# Partial purification of ALP from serum of obesity patients in Tikrit city

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**Abstract**

This study was done for partial purification of Alkaline phosphatase from the serum of obesity patients using the ion exchange chromatography technique, using DEAE- cellulose, and the Gel filtration technique, using Sephadex G 100. The results showed three peaks for purified ALP after elution by tris - HCl buffer solution. The first peak appeared between (3-10) tubes, the second active peak appeared between (13-15) tubes, and the third active peak appeared between (21-23) tubes. In the ion exchange technique, the ALP activity was still higher than normal level, but there was a decrease as compared with ALP activity in crud enzyme; the decrease was from (120 IU/l) after the ammonium sulphate concentration step to (85, 63, 71 IU/l) after ion exchange chromatography. The results of gel filtration indicate that three protein peaks also appear due to the enzyme alkaline phosphatase and the enzyme ALP. Upon tracking the activity of the ALP enzyme, it is found that the peaks give the high specific activity of the enzyme, reaching (77, 59, and 37) enzyme unit/mg, respectively.

## 1. Introduction

Obesity has reached epidemic proportions that affect different ethnic, age and socioeconomic groups [1]. A survey conducted by the Global Burden of Disease encompassing 195 countries revealed that, in 2015, the obese population doubled in about 70 countries compared to the findings of a similar survey performed in 1980, reflecting more than 603 million adults worldwide [2]. Body Mass Index (BMI) is a measure of weight adjusted for height. It is calculated as weight in kilograms divided by the square of height in meters (kg/m2). Obesity has been defined based on an arbitrary cut-off of BMI >30 kg/m2[3].

### Etiology

The etiology of obesity can be studied from a phylogenetic and/or ontogenetic perspective. From a phylogenetic perspective, the human race seems to inherit evolutionary traits that are predisposed to conserve fat storage as a survival trait [4]. There are different types of influences that may or may not corroborate with the establishment of obesity during the developmental process of each individual, being a consequence of the interaction between genetic load and intrinsic and/or extrinsic factors. Egger and Swinburn proposed a cological model to understand the origin of obesity that encompasses three major influences: environmental, biological and behavioural [5].

Environmental and biological influences undoubtedly impact human behaviour, and in obesity, some behavioural variables may be compromised [6, 8].

### Pathophysiology of obesity

The pathophysiological condition of obesity is directly related to location and accumulation of white adipose tissue, which determines the onset of comorbidities related to obesity. Subcutaneous adipose tissue (SAT) differs greatly from visceral adipose tissue (VAT) [9].

The SAT is present in the most superficial layers of the body with protective metabolic characteristics, since it is less metabolically active and is responsible for capturing free fatty acids (FFA) and triacylglycerol (TAG) excess from the circulation. Despite being responsible for lipid storage, adipocytes of SAT have a determined capacity, which when exceeded, this tissue begins to lose its protective character and the fat starts to accumulate in other sites unsuitable for storage, such as the liver, skeletal muscle and heart, contributing to the development of comorbidities [10, 11]. On the other hand, VAT is located close to organs, which potentiate its metabolic activity [5].

Chronic positive energy balance increases adiposity, which, to a great extent, impairs blood flow, and as such, some regions of adipose tissue may suffer from hypoxia. This impairment in blood perfusion results in the activation of transcription factors related to endothelial growth and hypoxia, such as hypoxia-inducible factor 1-alpha (HIF-1α), which activates the production of chemoattractant proteins and leptin [12].

These factors lead to greater infiltration of different immune cells into adipose tissue and produce more cytokines such as TNF-α and IL-6, which will increase lipolysis as an attempt to reduce adipocyte size and increase oxygen supply. At the same time, a higher release of TNF-α activates adipocyte apoptosis attracting more monocytes, lymphocytes and other immune cells within the adipose tissue, potentiating immunometabolic stress, figure (1) [5, 13].

