SPECTRAL STUDIES ON ISOLATED (^{125}I-ANTI CA 15-3 ANTIBODY/ CA15-3) COMPLEX FROM BENIGN AND MALIGNANT IN HUMAN BREAST TUMORS [+]

دراسات طيفية للمعقد المعزول (^{125}I-Anti CA 15-3 Antibody/ CA15-3)من مجانسات اورام الثدي الحميدة والخبيثة في الانسان

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Abstract:

Gel filtration technique was used to separate ^{125}I-anti CA 15-3 antibody bound to isolated CA 15-3 complex using benign and malignant breast tissue homogenate (as CA 15-3 source) from unbound (Free) ^{125}I-anti CA 15-3 antibody. The characterization of the complexes(^{125}I-anti CA15-3 antibody/ CA15-3) from both benign and malignant breast tumors was carried out through the ultraviolet spectroscopic studies. Factors affecting the absorption properties of the two types of complexes such as pH, solvent polarity, spectrophotometric pH titration, and thermal stability in the presence of different concentrations of sodium chloride have been studied. Spectrophotometric pH titration of the two types of the complexes show that about (41.43%) and (44.29%) of histydyl residues are located on the surface of the two types of protein complexes (benign and malignant) respectively, while (40%) and (50%) of tyrosyl residues are buried interiorly in the complexes of (benign and malignant) respectively.

المستخلص:

استخدمت طريقة الترشيح الهلامي لفصل المعقد (^{125}I-anti CA15-3 antibody/ CA15-3) عن الضاد المتخصص ^{125}I-anti CA 15-3 antibody ،استخدم المستضد الكاربوهيدراتي CA 15-3 المعزول من مجانسات اورام الثدي الحميدة والخبيثة . اجريت دراسة طيفية توصيفية للمعقد (^{125}I-antiCA15-3antibody/ CA15-3) في كل من الاورام الحميدة والخبيثة. درست العوامل المؤثرة خواص امتصاص المعقدين المعزولين من الاورام الحميدة والخبيثة مثل pH ، قطبية المذيب ، المعيرة الطيفية للـpH ، والاستقرار الحراري بوجود تراكيز مختلفة من كلوريد الصوديوم. اوضحت نتائج المعايرة الطيفية لكلا المعقدين بأن (41.43%) و (44.29%) من ثمالات الهستدين تقع على السطح في كلا المعقدين المعزولين من الاورام الحميدة والخبيثة على التوالي. بينما (40%)و (50%) من ثمالات التايروسين تكون مطمورة الى الداخل في كلا المعقدين على التوالي.

Introduction:

[+]**Received on 21/10/2009 , Accepted on 26/5/2010 .**
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Molecules absorb light; the efficiency of absorption depend on both the structure and environment of the molecule making absorption spectroscopy a useful tool for characterizing both small and large molecule.
The ultraviolet absorption spectra of protein solutions in the region 250 to 310 nm are contributed from phenylalanyl, tyrosyl and tryptophanyl residues. But at the shorter wavelengths the contributions come from other groups such as histidyl residues and the peptide bond [1]. Changes in the environment of these chromophores can lead to alteration in the absorption spectrum, and the conformational changes of a protein may also involve environmental changes of its chromophoric groups [2]. A variety of environmental changes (e.g. pH, temperature) can affect the absorption spectrum if the interaction of chromophore and perturbing agent affects the ground and excited states, the altered spectrum of the chromophore can be shifted to longer (red shift) or shorter (blue shift) wavelengths. The shift may or may not be accompanied by a change in intensity of the spectrum [3,4]. Saif-Alla, P.H., studied the UV spectra of h-PRL-antibody complex and CA15-3 molecule [5].
Interaction of h-CA 15-3 partially purified from benign (fibroadenoma) and malignant (IDC) tissues homogenate with its antibody is an example of protein-protein association. Although several new immunochemical techniques were developed to study such interactions, UV [6,7] spectral remain as one of the most important methods in immunology because it provides a sensitive and quantitative measurements for the study of antibody structure and its specific ligand binding [8,9].
Very limited work concerning the physical properties of CA 15-3 specially those related to UV spectroscopy has been done, also the UV studies on CA 15-3 antibody interaction are not wide spread. Hence, this work is planned to study the association of the partially purified h-CA 15-3 and its antibody at different conditions.

Materials and Methods:

Chemicals
All chemicals and reagents used in this study were of analytical grade, Immunoradiometric assay kit for CA 15-3 level from Diasorin Inc. (USA). Urea, $ZnCl_2$, $CaCl_2$, NH_4Cl, NaBr, ethylendiamine-tetraaceticdisodium salt (EDTA) from Fluka:(Switzerland). $CuSO_4.H_2O$, NaK-tartarateglycine, NaOH, HCl, $NaCO_3$, NaF, NaCl, NaI, Na_2HPO_4, NaH_2PO_4 from BDH,limited,Poole (UK). Folin-Ciolteau from E. Merck AG. Dastmstapt, finally Blue dextran (2000), and sepharose CL-4B from Pharmacia fine chemicals (Sweden).

Instruments
Gamma counter type 1270-rack gamma II , Spectrophotometer ultra space type 4050 and Combicold rack were from LKB, UV-210 a double beam spectrophotometer from Shimadzu. pH-meter from Pye-Unicam. Cooling centrifuge; with a maximum speed 5000 r.p.m. from Hettich. Cooling centrifuge type 202-MK; with a maximum speed 13500 r.p.m. from Sigma. Memmert water bath, memmert incubator from West Germany. SM-shaker from England.

Patients
Two groups of breast tumors patients were included in this study.
Group I : Consisted of 40 patients with benign breast tumors
Group II : Consisted of 32 premenopausal patients with breast cancer (IDC)..
All patients were admitted for treatment to (Baghdad Teaching Hospital), (University Hospital, Al-Nahrain College of Medicine), (Nursing Home Private Hospital) and (Al-Arabi

Private Hospital). Patients suffered from any disease that may interfere with this study were excluded. All surgical operation of breast tumors were carried out under the supervision of the following surgeons:Dr. Saab Sedeq, Dr.Munthir Al-Aubaidi, Dr.Azam Qanbar Agha, Dr. Abd Al-Salam Al-Tai, Dr. Zuhair Abid Al-Hadi. The host information of all patients and normal healthy subjects is summarized in table (1).

Table (1): The host information of breast tumors patients and healthy subjects studied.

Group	Patients	No.	Age	Type of tumor	Metastases
I	Benign breast tumor	40	18-42	fibroepithelial tumor (fibroadenoma)	–
II	Premenopausal malignant breast tumor	32	34-52	Infiltrative Ductal carcinoma	2 lymph nodes

Collections of Specimens

The tumors tissues were surgically removed from breast tumor patients by either mastectomy (cancer patients) or lumpectomy (benign tumor patients). The specimens were cut off and immediately rinsed with ice-cold isotonic saline solution. They were collected individually in plastic receptacle and stored at –20 ^{o}C until homogenization.

