zn^{+2}) did not show any effect upon the enzyme [44] .

Table (3 -4): Percentage of activation of ALP purified using metal ions at different concentrations

Metal ion	Activation %			
	1mM	3mM	5mM	10mM
Co^{+2}	34.56	46.45	53.95	62. 67
Mg^{+2}	29.82	36.83	44.93	55.35
Ni^{+2}	32.76	43.22	54.68	65.83

Table (3 -5) : Percentage of inhibition of ALP purified using metal ions and EDTA at different concentrations

Metal ion	Inhibition %			
	1mM	3mM	5mM	10mM
Zn^{+2}	12.4	21.37	43.74	53.86
Cu^{+2}	10.73	53.45	60.87	71.65
EDTA	13.3	50.74	73.64	82.97

ALP is one of the enzymes that metalo – dependent therefore it activity is reduced at adding EDTA to enzyme blending interaction for linking with EDTA with metallic ions (Mg^{+2} and Zn^{+2}) existed in active site of the enzyme and formation of chelating complex between EDTA and ions of (Zn^{+2}) and (Mg^{+2}). inhibition of enzyme at concentration 10mM at rate of (82.97%) and these results identical with other previous studies in chelating factor has inhibited (EDTA) alkaline phosphatase in Aspergillus caespitosus [45] , and strongly inhibited the purified enzyme from the goat kidney [46], besides what it was reached is similar to Mahesh et al.[30],and Al-Taii [21] .

Wang et al.,[47] has proposed that inhibition ALP (intestinal) occurs with competitive complex mechanism because the complex formed (EDTA – Enzyme) which leads to change in enzyme form by displacing (Zn^{+2}) from the enzyme .

3.3.2. The Effect of some Amino Acids on Purified ALP

The effect of some amino acids has been studied upon the velocity interaction enzyme ALP which is partially purified from serum breast cancer patients in which these amino acids (Cys ,Trp , Phe , Leu) with concentration of (1,3,5) mM on inhibition of enzyme with uneven inhibition rate , the highest inhibition degree at concentration 5mM (51.42% , 56.67% , 63.7% , 80.2%) for acids(Phe , Trp , Leu and Cys) respectively .Table (3 -6) illustrates the percentage of amino acids inhibition in above – mentioned concentration .

Table (3 - 6) : Percentage of inhibition of ALP purified using Amino acids at different concentrations

Amino acid	Inhibition %		
	1mM	3mM	5mM
Cys.	45.7	64.58	80.2
Leu.	42.4	56. 86	63.7
.Phe	36.1	44.92	51.42
Trp.	38.7	47.32	56.67

What has been reached is similar to a comparative study 5'-nucleotidase and ALP in human's placenta [48] . Also inhibited the two acids Phe , Leu the ALP enzyme in human's sperm tumor [49]. Regarding inhibition of enzyme by amino acid Cys. Agrees with what has been reached by Funk Kannampuzha et al.,[50] .

4. Conclusions:

Obtain three isoenzyme of the ALP enzyme when separated by ion exchange chromatography. Obtain (Km, Vmax), Optimal temperature, pH , incubation time and optimum substrate concentration for purified isoenzyme action, and the calculated M.wt was 97

KD. Activation isoenzyme purified by metal ions (Co + 2, Mg + 2 and Ni+2) and inhibited by Zn + 2 , Cu + 2 and EDTA. Inhibition isoenzyme purified by some amino acids (Phe., Cys., Leu., Trp.) .

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