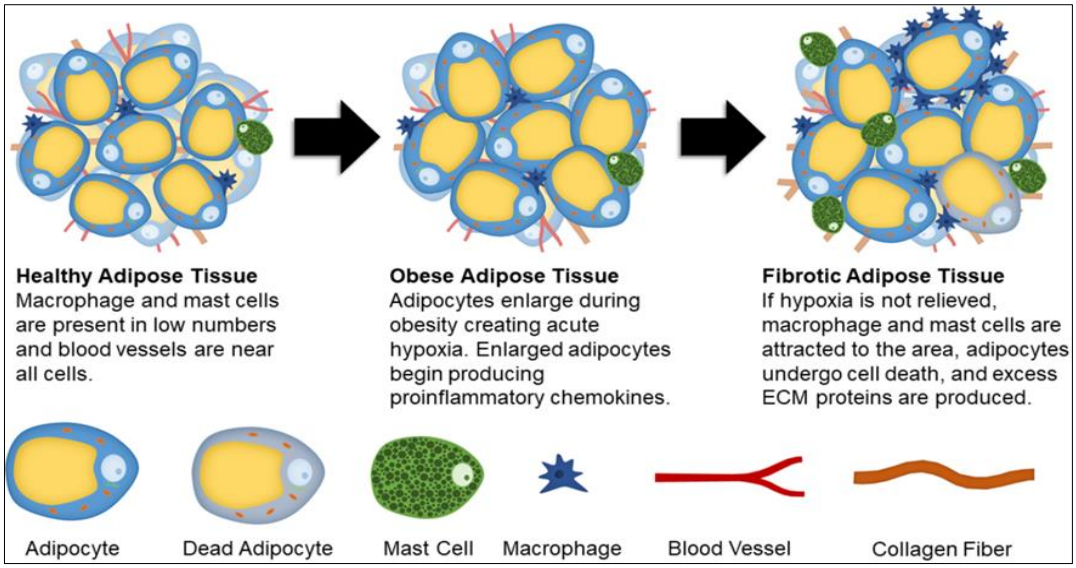


**Fig 1:** Inflammation by hypoxia of adipose tissue [14]

In addition to adipose tissue hypoxia, endotoxins can trigger inflammatory pathways. High fat intake increases gut permeability, which allows products of Gram-negative bacteria, known as lipopolysaccharides (LPS) to enter the bloodstream and infiltrate body tissues. LPS endotoxin is able to bind to receptors known as Toll-like (TLR), which are abundant in immune cells, triggering pathways related to expression, production and secretion of inflammatory cytokines [5]. Another pathway responsible for chronic low-grade inflammation is related to signaling pathways activated by FFA, particularly saturated fatty acids, which may come from dietary sources, or due to positive inflammation feedback itself [15, 16].

**Effects of obesity on health and metabolism**

**a) Diabetes mellitus, insulin resistance, and the metabolic syndrome**

The risk of type 2 diabetes mellitus increases with the degree and duration of obesity, and with a more central distribution of body fat. Both increased insulin secretion and insulin resistance result from overweight [17]. In addition, in obesity, insulin resistance (IR) may become systemic due to inflammation of adipose tissue. IR is the failure of response to insulin in tissues [18]. In obesity, IR seems to function by minimizing adipocyte anabolism in order to avoid further energy storage. However, this condition forces the pancreas to produce a surplus of this hormone, leading to long-term hyperinsulinemia [12, 19].

**b) Hypertension**

Blood pressure is often increased in overweight individuals. Overweight and hypertension interact with cardiac function. Hypertension in normal-weight people produces concentric hypertrophy of the heart with thickening of the ventricular walls [20]. In overweight individuals, eccentric dilatation occurs. Increased preload and stroke work are associated with hypertension. The combination of overweight and hypertension leads to thickening of the ventricular wall and larger cardiac volume [21].

**c) Kidney and urological disease**

Overweight may also affect the kidneys. An obesity-related glomerulopathy characterized as focal segmental glomerulosclerosis has increased. More recently, an increase in body weight, even in the normal weight range, is associated with an increased risk of chronic kidney disease. Obesity is an important risk factor for urinary incontinence that can be reversed by weight loss [22].

**d) Gastrointestinal diseases**

Overweight may also be a contributing factor in gastro-esophageal reflux disease [23].

