Research Article   ZOOLOGY

Isolation of Alpha fetoprotein from colorectal tumor homogenates and optimizing the binding conditions with its ^{125}I-antibody

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ABSTRACT

The aim of this work is to partially purify Alpha fetoprotein (AFP) from homogenates of human colorectal (colon and rectum) tumors. The homogenates used in this work were collected from two patients groups. Group I consists of 13 patients with benign colorectal tumor and group two (II) consists of 21 patients suffering from colorectal cancer. The results revealed that the elution profile gave two peaks by using Sephadex G 200, the first peak with high molecular weight representing the complex and the second peak represents a free antibody.

The optimum conditions of binding of the partially purified AFP with ^{125}I-anti AFP antibody were carried out. Protein amounts (25μg.ml^{-1}) for benign and (18μg.ml^{-1}) for malignant, tracer antibody (1.44 mg/ml) for benign and (2.16mg/ml) for malignant group. The optimum pH was 7.4 for benign and malignant. The optimum time and temperature were (180 min and 37°C) for benign and (180 min and 25°C) for malignant groups.

INTRODUCTION

Alpha-fetoprotein (AFP) is a glycoprotein with a molecular mass of 68kDa. It consists of a single polypeptide chain and is approximately 4% carbohydrate. AFP is synthesized in large quantities during embryonic development by the liver. It is one of the major proteins in the fetal circulation, but its maximum concentration is about 10% that of albumin [1]. AFP is a secretory protein with structure and physicochemical properties similar to serum albumin (SA) [2]. The main properties of AFP are high affinity for polyunsaturated fatty acids (105 times higher than SA) and ability to bind estrogens. Some data indicate that AFP participates in immune response regulation [2-4].

Different studies confirm that the level of AFP is elevated in tumors of Gastrointestinal Tract (GIT) systems [5]. Alpha fetoprotein is found in everyone's blood. Higher levels are found in the blood of pregnant women, fetus, and young children. AFP levels can also elevate in the blood of people with certain diseases and conditions. In adults who are not pregnant, the AFP levels are elevated with certain cancer [6]. The normal production of AFP is from liver and yolk sac in fetus but some other tissues produce AFP in cases of tumors like colon, stomach, pancreas [7].

The binding studies of anti AFP antibody with AFP had been carried out [8] with fully optimization of conditions. So we are trying here to examine the optimum conditions of binding reaction after isolation and partially purification of AFP from tumor homogenates as a step for using this protocol for characterization and diagnosing the tumor.

2. Materials and Methods

Chemicals:

All chemicals and reagents used in this study were of analar grade and were used without further purification. Bovine Serum Albumin (BSA) ,Tris (hydroxy methyl amino methane) hydrochloride, EDTA, and Sucrose were obtained from Fluka company, Switzerland. CuSO4.5H2O, Na,K–tartrate ,NaOH , HCl , Na2CO3, and Folin – ciocalteaue were obtained from BDH limited pool, U.K. Immunoradiometric assay for AFP was purchased from Immunotech Bechman (France).

Instruments:

The instruments used in this work: LKB gamma counter type 1270-rack gamma II, Switzerland. Pye-Unicom pH meter, LKB ultracentrifuge type 2332, Cintra 5 UV/visible Spectrophotometer, SM–Shaker,England, PH   M62 Standard

Bilal J.M. Al-Rawi., Isolation of Alpha fetoprotein from colorectal tumor homogenates and optimizing the binding conditions

147

pH meter, Denmark. Sartorius analytical balance BL 210 S, Germany. Memmert water bath, and Memmert incubator.

Patients

A total of 34 colorectal patients involved in this study with benign and malignant tumors subjected to curative surgery. Their mean age was 49 years ranges (16–66 years). Two groups of colorectal tumor patients were involved in this study; one group with benign colorectal tumors and the other was with malignant.

According to the histopathological examination of the resected pieces, the patients were grouped into the following:

Group (I): Consisted of 13 patients with benign colorectal tumors, the range of their age were (21 -38). Group (II): Consisted of 21 patients with colon and rectum cancer. the range of their age were (41 -66).

