Separation and Characterization of Free-PSA and Its Complexes in Benign and Malignant Uterus Tumors

Jwan A. Al-salihi

Department of Chemistry, College of Science, University of Baghdad, Baghdad-Iraq.
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Abstract

Gel filtration chromatography was used to separate free ^{125}I-anti total PSA antibody from the complexes of ^{125}I -anti total PSA antibody with the PSA immunoreactive isoforms [Free-PSA (F-PSA) and the complex of PSA with α_1-antichymotrypsin (PSA-ACT)] in benign and malignant uterus tumors tissues in order to estimate the ratio of (F-PSA\ t-PSA) or F-PSA%. The data obtained revealed that these ratios are different in benign patients from those of malignant patients.
Spectrophotometric studies using UV. range (200-350 nm) was performed on PSA, anti total PSA antibody, and their complexes in both cases . The results revealed the presence of more than one type of complexes from the same tissue homogenate in both cases.
Polyacrylamid electrophoresis was also performed to characterize the differences and the similarity between the PSA complexes in tissue homogenates of benign and malignant uterus tumors.

الخلاصة

تم استخدام كروموتوكرافيا الترشيح الهلامي لفصل الضد الموسوم بنظير اليود المشع (125) الحر غيـر المرتبط عن المرتبط مع متناظرات المستضد النوعي البروستاتي الفعالة مناعيا (المستضد النوعي البروسـتاتي الحر F-PSA والمستضد النوعي البروستاتي المرتبط بمثبط البروتييزPSA-ACT) في مجانس انسجة اورام الرحم الحميدة والخبيثة وذلك لتقدير النسبة المئوية للمستضد النوعي البروستاتي في تلك العينات. وقـد بينـت النتائج المستحصلة ان هذة النسبة تختلف في المرضى المصابين باورام الرحم الحميدة عنها فـي اورام الـرحم الخبيثة.

كما اجريت دراسة طيفية في منطقة الاشعة فوق البنفسجية باستعمال مدى من الاطوال الموجية تتـراوح بـين (200–350 نانومتر) لكل من المستضد النوعي البروستاتي وضدة الموسـوم بنظيـر اليـود المـشع (125) ومعقداتهم. وقد دلت النتائج على وجود اكثر من نوع من المعقدات في مجانس الانسجة فـي كلتـا الحـالتين المرضيتين. وتم استخدام تقنية الترحيل الكهربائي بهلام الاكريل اماييد لتوصيف مدى التشابة والاختلاف بـين معقدات المستضد النوعي البروستاتي ومجانسات الانسجة لكل من اورام الرحم الحميدة والخبيثة.

Introduction

Prostate specific antigen (PSA) is a 33-34 KD single chain glycoprotein, 3.1S sedimentation coefficient and isoelectric point at pH 6.8 -7.5 [1,2]. At least five PSA isoforms, based on differences in isoelectric

point have been described[3], two biologically active forms are differing in the degree of glycosylation ranging from non-glycosylated to fully glycosylated[4], and three biologically inactive or "nicked" forms[5].

PSA exists in serum in three main forms – a protein bound, complexed to α2-macroglobulin and α1-antichemotrypsin (PSA-ACT) and free unbound form (F-PSA). The two later forms are immunologically detectable[6,7].

Among the chromatographic techniques employed for protein purification, gel filtration is unique in that fractionation is based on the relative size of protein molecules[8]. Also electrophoresis of protein in polyacrylamide gel has provided to be one of the most useful analytical and preparative techniques. Hence proteins are separated on the basis of both size and charge. They have the advantages of high resolution and sensitivity[9]. There were many studies using polyacrylamide gel electrophoresis (PAGE) on PSA[10], and (PSA-ACT) complex in serum[11], prostatic fluid[12], seminal fluid[13], of prostatic diseases[14], and in breast diseases[15].

Generally, several methods have been developed to determine the structures of protein molecules in solution, such as: hydrodynamic, thermodynamic, optical and electrochemical methods, optical rotation, infrared absorption spectra and ultra violet spectra[16]. UV. Spectral method remain one of the most important spectral methods applied in immunology because it provide a sensitive, quantitative methodology for the study of antibody structure and specific ligand binding[17].

The first part of this study was directed toward separation of unbound PSA from its complexes form in both benign and malignant uterus tumors using gel filtration technique. Electrophoresis technique were applied in order to detect any changes in the bands of PSA after the binding with its antibody in benign and malignant uterus tumors in the comparable with crude tissues homogenates. While the second part of this study deals with the structural studies of PSA, ^{125}I-antitotal PSA antibody, and their complexes using UV spectroscopic technique.