PBS–Buffer

Phosphate –buffered saline (PBS) 0.05 M, PBS buffer pH 7 containing 0.02% sodium azide was prepared as following:

A: Disodium basic phosphate (0.5M); 7.0980g Na_2HPO_4 and 9.0g of NaCl
were dissolved in a final volume 1L deionized distilled water.

B:.Monobasic sodium phosphate (0.15M) 5.9990g of NaH_2PO_4 and 9.0g NaCl
were dissolved in a final volume 1L deionized distilled water.

Phosphate buffer saline pH 7 was prepared by mixing a volume of solution A with appropriate amounts of solution B to obtain the required pH.

Preparation of Breast Tumors Tissue Homogenates

The frozen tissue were weighed, sliced finely and scalped in Petri dish standing on ice bath, and then homogenized with fivefold volumes of PBS buffer pH7.2, using manual homogenizer [10]. The homogenate was filtered through four layers of nylon gauze in order to eliminate fibers connective tissues, and then centrifuged at 4000 r.p.m for 45 min. at 4 ^{o}C in order to precipitate the remaining intact cells and the intact nucleus. The supernatant fraction at this speed was separated, divided in aliquots and freeze -20 ^{o}C until use.

Methods

Isolation of CA15-3 by Sepharose CL-4B Column

This part was carried out according to Al-Rubae'i thesis [11].

Gel Filtration Technique for Separation of Free and Bound ^{125}I -Anti CA 15-3 Antibody

Preparation of the Column

The dimensions of the column were chosen according to the following equation [12].

Diameter= $\sqrt{\frac{m}{10}}$ Where: m = amount of protein in mg.

L = 30 x diameter ***Where: L : length of the column***

Preparation of the Gel

The gel was prepared by allowing the pre-swollen gel to swell again in PBS buffer (0.05 M) pH 7.0, 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 and slurry was left for 24 hrs to equilibrate with buffer. The swollen gel was suspended and carefully poured into a vertical glass-column (0.7 x 30 cm) down the wall using a glass rod. After the gel has settled, the column was equilibrated with PBS for 24 hrs.

Void Volume Determination

The void volume of the column was determined by using blue dextran 2000 at concentration of 2 $mg.mL^{-1}$ dissolved in PBS buffer pH 7.0 , then the elution was carried out with the same buffer at a flow rate of 20 $mL.hrs^{-1}$. Fractions of 2 mL were collected and their absorbance was measured at 600 nm. Figure (1) shows the elution profile of blue dextran 2000. The volume of the buffer required to elute the blue dextran which represents the void volume was (6 mL).

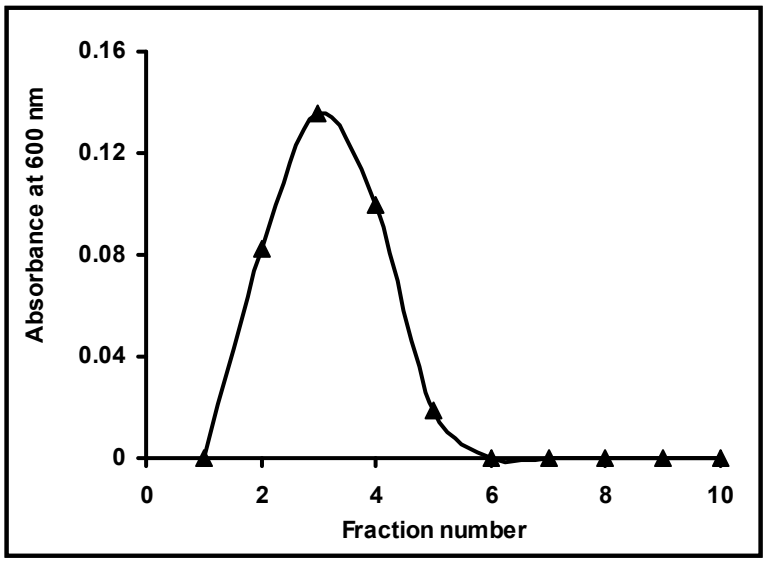


Figure (1): The elution profile of blue dextran 2000.

Column-Calibration

The column was calibrated by gel filtration kit, purchased from pharmacia fine chemicals which contained standard proteins. Standard protein(Thyroglobulin, Ferritin, Catalase, Aldolase) solutions were prepared according to the manufacturers instructions, then applied through two 0.5 mL portions, proteins 1 and 3 in the first portion, proteins 2 and 4 in the second portion. Elution was carried out with PBS buffer at a flow rate of 20 $ml.hrs^{-1}$. the

absorbance of the fractions collected was measured at 280 nm to evaluated the elution volume (Ve) of the standard protein.

Separation Procedure of (^{125}I-Anti CA15-3 Antibody/CA15-3) Complex

A) Partially Purified CA15-3 from Benign Breast Tumor (Fibroadenoma) and its Antibody ^{125}I -Anti CA 15-3

Reagents

Buffer PBS 0.15M, pH 7.0 containing 0.02% sodium azide was prepared as described previously.

Procedure

1- Partially purified CA 15-3 (475µL) containing (0.665 mg. mL^{-1}) was incubated with 120 µL of ^{125}I-anti CA 15-3 antibody (0.8412mg. mL^{-1}) and complete the reaction to a final volume of 700 µL with PBS buffer 0.15 M pH 7.0. The tubes were incubated for 90 min. at 37^{o}C.

2- At the end of incubation, the mixture was applied to the surface of a sepharose CL-4B (1x30 cm) with a bed volume (23.5 cm^3) equilibrated with PBS buffer 0.15M, pH 7.0. Elution was carried out using the same buffer to separate CA 15-3 bound to ^{125}I-anti CA 15-3 antibody from unbound (Free) CA 15-3 and ^{125}I-anti CA 15-3 antibody with a flow rate (1 mL per 7 min), and fraction volumes of 1 mL were collected.

3- The radioactivity of each fraction was counted by gamma counter for one minute.

4- Protein concentration was measured at 280 nm.

5- One hundred and twenty microliters of ^{125}I-anti CA 15-3 antibody (0.84 mg. μL^{-1}) was completed to 700 µL with PBS buffer (0.15M, pH7.0), then this volume was injected to the column as mentioned in step2, then steps 2,3 and 4 were repeated.

Calculations:

1. Radioactivity (c.p.m) of each eluted fraction was plotted against the fraction number.
2. The absorbance of each eluted fractions was measured at 280nm, and the absorbance was plotted against the fraction number.
3. The percent radioactivity was calculated by dividing the sum of the radioactivity of the fractions under each peak by the sum of radioactivity of all peaks appeared in the profile:
4.

$$\text{Percent radioactivity of each peak} = \frac{\text{Radioactivity per peak (c.p.m)}}{\text{Sum of radioactivity of all peaks (c.p.m.)}} \times 100$$

B) Partially Purified CA15-3 from Premenopausal Malignant Breast Tumors (IDC) and Its Antibody ^{125}I-anti CA 15-3

Reagents

Buffer PBS 0.15 M, pH 7.0 containing 0.02% sodium azid was prepared as described previously.