The patients were admitted for treatment and diagnosis to the following hospitals in Baghdad:

Iraqi College of Medicine, University Hospital, Baghdad Teaching Hospital.AL–Kindy University Hospital, and Al–Yarmook University Hospital.

Patients with diseases that may interfere with this study were excluded. All surgical operations of malignant and benign tumors were done under the supervision of surgeons:

Dr. Zuhair AL–Bahraini, Dr.Saaeb Sedeq, Dr.Faleh AL–Aubaidy,
Dr.Abd AL–Salam AL–Tai, and Dr.Ma'ad Medhat.

1- Specimens Collection:

The specimens were surgically removed from patients of colon and rectum (CR). They were immediately rinsed with ice–cold saline solution, and immersed in the same solution. They were collected and stored at –20oC until homogenization. The weight of resected tissue samples range between (1.6-18) gm.

2- Preparation of Tissue Homogenate (9):

The frozen tissue were washed with ice-cold normal saline and then weighed. The samples were minced, pulverized, with a scalpel scissors in the Petri dish on ice bath, and then homogenized at 4oC in tris buffer (0.05M , pH 7.4) with ratio of 1 : 4 (weight : volume) using normal homogenizer . The homogenates were filtered through a nylon mesh sieve in order to eliminate fiber connective tissue, and then centrifuged at 4oC. The supernatants and pellets were considered cytosolic and nuclear fractions respectively. The pellet (sediment) was discard, and the cytosolic (supernatant) was used in experiments involved cytosolic cancer antigen AFP source.

Solutions:

TES Buffer solution (0.05M, pH 7.4) was prepared as follows: (3.0285gm) of tris (hydroxy methyl amino methane), 0.93060 of Ethylene diamine tetra acetate disodium salt (EDTA) and (42.7875gm) of sucrose were dissolved in 400 mL of deionized distilled water, then the pH was adjusted with HCl (1M) at 7.4 and the solution was completed to 500 ml with deionized distilled water.

Protein Determinations:

The method of Lowry (10) was used to determine total proteins in tissue and sera, using bovine serum albumin (BSA) as standard protein.

3- Partial Purification of AFP by Sephadex G 200 Column:

Preparation of The Column:

The dimensions of the column were chosen according to the following equation (11, 12):

$$\text{Diameter} = \sqrt[3]{m/10}$$

Where:

m: amount of protein in mg.

L= 30x diameter

Where

L: length of column.

Preparation of The Buffer:

Tris buffer (0.05M) was prepared by dissolving 3.0285 gm of tris (hydroxy methyl amino methane, 0.9306 gm of EDTA and 0.1 gm of sodium azide in 400 ml, the volume was completed to 500 ml with deionized distilled water, the pH was adjusted to 7.2.

Preparation of Gel:

The gel was prepared by allowing the preswollen gel to swell again in tris buffer pH7.2, then left to settle and the excess of buffer was decanted. The step was repeated several times. Suction was then used to degas the gel then the slurry was left for 24 hrs. to equilibrate with buffer.

The swollen gel was suspended and carefully poured into vertical glass column (0.9 x 27) down the wall using a glass rod. After the gel had settled the column was equilibrated with this buffer for 72 hrs.

4- Determination of The Void Volume:

The void volume of the column was determined using blue dextran 2000 at concentration of 2mg.ml–1 dissolving in tris buffer pH 7.2, the elution was carried out with the same buffer at a flow rate of 10ml.hr–1.   Fractions of 1ml were collected and their absorbance was measured at 600nm.

5- Purification Procedure:

Reagents:

Tris buffer pH (7.2) contained 0.02% sodium azide was prepared as described previously in experiment (Preparation of the buffer).

Procedure:

The sample of tissue homogenate (720 μl) of colon and benign tumor containing approximately (8 mg) proteins was applied to the surface of the gel. The elution was carried out using tris buffer (pH7.2) with a flow rate of 10 ml.hr -1, and fraction of one (ml) was collected, the elution was made at room temperature.