**e) Liver and gallbladder disease**

- Dyslipidemia, which is characterized by hypertriglyceridemia, reductions in high-density lipoprotein cholesterol (HDL-c), and increase in VLDL and low-density lipoprotein cholesterol (LDL-c), is an important comorbidity that is frequently found in NAFLD patients. Emerging data suggest that lipid profile parameters may be associated with NAFLD severity and the development of NASH and liver fibrosis [24].
- The total proteins in serum are made of albumin, globulin and fibrinogen. Research on human proteins has been directed toward problems of physiological and biochemical interest. The serum proteins play important roles as biological buffer systems, maintenance of osmotic pressure of blood, control of blood pH, transport of lipids, in immunological reactions and other functions [25].
- Alkaline Phosphatase (ALP) is a membrane associated enzyme that catalyzes the release of phosphate via cleavage of the phosphate ester bond (E.C.3.1.3.1). The enzyme is found in virtually all forms of life, spanning bacteria to mammals, and in all species the active site of the enzyme contains two Zn2+ and one Mg2+ [26]. As indicated by its name, the pH optimum of this enzyme seemingly is in the alkaline range (pH 8.0 to 10) [27]. In humans, ALP is a family of four isoenzymes with differing amino acid sequences indicating that four different genes

code for ALP. These four isoenzymes commonly are identified by their primary tissue of origin: placenta, intestine, germ cells, and "tissue non-specific", so named because high concentrations of this isozyme are found in liver, bone, and kidney [26]. Intravenous infusion of various isoforms of ALP have been used to treat a variety of disease states. The only approved use of this enzyme is an ALP modified to improve its "bone seeking potential" in children with hypophosphatasia. Other conditions in which ALP has been employed with variable/questionable benefit include: infant cardiopulmonary bypass (to enhance the conversion of adenosine monophosphate to adenosine), acute kidney injury, sepsis, and inflammatory bowel disease [27].

## 2. Materials

Sodium hydroxide, Tris-HCl, Hydrochloric acid were purchased from Sigma-Aldrich, Glass wool, DEAE-Cellulose, ammonium sulphate was purchased from AppliChem. Sephadex G-100 was purchased from Pharmacia Fine Chemicals, ALP Kit, AST Kit, ALT Kit, Glucose Kit, Total Protein Kit, cholesterol Kit, HDL-C Kit, LDL-C Kit, VLDL-C Kit, Triglycerides Kit were purchased from Biolabo, all chemicals were high purity, analytically and were used as received without further purification. Deionized water was used was used in the preparation of buffer solution.

### Preparation of samples

Blood samples were taken from one hundred (55 female, 45 male) from obesity patients 60 and control 40 from Salah el-din general hospital during the months of December 2021 to March 2022, the participants ages ranged from (20 to 60) years and divided into two groups:

- **Group 1:** There were 60 of obesity participants in total, (35 female and 25 male), ages ranged from (22-60) years, all of them are obesity patients.
- **Group 2: Control:** The control group included a total of 40 participants (20 male and 20 female) apparently healthy; ranged from (28 to 60) years.

For specimen collection (5ml) disposable plastic syringes used to draw of venous blood from each group. After coagulation, serum was separated by centrifugation for 10 minutes at (6000) rpm. Serum was obtained and stored at -20C in small epindroof tubes with a capacity of 1.5 mL until analysis.

The Data were collected from participants as in the table (1) below:

**Table 1:** The data of participants

| No. | Name | Gender | Age | Length | Weight | Marital status single or married | Living | Comor-bidities |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

### Partial purification of ALP enzyme from serum of obesity patients

### Precipitation of enzyme by ammonium sulphate

Solid ammonium sulphate is added gradually to the 10 ml blood serum at a saturation rate of 60%, by adding 6.5 g under cooled conditions, and the addition is gradual with continuous stirring for 60 minutes at a temperature of 4°C, and then leaving the solution for 24 hours at 4°C (in the refrigerator) to complete the precipitation process. The precipitation process with ammonium sulphate is the first stage of purification which the proteins present in the blood serum are deposited depending on the degree of saturation of the blood serum with ammonium sulphate [28], as ammonium sulphate competes with proteins for dissolution and interaction with water molecules, so protein molecules tend to aggregate with each other at high concentrations due to the strong protein-protein interactions that overcome protein-solvent interactions.