Procedure

1. Four hundred and twenty four microliters of partially purified CA 15-3 (0.147 mg. mL^{-1} protein) and incubated with 106 µL of ^{125}I-anti CA 15-3 antibody (0.743 mg.mL^{-1}) in a final

volume 700 mL with PBS buffer 0.15M pH 7.0. The tubes were then incubated for 150 min at 15^{o}C.
2. Steps 2,3,4 and 5 in section (A) were repeated.

Calculation:

The same calculation that mentioned in section (A) was used to calculate the radioactivity; protein was measured at 280nm and the percent of radioactivity of each peak was determined.

The UV Spectrum of (^{125}I-Anti CA 15-3 Antibody/CA15-3) Complex from Benign and Malignant Breast Tumors

The gel filtration profile in section (A&B) gave two peaks. The fractions under each peak were pooled and the absorption spectrum was scanned in UV Region against the appropriate blank in the reference beam.

The UV. Spectrum of ^{125}I-Anti CA 15-3 Antibody

Half milliliter of ^{125}I-anti CA 15-3 antibody was placed in a 0.25 cm cuvette in the sample beam and the absorption spectrum was measured immediately against an appropriate blank in the reference beam.

The UV Spectrum of Partially Purified CA 15-3

Half milliliter of partially purified CA 15-3 from benign (Fibroadenoma) and malignant (IDC) breast tumors was placed in a 0.25 cm curette in the sample beam and the absorption spectrum was measured immediately against an appropriate blank in the reference beam.

Factors Affecting the Absorption Properties of (^{125}I-Anti CA 15-3 Antibody /CA 15-3) Complex from Benign and Malignant Breast Tumors

The pH Effect on the Complex

Reagents:

1. KCl-HCl buffer (pH 2) was prepared as follows:

Solution A: Potassium chloride (0.15M), 1.11825 gm was dissolved in a final volume of 100mL deionized distilled water.

Solution B: Hydrochloric acid (0.15M).

The required pH (2.0) was prepared by mixing a volume of solution A with an appropriate amount of solution B to obtain the required pH.

2. Citrate-phosphate buffer at different pH was prepared as follows:

Solution A: Citric acid (0.15M); 2.8815 gm citric acid dissolved in 100mL deionized distilled water.

Solution B: Dibasic sodium phosphate (0.15M); 2.1294 gm of Na_2HPO_4 was dissolved in a final volume of 100 mL deionized distilled water.

Working buffer pH (4 and 6) was prepared by mixing a volume of solution A with an appropriate amount of solution B to obtain the required pH.

3. Phosphate buffer at different pH values was prepared as follows:

Solution A: Dibasic sodium phosphate (0.15M), 2.1294 gm Na_2HPO_4 was dissolved in a final volume of 100 mL deionized distilled water.

Solution B: Monobasic sodium phosphate (0.15M), 1.7997 gm NaH_2PO_4 was dissolved in a final volume of 100 ml deionized distilled water.

Phosphate buffers at different pH rang (7-8) were prepared by mixing a volume of solution A with an appropriate amount of solution B to obtain the required pH.

4. Glycine - NaOH buffer was prepared as follows:

Solution A: Glycin (0.15M); 1.12575gm $C_2H_5NO_2$ was dissolved in a final volume of 100 mL deionized distilled water.
Solution B: Sodium hydroxide (0.15M); 0.6gm NaOH was dissolved in a final volume of 100 mL deionized distilled water.
Working buffer pH (9-11) was prepared by mixing a volume of solution A with an appropriate a mount of solution B to obtain the required pH.

Procedure
Two hundred and fifty microliters of pooled fractions under the first peak that represent (^{125}I-anti CA 15-3 antibody/CA 15-3) complex, was completed to 500µl with different buffers at different pH values (4 to 11), then each sample beam and the buffer at the adjusted pH in the reference beam. The absorption spectrum was scanned.

Calculations
The molar absorption coefficient (□) for (^{125}I-anti CA 15-3 antibody/CA 15-3) complex at 278 nm was calculated from Lambert-Beer's law.

Effect of Solvent Polarity on UV Spectra of the Complex

The effect of 20% ethanol, and the same amount for ethylene glycol, glycerol, sucrose, urea, dimethyl sulphoxide, dioxane, and polyethylene glycol; on the complex. Two hundred and fifty microliters of complex from benign and malignant breast tumors of pooled fractions under the first peak were completed to 500 µL with phosphate buffer containing any of the following solvent at pH 7.4 in the test cell and the 20% ethanol, ethylene glycol, glycerol, sucrose, urea, dimethyl sulphoxide, dioxane, and polyethylene glycol was adjusted and placed in the reference cell using 0.25 cm cuvette (i.e., the experiment was repeated by using solvents individually).

Calculations
The absorption spectrum of each sample was scanned immediately in the area of (200-350 nm).

Spectrophotometric pH Titration on the Complex

A series of complex from benign (Fibroadenoma) and Malignant (IDC) breast tumors (250 µL) were completed to 500 µL with buffer at pH ranging from 8 to 11. The maximum absorbance of each sample was measured at 295 nm; the absorbance of λ_{max} at each pH value was plotted versus the corresponding pH. Other series of complexes isolated from benign (Fibroadenoma) and malignant (IDC) breast tumors (250 µL) were completed to 500 µL with buffer at pH ranging 4 to 8. The maximum absorbance of each sample was measured at 211nm. The absorbance of λ_{max} at each pH value was plotted against the corresponding pH.

The Effect of NaCl Concentration on the Thermal Stability of the Complex by UV Spectral Studies

Reagents
Twenty percent ethylene glycol buffur was prepared by dissolving 20mL of ethylene glycol in 80mL of phosphate buffer. NaCl (0.01M) in 20% ethylene glycol was prepared by dissolving 0.05844 gm of NaCl in 100mL of 20% ethylene glycol buffur, while NaCl (0.1M) in 20% ethylene glycol was prepared by dissolving 0.5844 gm of NaCl in 100mL of 20% ethylene glycol buffer.
Procedure
Two hundred and fifty microliters of complex from benign (Fibroadenoma) or malignant (IDC) breast tumors were completed to a final volume 500 µL with 20% ethylene glycol

buffer pH7.4 containing 0.01 M NaCl Each mixture was placed in 0.25 cm cuvette in the sample beam and the buffer at the adjusted pH in the reference beam.The absorbtion was measured at the wavelength of (292 and 295 nm) at different temperatures 20, 30, 40, 50, 60, 70^{o}C. The experiment was repeated for each complex with another solution (20% ethylene glycol 0.1 M NaCl), at 295 nm.

Calculations

The absorbance of each complex was plotted against the different temperatures at two wavelengths (292 and 295 nm).

Effect of Urea, KCl and (Urea, KCl) Mixture on the Spectrum of the Complex

Reagent

1. Eight molar of urea was prepared by dissolving 24.02gm of Urea in a final volume of 50 mL of PBS buffer at pH 7.4.
2. KCl (0.03 M) was prepared by dissolving 0.2737gm of the salt in a final volume of 50mL of corresponding buffer.
3. PB buffer solution was prepared as described previously.