Calculations:

In each fraction, the protein concentration was determined using the absorbance in UV region at 280 nm, then the accurate protein concentration was determined according to Lowry method (10). The total binding of each fraction was estimated using the optimum condition of crud homogenate which had been got previously (9). The binding of each fraction was calculated and plotted against the elution volume. The specific binding activity percent was estimated from the following equation:

$$\text{Specific binding activity} = \frac{\text{Total binding B/T}}{\text{mg of protein}} \times 100$$

6- Dialysis for Concentration

After preparing a dialysis tube, the fraction that contained high levels of the binding activity were collected, pooled, and concentrated by dialyzing against sucrose at 4oC for 2 hours, to obtain the required concentration to be used in the next experiments.

7- The Choice of Optimum Conditions For The Binding of The Partial Purified AFP To 125I– Anti AFP Antibody:

The Choice of The Optimum Protein Concentration:

1. A volume of 40 μl of 125I–anti AFP Antibody (2.38 mg.ml–1) was added to increasing amounts (5, 10, 15, 20, 25 and 30 μg.ml–1) of a pooled fractions under the first peak of the partial purified colon cancer and (9, 18, 27, 36, 45 and 54 μg.ml–1) of a pooled fractions under the first peak of benign colorectal tumor, the volume was completed to 250 μl with 0.05 M of tris buffer pH 7.8.
2. All tubes was incubated for 4 hours at 25°C.
3. Two additional tubes containing 40 μl (2.38 mg.ml–1) of 125I–anti AFP Antibody only for total activity were set–aside until counting.
4. After incubation, the tubes were centrifuged at 4000r.p.m for one hour at 4oC using cooling centrifuge.
5. The supernatant was decanted and the radioactivity of the complex formed was counted.
6. The (B/T)% was calculated as described previously, then was plotted against protein concentration.

Solutions:

This buffer (0.05 M) was prepared by dissolving 0.6057 gm of tris (hydroxy methyl amino methan in 50 ml of distilled water and the pH was adjusted with HCl (1 ml) at pH (7.8). The volume was completed to 100 ml with distilled water.

The Effect of 125I–anti AFP Antibody:

1. Increasing volume of 125I–anti AFP Antibody (10, 20, 30, 40, 50, and 60 μl) containing (0.720, 1.44, 2.16, 2.88, 3.60 and 4.32 mg.ml–1) was incubated with 25 and 18 μg.ml–1 of partially purified AFP for the benign case and malignant respectively, the volume was completed to 250 μl with 0.05M tris buffer pH 7.8.
2. All tubes were incubated for 180 min at 25°C.
3. After incubation, the tubes were centrifuged at 4000r.p.m for one hour at 4oC using cooling centrifuge.
4. The supernatant was decanted and the radioactivity of the complex formed was counted.
5. The (B/T)% was calculated as described previously, then was plotted versus 125I-anti AFP antibody concentration.

The Choice of Optimum pH:

1. To choice the optimum pH for the partially purified AFP from colon colorectal tumor homogenate

(25μg.ml–1) and (18 μg.ml–1) of the benign and malignant of partially purified AFP were incubated with (30, 20 μl) (2.16, 1.44 mg.ml–1) respectively of 125I–anti AFP Antibody. The volume of all tubes was completed to 250 μl with tris buffer (0.05M) of different pH (6.8–8.0).
2. All tubes were incubated for 180 min at 25°C.
3. After incubation, the tubes were centrifuged at 4000r.p.m for one hour at 4oC using cooling centrifuge.
4. The supernatant was decanted and the radioactivity of the complex formed was counted.
5. The (B/T)% was calculated as described previously, then was plotted against the corresponding pH.

The Time Course of Partially Purified AFP:

1. To determine the time course of the partially purified AFP from colorectal tumor homogenate, 25 μg.ml–1 protein of the benign case of partially purified AFP was incubated with 30 μl of 125I–anti AFP Antibody, while 18 μg.ml–1 protein of the malignant case of the partially purified AFP was incubated with 20 μl of 125I–anti AFP Antibody, the volume was completed to 250 μl with tris buffer (0.05M, pH 7.4).
2. All tubes were incubated at 25ºC at different time intervals (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10) hours.
3. To determine the time course of the two groups of partially purified AFP at different temperature, steps 1,2 in the same experiment were repeated at different temperature (4, 37and 45ºC).
4. After incubation, the tubes were centrifuged at 4000r.p.m for one hour at 4oC using cooling centrifuge.
5. The supernatant was decanted and the radioactivity of the complex formed was counted.
6. The (B/T)% was calculated as described previously, then was plotted against the time at different temperature.
7. The concentration of (125I–anti AFP / AFP) complex formed after time t was calculated from the following equation:

$$^{125}I\text{-}anti\,\text{AFP}/\text{AFP} = \frac{\text{count (c.p.m) of }^{125}I\text{-anti AFP specificaly bound after time(t)}}{\text{Total counts (c.p.m) of }^{125}I\text{-anti AFP used in the incubation}} \times \text{ }^{125}I\text{-anti AFP}$$