### Dialysis

The dialysis has been carried out by placing the protein solution (Precipitate) into the washed dialysis tubes and tightly tied from the bottom in advance, and after placing the sample in the tube, the top of the tube is tied with a thread tightly, then washed with distilled water and dipped in a volumetric container containing 2.5 liters of a buffer solution 10 mM Tris-HCl pH 7.2.

The dialysis process has been carried out at 4°C, and the dialysis continued for 24 hours, taking into account the replacement of the dialysis solution three times, and then left for the next day, the ions resulting from the sedimentation salt as well as from the proteins molecular weights less than 10 kDa are removed.

### Preparation of DEAE- cellulose ion exchange column

Fifty grams of diethylaminoethyl-cellulose (DEAE-Cellulose) anion exchanger resin has been weighed, then the tris-HCl buffer at a concentration of 10 mmol /L and a pH of 7.2 (prepared by adding 1.5760 g of tris-HCl, dissolved in deionized water and adjust by NaOH) is added as 20 ml/g dry weight of the resin.

A glass column with dimensions of 2.3×70 cm is used and the column is filled with DEAE-Cellulose by pouring it gently in the column, after that most of the excess buffer solution has withdrawn from the column and then another amount of DEAE-Cellulose is added. This process is repeated until the level of the resin reached a height of 20 cm.

Then it is allowed to stack by allowing the buffer solution to descend from the lower hole of the column, taking into account keeping its level constant to the required height by adding a new number of suspensions (DEAE-Cellulose) whenever its level decreases due to its compactness, the column was left for 24 hours at 4°C for stability, the separation column has been put in a cold room during use, to preserve the enzyme activity during the purification process [28].

After preparing the column DEAE-Cellulose, the sample solution is passed by injecting it quietly at the top of the column. The filtrated portions are collected in test tubes (5 mL per tube) by manual method at the flow rate of the solution 1mL/min. The filtered protein fractions are collected and then measured.

**Preparation of sephadex gel filtration column**

5 g of Sephadex G100 Gel has been prepared according to the instructions of Pharmacia Fine Chemical Company. The gel was suspended in 10 mM Tris-HCl pH 7.2 and, 100 ml of deionized water was added and mixe well and left for 24 hours at 4°C, and the gases also removed from the gel granules because their presence reduces the velocity of the osmotic solution flow through the column. After that, the gel was poured into a 2.3×15 cm glass column. The equilibrium of the column was carried out by adding enough amount of 10mM Tris-HCl buffer pH 7.2 until the pH of the leaching fractions become 7.2 with a rate of flow of (1ml/min). After column preparation, the concentrated enzyme solution produced from dialysis methods was introduced into the column and eluted with 10mM Tris-HCl of buffer pH 7.2 at a flow rate of 1ml/min. The eluted fractions are of 5mL volume. The total protein in each portion was measured at 280 nm. The volumes of the peak fractions containing enzyme activity were measured. Protein levels in peak fractions were also estimated to determine the specific activity of an enzyme. The purified fraction's enzyme activity and protein content were determined, and the collected fraction (purified enzyme) was kept frozen for characterization purposes.

**Determination of ALP by HPLC**

The analyte was separated by a reversed-phase HPLC using C18-ODS (20 cm × 4.6 mm) column, and applying an isocratic mobile phase composed of distilled water: acetonitrile (50:50 v/v) at flow rate 1ml/min the absorbance was measured at a wavelength of 319nm.

**Results and discussions**

**Concentration of ALP by precipitation by ammonium sulphate**

Protein precipitation by salting out technique using of inorganic salts such as ammonium sulphate [$NH_4(SO_4)_2$] was carried out with constant gentle stirring [29, 30]. This occurs by using varying saturating ratio to concentrate and precipitate the sample by gradual dissolving of calculated amount of solid ammonium sulphate, added slowly on ice bath [31].

The results in table (2) show that most of proteins in the sample are precipitated at partial purification in saturated degree (60 %) and this result in an increase specific activity for partial purification ALP from (1.948U/mg) in crude sample was concentrated to (1.95U/mg) after first step of purification. The purification also increases after precipitating from (0 to 1.94). 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 solution, 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. The results indicate that the salting-out is a very useful procedure to assist the purification of a given proteins.