Procedure

Two hundred and fifty microliters of complex isolated from benign (fibroadenoma) and malignant (IDC) were pipetted in a set of three tubes. The volume was completed to 500 µL with PBS buffer at pH 7.4 contains (0.03 KCl, 8 M urea and mixture 1:1 of both 0.03 KCl and 8M Urea) respectively, then each sample was placed in 0.25cm cuvette in the sample beam and the buffer at the same pH in the presence of the same salt in the reference beam.

Calculations

The absorption spectrum of each sample was scanned immediately in the area of (200-350 nm).

Results and Discussion

Protein UV light maximum absorption is at approximately 280nm, caused by tryptophan, tyrosine and (to a lesser extent) phenylalanine residues, and at lower wavelength (215-230 nm) due to polypeptide chain backbone. Absorbance at 280 nm varies for each protein. The absorbance at lower wavelengths is directly related to the amount of polypeptide material and is usually considerably more sensitive than at 280nm.however,many buffers and other molecules also absorb at these lower wavelengths (phosphate and tris buffers are acceptable but the preservative sodium azide absorbs strongly). Absorbance at 215-230 nm is useful for monitoring peptides that may not contain tryptophan or tyrosine [13].

Gel Filtration Technique for Separation of Free and Bound ^{125}I-Anti CA15-3 Antibody

Figure (2) and (3) show the results of gel filtration technique to separate ^{125}I-anti CA 15-3 antibody bound to partially purified CA 15-3 from benign (Fibroadenoma) and malignant (IDC) breast tumors respectively. The profile of separation revealed two peaks. The first peak represents (^{125}I-anti CA 15-3 antibody/CA 15-3) complex, the second peak represents the unbound (Free) ^{125}I-anti CA 15-3 antibody. Figure (4) show the gel filtration profile of ^{125}I-anti CA 15-3 antibody, the results revealed only one peak in the same position of the second peak of figures (2) and (3), which represent the unbound ^{125}I-anti CA 15-3 antibody. The percent of ^{125}I-anti CA 15-3 antibody/ CA 15-3) complex was 49.74% in benign (Fibroadenoma) breast tumors patients, while the percent of complex was 56.20% in

malignant breast tumors patients (IDC). On the other hand the percent of ^{125}I-anti CA 15-3 antibody was 34.40% in benign breast tumors (Fibroadenoma) and 31.42% in malignant breast tumors (IDC). This is because the epitope of CA 15-3 in malignant breast tumors was higher than in benign breast tumors.

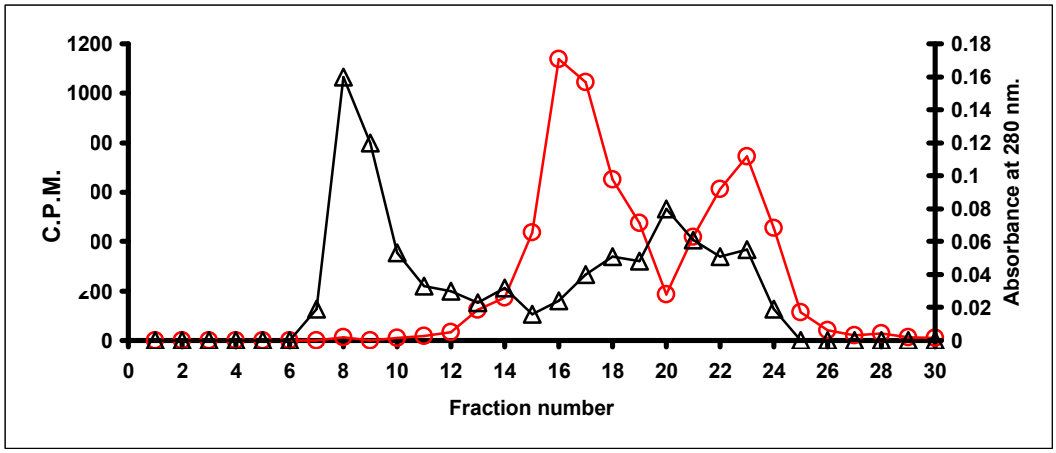


Figure (2): The elution profile of the isolated complex (^{125}I-antiCA15-3 antibody/CA15-3) and free antibody in benign breast tumors on Sepharose CL-4B. (○) radioactivity, (△) protein.

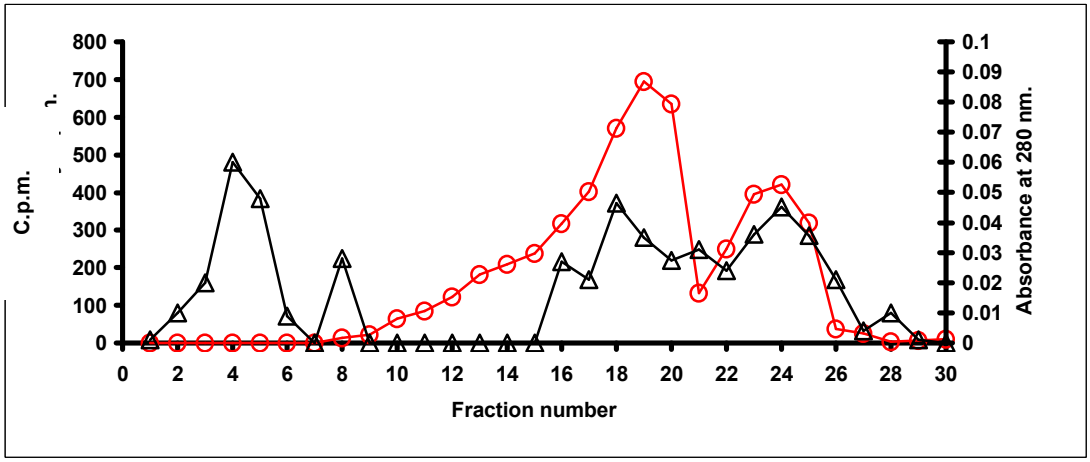


Figure (3): The elution profile of the isolated complex (^{125}I-antiCA15-3 antibody/CA15-3) and free antibody in malignant breast tumors (IDC) on Sepharose CL-4B, (○) radioactivity, (△) protein.