Concentration of ^{125}I-anti AFP in mg.ml^{-1} after time(t) in the incubation (mg.ml^{-1})

RESULTS and Discussion

Partial Purification of AFP:

Isolation of cytosol AFP antigens was performed by gel exclusion chromatography technique. Colorectal tumor homogenate was applied to Sephadex G 200 (0.9x27 cm). Figure (1) shows the elution profile of blue dextran 2000.The volume of the buffer required to elut the blue dextran, which represents the void volume, was (10 ml). The use of blue dextran is because the high molecular weight (2 000 KDa) to make sure that the gel packing is suitable for isolation different protein from each other's depending on the difference in molecular weight [13].

The elution profile of AFP from malignant colorectal tumor and benign was illustrated in figure (2). The resultant fraction

Bilal J.M. Al-Rawi., Isolation of Alpha fetoprotein from colorectal tumor homogenates and optimizing the binding conditions

149

of the homogenate were collected, polled and detected for the binding with ^{125}I–anti AFP Antibody. All trials of gel filtration revealed two peaks profile. The first peak represents ([125]I- anti AFP antibody / AFP) complex, while the second peak represents unbound (free) ^{125}I-anti AFP antibody. The difference in molecular weight is the principle of isolation in gel filtration and depend on the exclusion. So the first peak is for the complex of AFP with its antibody which have high mass comparing with AFP which has the second peak. The binding method in ImmunoRadioMetricAssaay IRMA is highly specific and highly sensitive because it established on the immune reaction and monitored by estimating the radioactivity [14]. Accordingly, only two peaks had been got.

The Choice of the Optimum Conditions For The Binding of Partially Purified AFP with ^{125}I–anti AFP Antibody:

The Choice of the Optimum Protein Concentration:

Figure (3) shows the optimum protein concentration for the isolated AFP of the malignant and benign colorectal tumor homogenate. This experiment was carried out by adding increasing amounts of the isolated forms to fixed amounts of ^{125}I–Antibody to produce (^{125}I–anti AFP Antibody/ AFP) complex. The maximum binding occurred at 25 μg.ml^{-1} for (benign) isolated form, while 18μg.ml^{-1} was the optimum protein concentration for the binding of (malignant) isolated form. Further additions of AFP gave rise to solubilization of complex formed [15]. The excess of added antigen, which is represented by excess of protein, gives small chance to make a lattice between antibody and antigen [16]. The difference in results between two types of tumor homogenates gave different results because the whole environment is different [17].

The decrease in the binding after reaching the maximum binding may be due to the solubilization of the complex formed by the excess of AFP added [18], or may be due to the conformational changes in AFP and ^{125}I–anti AFP Antibody rather than the formation of reversible inactive (^{125}I–anti AFP Antibody / AFP) complex [19], another auther reported that when the precipitation of the complex out of the solution, due to the multivalent nature of both molecules [20]. The radioactive antibody has two binding sites, it can cross link antigenic sites of two different AFP molecules and can form maximum amount of the complex and therefore maximum precipitate will occur [20].

The Effect of ^{125}I–anti AFP Antibody:

The effect of [125]I – anti AFP Antibody concentration on the binding with isolated forms of the benign and malignant colorectal homogenate is shown in figure (4).

The maximum binding obtained at 2.16 mg.ml^{-1} for (malignant) and 1.44 mg.ml^{-1} for (benign). It was found that the amount of [125]I – anti AFP Antibody required to bind with their isolated Antigen forms is less than in crude homogenate [8]. This may be due to the increment of the epitop (the part of an antigen molecule that binds to any single antigen combining site)[21].