Ammonium sulphate has been widely used in salting out for protein purification [32], because it is highly water soluble, forms two ions, a large amount of water is bond to each ammonium sulphate molecule. Therefore, as the number of ammonium sulphate molecules in solution increases, less water is available to interact with proteins that may be present [33].

**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 [34]. 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 [32].

The results in table (2) show that most of the proteins in dialysis this result caused an elevate in specific activity for partial purification ALP from (1.95U/mg) in precipitating to (3.653U/mg) after the second step in purification. This may be due to the instability of the enzyme or the 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, even though their concentration in the external water is very small. It may take up all the traces in a very large volume of water. This is not usually as important with crude preparations as in the later stages [32].

**Table 2:** Steps of ALP purification from serum of obese patients

| Purification steps | Volume ml | Activity iu/ml | Total activity | Protein concentration mg/ml | Specific activity u/mg | Recovery yield % | Fold of purification | Total protein |
|---|---|---|---|---|---|---|---|---|
| Crude | 10 | 150 | 1500 | 77 | 1.948 | 100 | 1 | 770 |
| Precipitation | 9 | 117 | 1053 | 60 | 1.95 | 70.2 | 1.001 | 540 |
| Dialysis | 10 | 95 | 950 | 26 | 3.653 | 63.33 | 1.87 | 260 |
| Ion exchange | 5 | 85 | 425 | 23 | 3.695 | 28.33 | 1.896 | 115 |
| Gel Filtration | 5 | 77 | 385 | 21 | 3.666 | 25.66 | 1.844 | 105 |

**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 exchanger. An ion exchanger usually consists of small spherical inert grains have a charged groups (functional groups) exist onto the grains'

surfaces; the charges of functional groups are neutralized by free counter-ions.

$$R+A^- + B^- \longleftrightarrow R+B^- + A^-$$

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 [35].

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

Figure (2) shows the presence of three peaks for purified ALP after elution by tris - HCl buffer solution. The first peak (elution peak) that appeared between (3-10) tubes, refers to elimination of disassociates proteins, the second active peak that appeared between (13-15) tubes, and the third active peak that appeared between (21-23) tubes where after ALP got eluted by tris - HCl buffer solution. This peak indicated that the ALP has been purified to maximum homogeneity. These results are in agreement with study of AL-Taii [36] who uses the same column, after second purification steps, the ALP activity was still higher than normal level, but there was a decrease as compared with ALP activity in crud enzyme, the decreasing was from (150 IU/l) after ammonium sulphate concentration step to (85, 63, 71 IU/l) after ion exchange chromatography as shown in table (2).

The purified ALP peak revealed specific activity (3.695IU/mg) with 2.95 folds 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) [37].