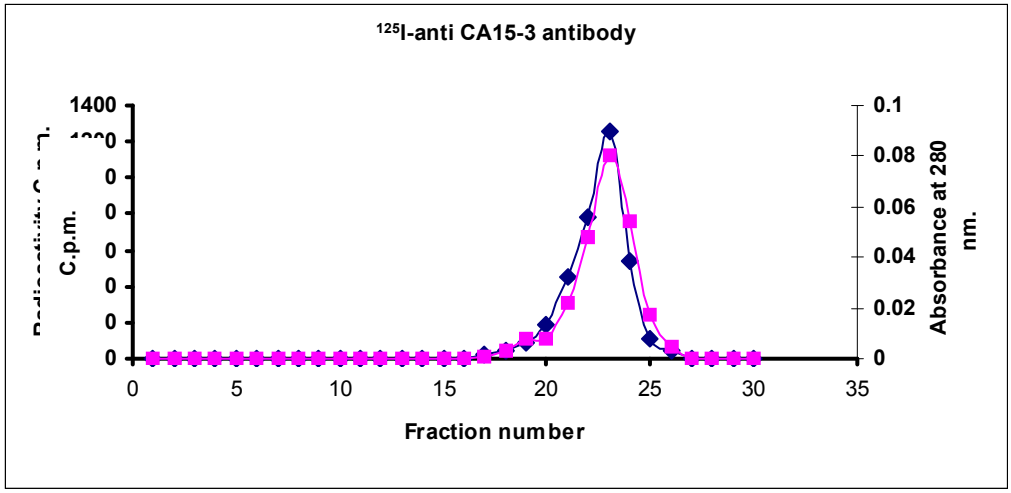


Figure (4): The elution profile of the 125I-antiCA15-3 antibody on Sepharose CL-4B, (■) radioactivity, (◆) protein.

The UV Spectra of Partially Purified CA 15-3, Anti CA 15-3 Antibody and (^{125}I-Anti CA 15-3 Antibody/CA 15-3) Complex Molecules

The UV spectra of partially purified h-CA 15-3, ^{125}I-anti CA 15-3 antibody and (^{125}I-anti CA 15-3 antibody/CA 15-3) complex were scanned from 200-350 nm to determine the absorption spectra, and the alternation in the UV spectra as a results of their interaction.

The UV Spectrum of Partially Purified CA 15-3

The UV spectra of partially purified h-CA15-3 in benign tumors (Fibroadenoma) and malignant tumors (IDC) at neutral pH shows that the λ_{max} for purified CA15-3 from benign (Fibroadenoma) consisted of two peaks; a large one at 208nm and smaller one at 270nm, while the UV spectra of purified CA 15-3 from malignant tumors (IDC) shows two peaks at 205 and 270nm as shown in table (2). Therefore it seemed that each human CA 15-3 has a characteristic spectrum and can be identified by its peaks, the first peak (at 208nm or 205nm) such results could be due to the amide group in polypeptide bond of h-CA 15-3 molecule with contribution of the histidyl residues [14], while the second peak (at 270) is assigned to the side chain chromophore of phenylalanine or tryptophyl residues [15].

The UV spectrum of ^{125}I-Anti CA 15-3 Antibody

The UV spectrum of ^{125}I-anti CA 15-3 antibody at neutral pH shows that the λ_{max} consisted one peak at 203.6nm, which is assigned to the amide groups in the polypeptide bond [14], with contribution of hisidyl residues [16] as shown in table (2) .

The UV spectrum of (^{125}I-Anti CA 15-3 Antibody/CA 15-3) Complex

The UV spectra of partially purified CA 15-3 extracted from benign (Fibroadenoma) and malignant (IDC) bound to ^{125}I-anti CA 15-3 antibody at neutral pH show that the λ_{max} is consisted of two peaks at (203.4nm and 274nm) in benign complex, while the λ_{max} is consisted of two peaks at (204.2 nm and 278nm) in malignant complex as shown in table (2). The first peak at (274nm or 278nm) is assigned to tyrosyl residues [15], it is very weak band and it seems that the tyrosyl residues in the benign or malignant complexes is located on the surface of protein complex.

The strong absorption of the second peaks (at 203.4 or 204.2nm) arises form electronic transition in the peptide backbone itself and is therefore sensitive to backbone conformation [14].

Table (2): The λ_{max} valves of (^{125}I-anti CA 15-3 antibody/CA 15-3) complex, partially purified CA 15-3 and unbound (Free) ^{125}I-anti CA 15-3 antibody in both cases benign and malignant breast tumors.

No.	Fractions	Benign λ_{max} (nm)	Malignant λ_{max} (nm)
1	CA 15-3 partially purified	208, 270	205, 270
2	^{125}I-anti CA 15-3 antibody	203.6	203.6
3	Complex	203.4, 274	204.2, 278

Factors Affecting the Absorption Properties of (^{125}I-Anti CA15-3 Antibody/ CA15-3) Complex from Benign and Malignant Breast Tumors

The Effect of pH on the Complex

The pH of the solvent determines the ionization state of ionizable chromophores in the proteinmolecule [15]. The UV spectrum of isolated (^{125}I-antiCA 15-3antibody/CA15-3) complex from benign (Fibroadenoma) and malignant (IDC) breast tumors was determined at different pH (2, 4, 6, 7, 7.4, 8, 9, 10, and 11). Table (3) shows the effect of different pH on both complexes. At an acidic pH 2 and neutral pH (7 and 7.4) the both complexes benign (Fibroadenoma) and malignant (IDC) have one maximum wavelength near 200 nm as compare to UV spectrum of h-CA 15-3 and the ^{125}I anti CA15-3 antibody. The λ_{max} of CA 15-3 (270 and 208 nm) in benign (Fibroadenoma) and its antibody λ_{max} (203.6) disappeared. The λ_{max} of CA 15-3 (270 and 205 nm) in malignant (IDC) and its antibody λ_{max} (203.6) also disappeared. The absorption near 200 nm is characteristic of the amide group in the polypeptide bond of the complex [16]. The blue shift is due to the increasing of hydrogen bond formed in the presence of highly positively charged state [17]. The disappearance of λ_{max} 280 nm of tyrosine and phenylalanine due to conformational changes and chromophore in native complex were buried in the interior of their complexes [18, 19]. Protein shows a strong absorption in range (180-225 nm), absorption at such wavelength arises from electronic transition in the polypeptide backbone itself and is therefore sensitive to back bone conformation [14]. At pH (4, 6, 9, 10, and 11) no band was observed and all peaks disappeared. The disappearance of the λ_{max} at these pH's may be due to conformational changes of the protein complex or dissociation complex [20].

Table (3): The effect of different pH on λ_{max} values of (^{125}I-anti CA 15-3 antibody/CA 15-3) complex.

pH	λ_{max} (nm)	
	(^{125}I-anti CA 15-3 antibody/CA15-3) benign complex	(^{125}I-anti CA 15-3 antibody/CA 15-3) malignant complex
2	**200**	**200**
4	**-**	**-**
6	**-**	**-**
7	**200**	**200**

7.4	**200**	**200**
8	**200**	**200**
9	**-**	**-**
10	**-**	**-**
11	**-**	**-**

Effect of Solvent Polarity on UV Spectra of the Complex

The immediate environment of a chromophore affects its absorption. The determination of whether an amino acid is internal or external by measuring the spectra of protein in a polar and non-polar solvent is called the solvent perturbation method [15]. In fact, proteins are rarely studied in completely non-polar solvents because most proteins are either insoluble or denatured in these solvents. However, significant solvent effects can be induced by use of a mixture of water and substance of a reduced polarity such as ethanol, ethylene glycol, polyethelyne glycol, sucrose, dioxane and dimethyl sulfoxide (DMSO) [15]. Several spectra changes were obtained in the precence of these perturbants, like the alteration of λ_{max} positions and intensities of protein spectrum and the appearance of new chromophores on the surface of the complex. These chromophores on the region of the protein disappeared in the absence of the solvent. One of the main assumptions of the solvent perturbation technique is that solvent alters the peak positions and intensities by altering the energy and probably of electronic transitions. Other considerations include the following:

a. Polarization effect
b. Change in permanent dipole moment during excitation, which will tend to produce either a short wave or a long wave shift depending on the nature of the electronic transition and wheather the solute is a hydrogen donor or hydrogen acceptor [21].