The binding increased when labeled antibody increased, then the binding percent decreased as the amount of ^{125}I–anti AFP antibody increased. The reason is due to the all-antigenic sites covered with antibody and the complex formation is inhibited [22]. These results indicate that the binding is principally dependent on the amount of the antibody in the reaction mixture [23], because one of the factors affecting the binding percent of Antibody – Antigen reaction is the concentration of the Antibody. According to the results of this experiment, the above concentrations of ^{125}I-anti AFP antibody were used in the subsequent experiments.

In spite of the specificity of IRMA technique, there is a difference between the crude homogenates binding and partially purified AFP binding. That is because the protein-protein interaction is affected by the vicinity of high protein concentration [24, 25].

The Choice of The Optimum pH:

In order to choice the optimum pH 25 μg.ml^{-1} and 18 μg.ml^{-1} for (benign, malignant) respectively of the two isolated forms of the colorectal tumor homogenate were incubated with 2.16,1.44 mg.ml^{-1} of ^{125}I–anti AFP antibody respectively. Figure (5) shows the optimum pH of the two isolated antigens forms. The results revealed that the optimum pH for both isolated antigens binding to its antibody was 7.4. The similarity in pH (7.4) suggests that the AFP isolated forms possess the same epitopes in both cases. That means the induction of protonation – deprotonation process occurs with the same changed polar groups on the amino acid residues present in the binding domain [26, 27].

The Time Course of Partial Purification AFP:

Figure (6) and (7) illustrate the time course of the binding of isolated antigen from malignant and benign colorectal tumor homogenate to their antibody. The malignant antigen binds to its antibody in highest state after 3 hours at 25 ºC, while benign Antigen binds after 3 hours at 37ºC.

The binding of [125]I – anti AFP Antibody to its Antigen is a time and temperature dependent process [28]. The decrease of the binding may be due to either the degradation of AFP or irreversible dissociation of the (^{125}I–anti AFP Antibody / AFP) complex. At higher temperatures, denaturation and destruction tertiary structure may occur leading to loss of activity and conformational changes. At lower temperature, heat is not enough to overcome the energy barrier, even for the catalyzed reaction [29]. Heating more than 45°C disrupt the folded structure of the protein by increasing the vibrational motions of atoms [30, 31].

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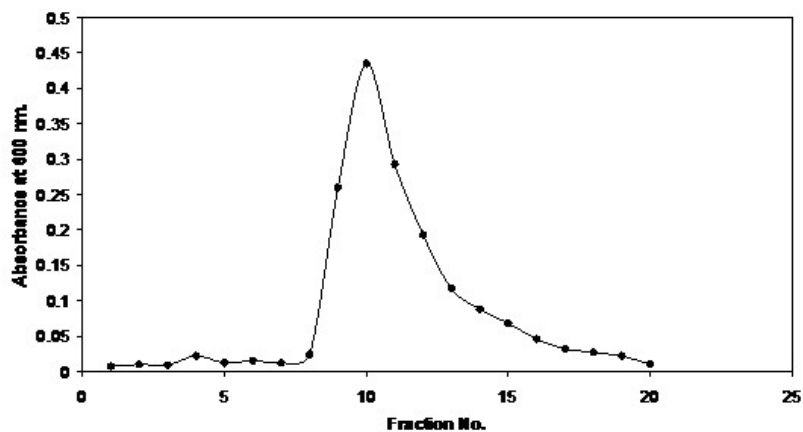George N. Somero, (1995); protein and temperature,Annual Review of Physiology, Vol. 57: 43-68.
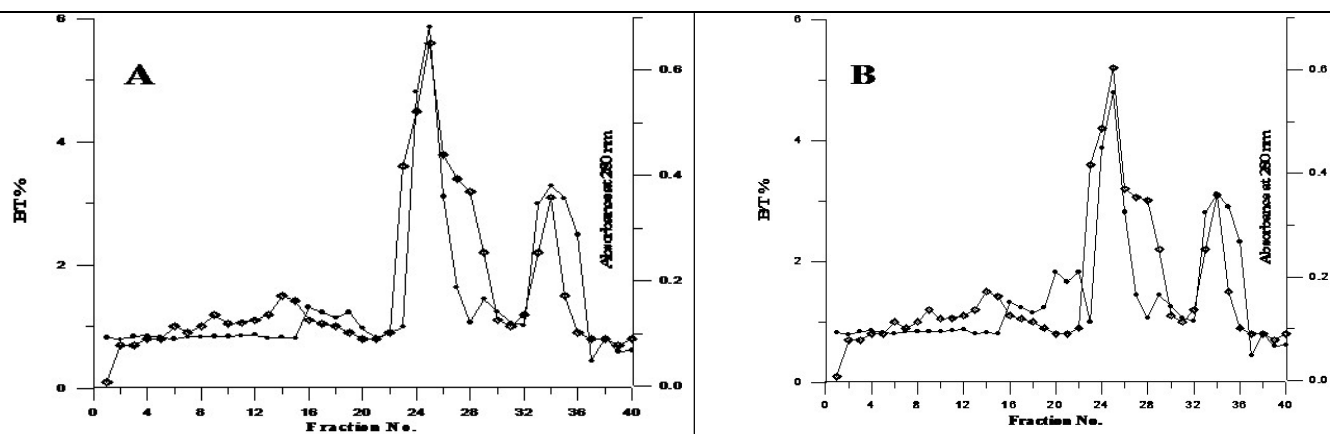
Figure (1): The elution of blue dextran 2000.