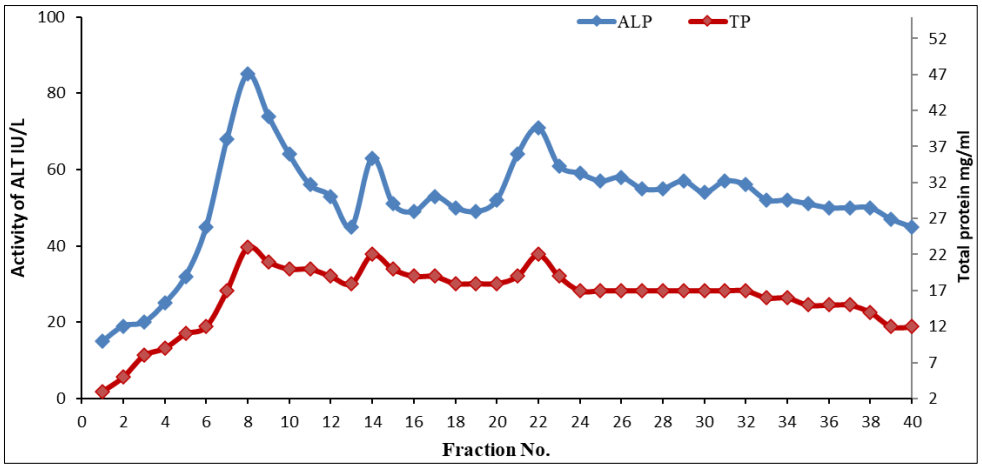


**Fig 2:** Results of DEAE-cellulose ion exchange chromatography for separation of ALP

**Gel filtration or size-exclusion chromatography**

It is used for separating the proteinous compounds obtained from the ion exchange process using a separation column with dimensions (2.3 × 20 cm) containing G-100. The results of gel filtration when passing the protein peaks (resulting from ion exchange) indicate the appearance of a three protein peaks, and its elution volume is (200 mL) (Fig. 4). Upon tracking the activity of ALP enzyme, it is found that the peaks give high specific activity of the enzyme, reaching (77, 59 and 37) enzyme unit/mg respectively, and figure. (3) below shows the results.

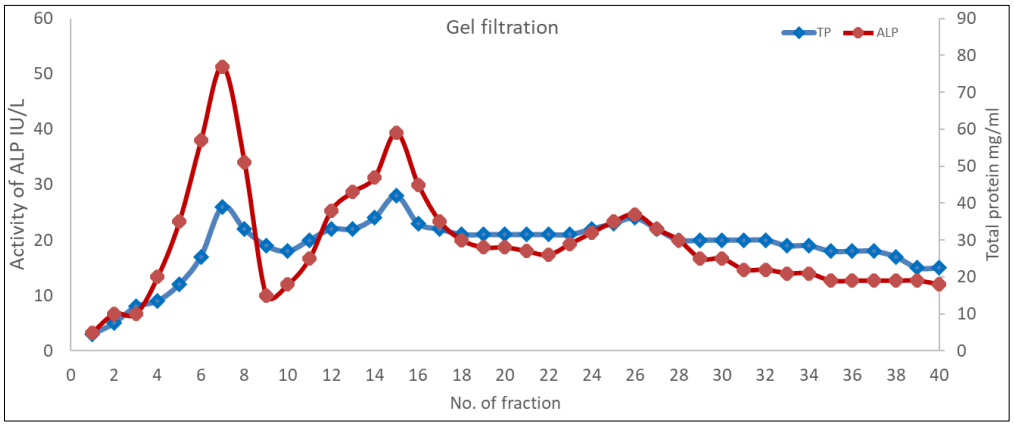


**Fig 3:** Results of Gel filtration chromatography for separation of ALP by Sephadex G100

**Evaluation of purification efficiency by HPLC**

The analyzes were performed by HPLC technique under the following operating conditions: reversed-phase column: C18 column (5µm, 150mm × 4.6mm ID).

Mobile phase flow velocity: grade water as solvent A and acetonitrile as solvent B in a 1:1 ratio

Detector: UV-vis spectrophotometer was used for testing samples at a wavelength of 319 nm.

temperature: 37 °C

The analysis results by HPLC technique showed that the standard ALP enzyme appears with a retention time of 4.10 minutes, as shown in Fig. (4).