The effects of different solvents on the (^{125}I-anti CA 15-3 antibody /CA 15-3) complex from benign (Fibroadenoma) and malignant (IDC) breast tumors at pH 7.4 were investigated. The data obtained are illustrated in table (4). It was found that one λ_{max} specific for the amide groups of polypeptide bond at pH 7.4, this shift toward the shorter wavelength is due to the n-π^* transitions in the presence of 20% ethanol, ethylene glycol and glycerol. In the presence of polyethylene glycol there was a significant red shift in the λ_{max} (204 nm) in benign (Fibroadenoma) complex and λ_{max} (205nm) in malignant (IDC) complex. When 20% Dioxane was used there were a significant red shift in the λ_{max} (220nm) of the amide bond at pH 7.4, which assigned to tyrosyl residue. The value of λ_{max} is for n-π^* transitions which occur at longer wavelength because the nonbonded electrons in the anion are available for interaction with the π electron system of the ring [21], while in the presence of 20% sucrose the complex has a slight blue shift and show λ_{max} at 202 nm and 201nm in both benign and malignant complexes. Finally the effect of 20% DMSO on the complex, show that the amide bands at pH 7.4 were disappeared, this may be due to the denaturation of protein complex in presence of 20% DMSO.

The application of spectrophotometric solvent perturbation on the complex is to determine the location of tyrosyl residues, whether they are buried and inaccessible or exposed and accessible to the solvent approach [22]. Laskowski [2] has listed the major assumptions of solvent perturbation experiments. There are: (1) buried chromophors are unperturbed, that is only the groups located on the surface or near the surface of the protein should experience the perturbing effects of the solvent; groups buried in the interior of the protein, not accessible to the solvent; which should not be affected and consequently could not contribute to the overall

spectral shift observed. (2) No conformational changes take place upon addition of perturbant, and (3) the solvation layer around the chromophore contains the same concentration of perturbation experiments when employed at convenient concentrations (often 20%), do not appear to produce conformational changes in most protein studied under reasonable conditions of pH, ionic strength, and temperature. This concentration is large enough to cause measurable shifts in the spectra of chromophoric residues. Conformational changes can be expected if perturbation is carried out under conditions in which the protein structure has marginal stability (low-or high pH for many protein) [2, 22]. Chromophore may not completely bury. It has been, distinguished between chromophores in crevices and chromophores that are partially buried [2]. The former are observed to be fully perturbed by perturbant solvent smaller than a certain critical size (e.g ethanol), but not by larger perturbants (e.g polyethylene glycol). The degree of exposure is thus determined only by the size of perturbant molecule (solvent) or by the size of the crevice in which the chromophore is located [22]. Partially buried chromophores on the other hand, show a degree of exposure that depends on the nature of the perturbant, rather than on its size. The observed degree of exposure decrease in the order:

Sucrose ≥ Glycerol ≥ Ethyleneglycol ≥ Methanol, Ethanol > Polyethylene glycol ≥ Dimethyl sulfoxide.

The first perturbant in this series modify the solvent nonspecifically, while the later ones in the series may specifically interact with chromophore [22]. When comparing the effects of the six solvents used, ethanol, ethleneglycol, glycerol, dioxane, polyethylene glycol and dimethylsulfoxide at pH 7.4 on the UV spectrum, especially on the shift of λ_{max}, which is due to tyrosyl residues. It seems that the maximum effect was observed in the presence of 20% dioxane as perturbant solvent, where there was a shift in the λ_{max} about 16nm, while minimum effect was observed in the presence of 20% polyethylene glycol where the λ_{max} remained unchanged. Since the change in the λ_{max} of tyrosyl residues does not depend on the size of the perturbant solvent, the tyrosyl residues showing the changes in λ_{max} ; absorbance must be partially buried.

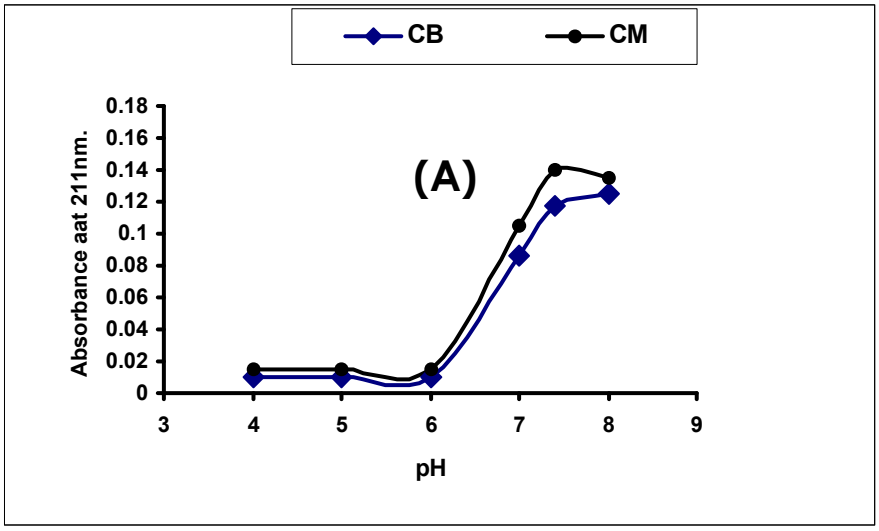
Table (4): The effect of 20% of ethanol, ethyleneglycol, glycerol, polyethylene glycol, sucrose, dioxane, DMSO and on the λ_{max} of (^{125}I- Anti CA 15-3 Antibody/ CA15-3) complex at pH 7.4.