Figure (2): The elution profile of Human AFP '(.) means absorbance and (◇) means B/T%'. (A) Benign colorectal tumor, (B) Malignant colorectal tumor
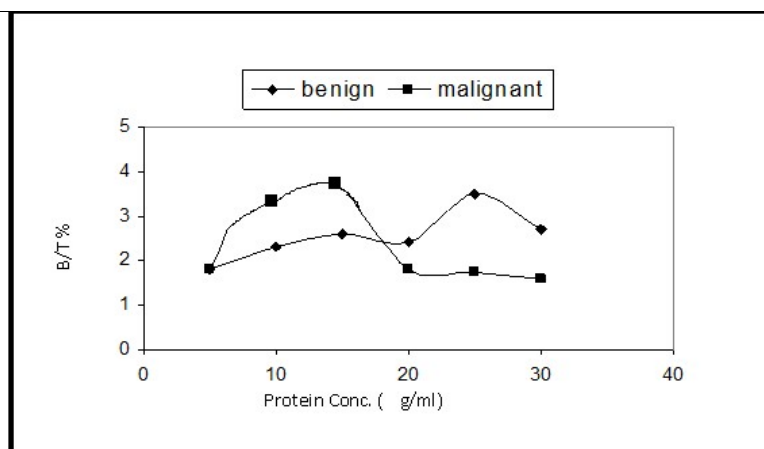
Figure (3) :Influence of Protein Concentration on the binding of 125I–anti AFP Antibody with partially purified AFP from Colorectal tumor homogenate.

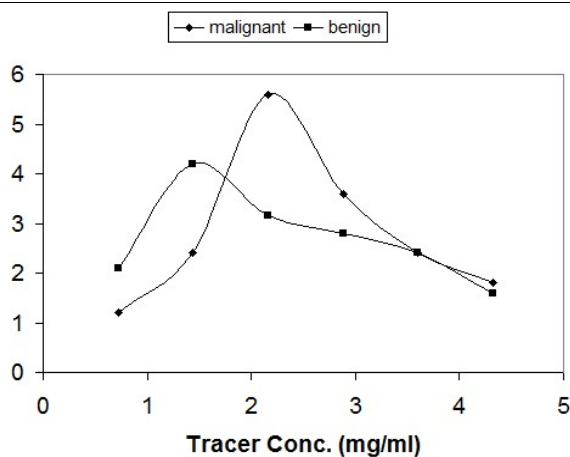
Figure (4): Effect of 125I–anti AFP Antibody concentration on the binding with partially purified AFP from Colorectal tumor homogenates.
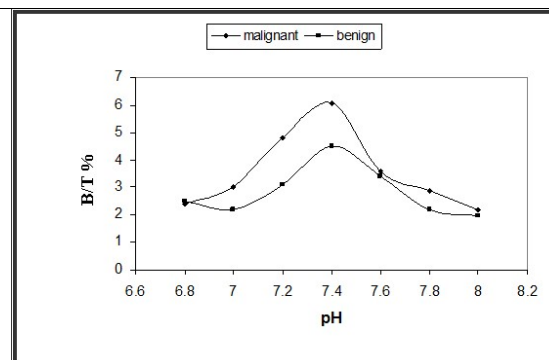