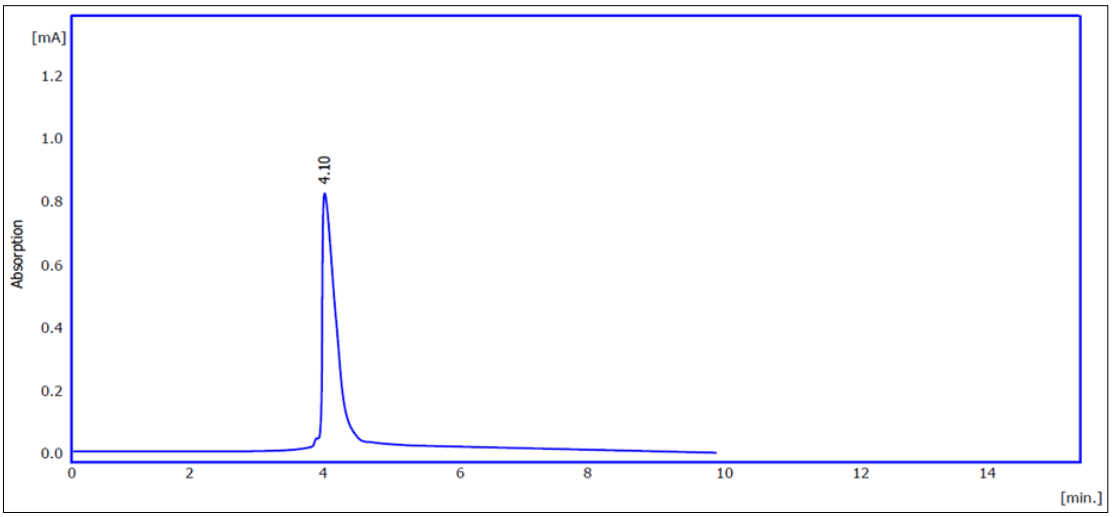


**Fig 4:** Chromatogram of standard ALP analysis

The results showed the presence of the ALP enzyme by 17.5% and a total percentage of 82.5% for the other enzymes, as there are five substances in the chromatogram, as shown in Fig (5).

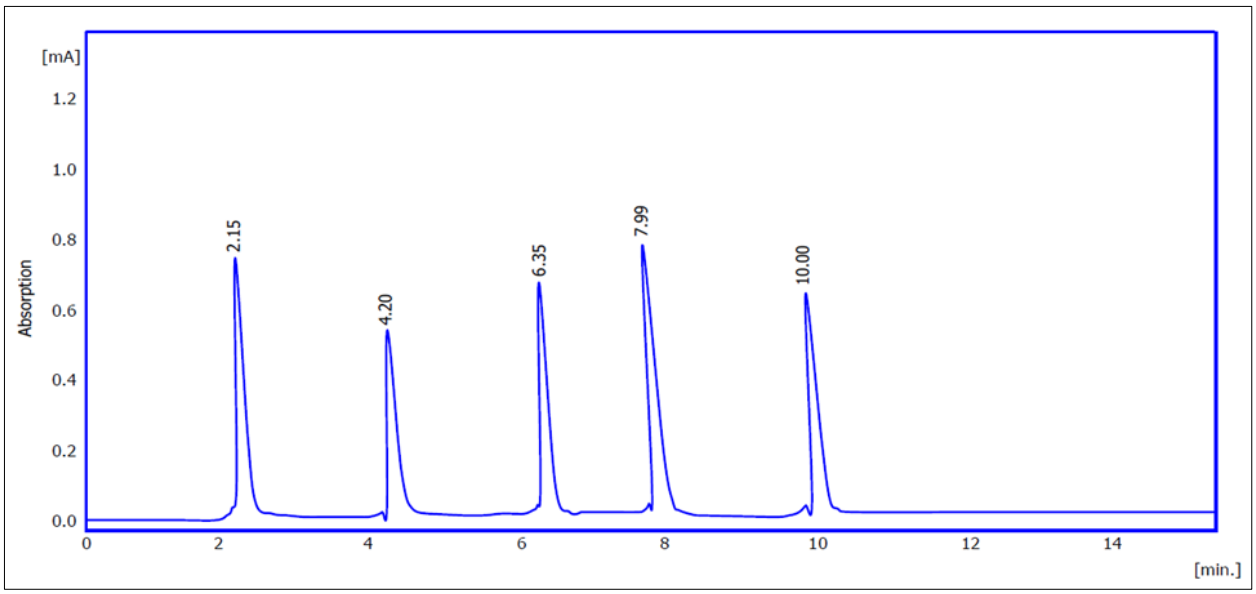


**Fig 5:** Chromatogram analysis of the purified enzyme by column No. (1) DEAE-cellulose.

**Conclusion**

The partial purification of Alkaline phosphatase from the serum of obesity patients was done by ion exchange chromatography and Gel filtration techniques. Where was deduced the ALP activity was still higher than normal level, but there was a decrease as compared with ALP activity in crud enzyme; the decrease was from (120 IU/l) after the ammonium sulphate concentration step to (85, 63, 71 IU/l) after ion exchange chromatography step. And gel filtration indicates that three protein peaks also appear due to the enzyme alkaline phosphatase and the enzyme ALP. Upon tracking the activity of the ALP enzyme, it is found that the peaks give the high specific activity of the enzyme, reaching (77, 59, and 37) enzyme units/mg, respectively.

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