Solvent of 20% of	λ_{max} (nm)	
	(^{125}I-anti CA 15-3 antibody/ CA15-3) benign complex	(^{125}I-anti CA 15-3 antibody/ CA 15-3) malignant complex
Ethanol	Near 200	200
Ethylene glycol	Near 200	Near 200
Glycerol	Near 200	Near 200
Polyethylene glycol	204	205
Sucrose	202	201
Dioxane	220	220
DMSO	-	-

Spectrophotometric pH Titration of the Complex from Benign and Malignant Breast Tumors

To study (^{125}I- anti CA 15-3 antibody/ CA15-3) complex structure, this requires the determination of pk_a values for proton dissiociation from ionizable amino acid side chains, because these values give an indication of the location of amino acid in the protein. This can often be done spectrophotmetrically because dissociation often changes the spectrum of one of the chromopores (tyrosyl) [15]. For proteins this usually amounts to the titration of the phenolic groups of tyrosine residues. By the measurement of the absorption at 295 nm (λ_{max} for the ionized form of tyrosine), or observation of histidine dissociation by measurment at 211nm. The titration curves of (^{125}I- anti CA 15-3 antibody/ CA15-3) complex from benign (Fibroadenoma) and malignant (IDC) for both histidyl and tyrosyl residues are illustrated in figure (5 A&B) respectively. Figure (5) shows that the pk_a for histidine is (6.69) for (^{125}I- anti CA 15-3 antibody/ CA15-3) complex from benign breast tumors, while the pk_a for histidine is (6.65) for (^{125}I- Anti CA 15-3 Antibody/ CA15-3) complex from malignant (IDC) breast tumors. From the same curve it could be concluded that about (41.43%) histidyl residues are located on the surface of the protein complex [23] of benign (Fibroadenoma), while about (44.29%) histidyl residues are located on the surface of the protein complex from malignant (IDC). The other residues are buried interior the benign and malignant complex. Figure (5B) shows that the pk_a value of the benign complex of tyrosyl residues is (8.9) and it's about (40%) at tyrosine residues are internal and a large arise in the absorbance at very high pH was observed. While in the malignant (IDC) complex the pk_a value of tyrosyl is (8.4) and it's about (50%) this indicates that the internal tyrosines have become exposed to the solvent, which is the protein complexes in folded (become denatured) [23].

The two curves also illustrated the low content of histidine compared to the high content of tyrosine in the benign and malignant complex.



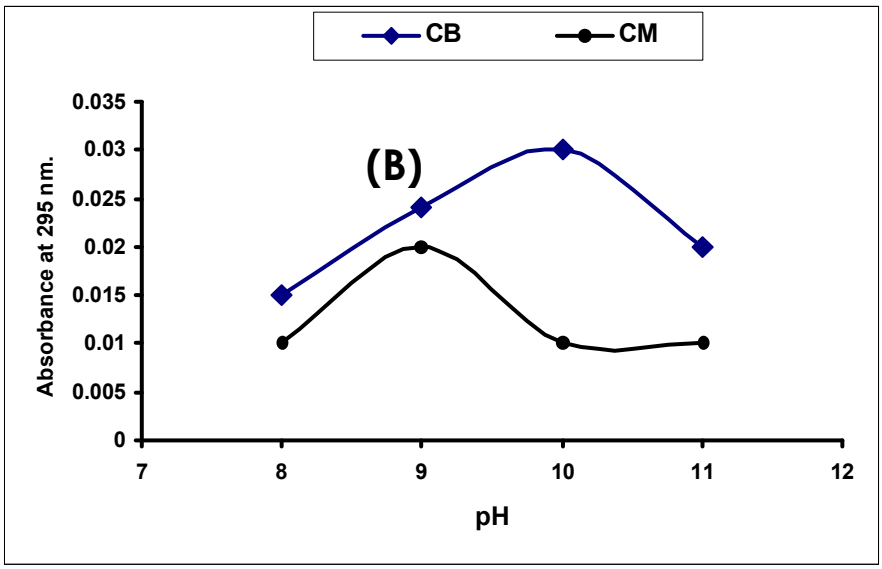


Figure (5): Spectrophotometric pH titration of ^{125}I-anti CA15-3 antibody/CA15-3 complex from benign and malignant breast tumors: (A) for histidine, (B) for tyrosine.
(CB): Complex of benign breast tumors, (CM): Complex of malignant breast tumors.

The Effect of NaCl Concentration on the Thermal Stability of the Complex by UV Spectral Studies

The effect of different concentrations of NaCl on the thermal stability of the protein complex isolated from benign and malignant breast tumors was examined in this experiment. The values of absorbance at λ_{max} (292, 295nm) for tryptophyl and tyrosyl residues respectively, in two different concentrations of NaCl 0.01 M and 0.1 M in 20% ethylene glycol buffer are shown in figure (6 A&B) and (7 A&B). The λ_{max} was used to examine if the protein contains internal tryptophans and tyrosines .

As shown in figure (6A&B), the absorbance of both tryptophane and tyrosine reach higher absorbance at 60^{o}C, in the presence of 0.01 M NaCl in benign and malignant complex. The increment in the absorbance of both tryptophyl and tyrosyl residues with increasing temperature could be due to that buried chromophores becomes exposed to the solvent during thermal denaturation [16].

Figure (7 A) shown the absorbance of tyrosin reach higher absorbance at 70^{o}C in the presence of 0.1 M NaCl in benign and malignant complex. On the other hand figure (7 B) shown the absorbance of tryptophane reach higher absorbance at 60^{o}C and 30^{o}C in benign and malignant breast tumors complexes in presence of 0.1 M NaCl respectively. Which means that the complexes were very stable at 70^{o}C in presence of higher concentration of NaCl, 70^{o}C was needed for unfolding benign and malignant complex at λ_{max} 292 nm and benign complex was more stable at 60^{o}C in presence of 0.1 M NaCl,while the temperature is decreased to 30 ^{o}C in the presence of 0.1M NaCl at λ_{max} 295. This is due to conformational changes required more energy 70^{o}C in presence of 0.1 M NaCl than in 0.01 M NaCl.

The decreased absorbance in presence of 0.1 M NaCl as compared with that in 0.01 M NaCl could be due to salt concentration. Each protein in solution containing salts will collect around it a counter ion atmosphere entriched in oppositely charged small ion (chloride ion, sodium ion) and such a cloud of ions will tend to screen the protein, the more effective electrostatic screening will be, and decrement in the absorption intensity will be observed [14].

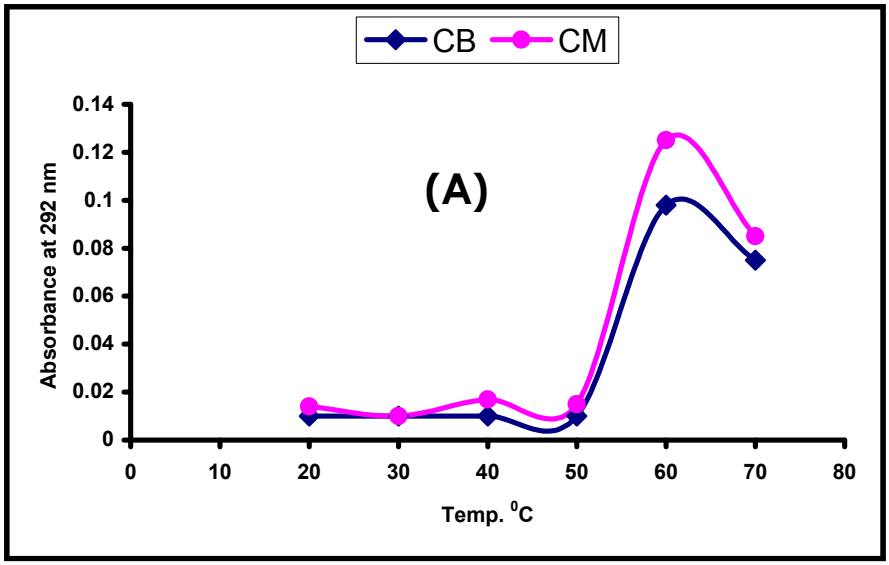


Figure (6 A): Thermal stability curve for benign and malignant at λ_{max} 292 in the presence of 0.01 M NaCl, (CB): Complex of benign breast tumors, (CM): Complex of malignant breast tumors.