Figure (5): pH effect on the binding of 125I–anti AFP Antibody with partially purified AFP from Colorectal tumor homogenates.
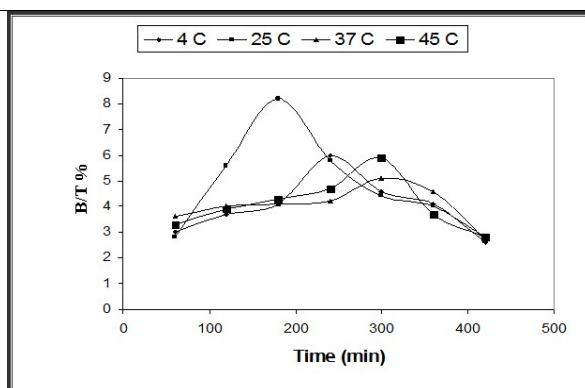

Figure (6): Time – Course of 125I–anti AFP binding to partially purified (malignant) Antigen from Colon Cancer.
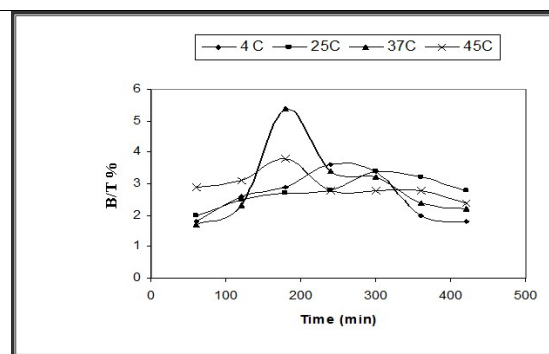

Figure (7): Time – Course of 125I–anti AFP binding to partially Purified (benign) Antigen from Colorectal tumor.

153

Bilal J.M. Al-Rawi., Isolation of Alpha fetoprotein from colorectal tumor homogenates and optimizing the binding conditions

الملخص العربى

عزل البروتين الجنيني الفا من مجانسات اورام القولون والمستقيم وضبط الظروف المثلى لارتباطه مع مضاده المعلم بنظير اليود المشع 125

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2 قسم الكيمياء، كلية العلوم / جامعة بغداد

يهدف البحث الى عزل البروتين الجنيني الفا (AFP) وتنقيته جزئياً من مجانسات اورام القولون والمستقيم البشرية. استخدمت مجموعتين من المرضى اذ تمت مقارنة النتائج فيما بينهما. شملت المجموعة الاولى 13 مريضاً مصاباً باورام القولون والمستقيم الحميدة بينما ضمت المجموعة الثانية 21 مريضاً مصاباً باورام القولون والمستقيم الخبيثة. دلت نتائج الفصل وجود قمتين عند استخدام هلام السيفاديكس (G 200)، اذ تدل القمة الاولى للمركب الاعلى وزنا وهو المعقد ما بين جزئية (AFP) والجسم المضاد المعلم باليود المشع 125 ،اما القمة الثانية فعائديتها لجزئية الجسم المضاد للبروتين الجنيني الفا المعلم باليود المشع 125 الحر غير المرتبط .

اجريت دراسات ضبط الظروف المثلى لارتباط البروتين الجنيني الفا مع ضده المعلم بالاشعاع، واتضح بان تركيز البروتين الامثل هو (25) مايكروغرام لكل ملليتر للاورام الحميدة و(18) مايكروغرام لكل ملليتر للورام الخبيثة. بينما كان تركيز المضاد المعلم باليود المشع 125 (1.44) مليغرام لكل ملليتر للورام الحميدة بينما (2.16) مليغرام لكل ملليتر للورام الخبيثة. درجة حامضية المحلول المثلى كانت 7.4 لكلا الحالتين. الزمن الامثل لفترة الحضن كانت 180 دقيقة لكلا الحالتين اما درجة الحرارة المثلى فهي 37 درجة مئوية للاورام الحميدة و25 درجة مئوية للاورام الخبيثة.