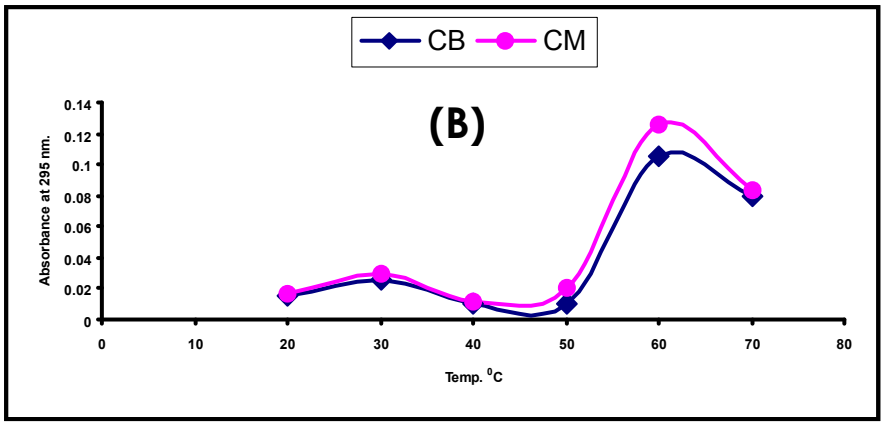
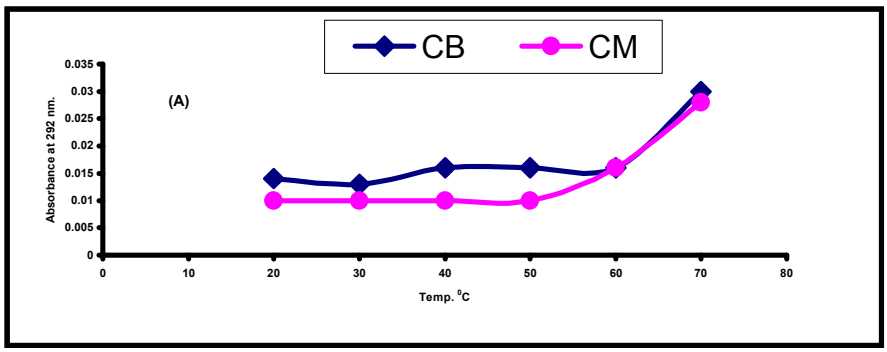


Figure (6 B): Thermal stability curve for benign and malignant at λ_{max} 295 in the presence of 0.01 M NaCl. (CB): Complex of benign breast tumors, (CM): Complex of malignant breast tumors.



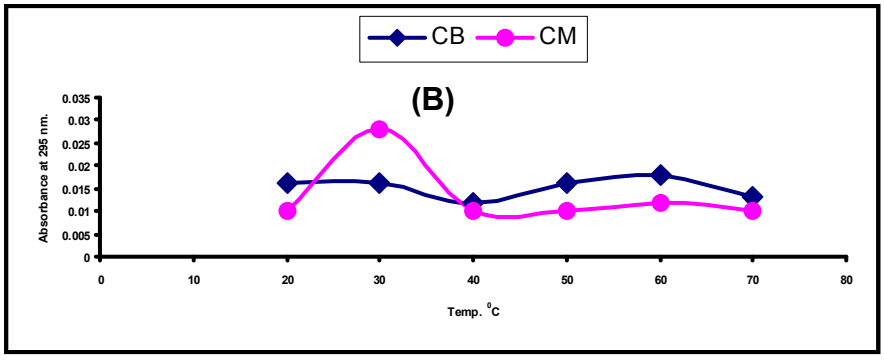


Figure (7): Thermal stability curve for benign and malignant: (A) at □max 292 in the presence of 0. 1 M NaCl, (B) at □max 295 in the presence of 0.1 M NaCl.
(CB): Complex of benign breast tumors, (CM): Complex of malignant breast tumors.

Effect of Urea, KCl and (Urea, KCl) Mixture on the Spectrum of the Complex

The effect of 8 M urea, 0.03 M KCl and a mix of 1:1 of 8 M urea and 0.03 M KCl on the λ_{max} of the benign (fibroadenoma) and malignant (IDC) complexes, were examined. The values of λ_{max} are illustrated in table (5). When table (5) is compared with table (2), it seems that the presence of 8 M urea at pH 7.4, there was a red shift of the λ_{max1} of polypeptide bond from 200 to 227.4 nm in benign complex and a red shift of λ_{max1} from 200 to 226 nm in malignant complex respectively. While λ_{max2} of aromatic amino acid i.e., tyrosine residues in both complexes was disappeared. The red shift is due to intramolecular hydrogen bonding between the oxygen of the amide group and the solvent [24]. When 0.03 M KCl was used, there was no alternation in the position of the λ_{max2} of the tyrosyl at pH 7.4 in both benign and malignant complexes. There was a slight blue shift (3-4nm) in the λ_{max1} of the polypeptide bond in the benign and malignant complex spectra respectively. On the other hand the λ_{max} of the aromatic ring of tyrosyl residues at (274 or 278nm) disappeared. Such blue shift can arise by introducing positive (K^+) or negative (Cl^-) charges near the chromophore (the amid group), which might interact with π -electron system of the amide group [2]. When 8 M urea was mixed with 0.03 M KCl there was significant red shift in λ_{max} (203.4 and 204.2nm) to λ_{max} (221.4 and 219.4nm) in both benign and malignant complexes. The same shift was observed when 8 M urea was used alone with each benign and malignant complexes, this mean that the red shift due to the effect of urea, but not to 0.03 M KCl. On the other hand, there was no alternation in positions of the λ_{max} of the tyrosyl residues near 278nm.As was seen,the changes in absorption were near 230 nm and near 280 nm. This was also observed by Glazer who that solvent perturbation or denaturation of protein poduces may changes in absorption near 230 nm and 280 nm. Some of this change in absorption may be produced by change in the n-π* absorption of poly peptide bond in protein either because of a change in their geometrical arrangement, or because of an environment changes [25].

Table (5): The effect of 8M urea, 0.03M KCl and mixture (urea+KCl) on the λ_{max} of the complex UV spectrum at pH 7.4.

Solvent	λ_{max} (nm)	
	(^{125}I-anti CA 15-3 antibody/ CA 15-3) Benign Complex	(^{125}I-anti CA 15-3 antibody/ CA 15-3) Malignant Complex
Urea 8M	227.4	226
KCl 0.03M	200	200
Urea+KCl mixture 1:1	221.4 , 278.6	219.4 , 278

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