Experimental
Chemicals

All laboratory chemicals and reagents were of analar grade: Tris (hydroxy methyl) amino methane, Na-K-tartarate, EDTA, $MgCl_2$, acrylamide, and N,N-methylene bis acrylamide were obtained from **Fluka-Switzerland company**, Dithiothreitol, polyethylene glycol, NaCl, ammonium persulfate, and N,N,N,N-tetramethylethylenediamine from **BDH-UK company**, bovine serum albumin (BSA) from **Sigma-USA company**, Urea from **May&Baker company**, Bromophenol blue from **Hopkins&William company**, Coomassie Brilliant blue from **Merk company**, and total PSA kit purchased from **Immunotech-Beckman Coulter Company-Czech Republic.**

Apparatus

The apparatus used during this study were: Analytic balance, LKB gamma counter type 1270-rack gamma II, Pye unican pH meter, Cooling centrifuge type Hettichi, memmert incubator, memmert water bath, orbital shaker, and spectrophotometer ultra.

Patients

Two groups of patients were included in this study. The first group involved 6 postmenopausal patients suffering from benign uterus tumors (age 55.7 years ± 1.7) while the second group included 8 postmenopausal patients suffering from malignant uterus tumors (age 56.4 years ± 2.3). The patients were clinically diagnosed by physicians and histologically proven by laboratory reports and were not taken any type of therapy.

Collection of Specimens and Preparation of Tissue Homogenate

The tumor tissues were surgically removed from uterus tumor patients by hysterectomy. The specimens were cut off and stored immediately at -20°C. When needed, the frozen tissue was pulverized on ice bath then homogenized at 4°C in TED buffer with a ratio of 1:3 (w:v) using a manual homogenizer. (TED buffer contains 0.01M tris(hydroxy methyl) amino methane, 0.15mM EDTA, and 1.2mM dithiothreitol). The homogenate was filtered through a nylon mesh sieve in order to eliminate filters of connective tissues then centrifuged at 1600 xg for 20 min at 4°C. The supernatant was used as a source of PSA in this study.

Methods

A: Separation of PSA Immunoreactive Isoforms (F-PSA) and (PSA-ACT) Complexes of Benign and Malignant Uterus Tumors Homogenates Using Gel Filtration Chromatography:-

1- A volume of 100µl (250µg protein) of ^{125}I-antitotal PSA antibody was incubated with 200µl (300µg protein) of benign uterus tissue homogenate for 1.5 hr. at 15 °C in a final volume of 1000µl with TED buffer pH 7.6. Two additional tubes, containing 100µl labeled antibody only for total activity computation were set aside until counting.

2- At the end of the incubation, the mixture was applied to the surface of a sephadex G-200 gel filtration column (1x27cm) equilibrated with TED buffer PH 7.6. Elution was carried out to separate F-PSA and PSA-ACT bound to ^{125}I-antitotal PSA antibody using the same above buffer with a flow rate of 4ml/hr and fraction volume of 1ml.

3- The Absorbance at 280nm was measured and the radioactivity of each fraction was counted in a gamma counter for 1min.

4- The experiment was repeated using 200µl (500µg protein) of ^{125}I-antitotal PSA-antibody incubated with 100µl (400µg protein) of malignant uterus tissue homogenate for 2.5 hrs at 15°C using TED buffer pH 7.2.

• Void Volume Determination

The elution volume of Blue Dextran 2000 is equal to the column void volume (V_o), and was determined as follows:
A fresh solution of Blue Dextran (2mg/ml) was prepared in the eluent buffer in a sample volume of 1-2% of the total bed volume, then applied to the column with a flow rate of 4 ml/h. , fractions of 1 ml were collected and their absorbance were measured at $\lambda = 600$ nm.

Calculation

1- The radioactivity (c. p. m) of each eluted fraction was plotted against fraction number.

2- All experiments gave three peaks profile. The first and second peaks represent the ^{125}I-Ab bound to (PSA-ACT) and ^{125}I-Ab bound to F-PSA, respectively while the third peak represents the unbound ^{125}I-Ab.

3- The percent radioactivity of each peak was calculated by dividing the sum of the radioactivity of the fractions under each peak by the sum of radioactivity of all three peaks appeared in the profile:

$$\frac{\text{Radioactivity per peak (c.p.m.)}}{\text{Sum of radioactivity under of all peaks (c.p.m.)}}$$

4- The percentage of free- PSA (f-PSA%) in each sample was determinated as follows.

$$F\text{-}PSA\% = \frac{\text{The radioactivity under (peak2)}}{\text{The sum of radioactivity under (peak1 + peak2 + peak3)}} \times 100$$

5- The percentage of bound PSA (bound PSA%) and (PSA-ACT%) in each sample was determined as follows.

$$Bound\text{-}PSA\% = \frac{\text{The radioactivity under (peak1 + peak2)}}{\text{The sum of radioactivity under (peak1 + peak2 + peak3)}} \times 100$$

$$PSA\text{-}ACT\% = \frac{\text{The radioactivity under (peak1)}}{\text{The sum of radioactivity under (peak1 + peak2 + peak3)}} \times 100$$

6- The percentage unbound PSA (unbound PSA%) in each sample was determined as follow.

$$Un\,bound\text{-}PSA = \frac{\text{The radioactivity under (peak3)}}{\text{The sum of radioactivity under (peak1 + peak2 + peak3)}} \times 100$$

B: Spectral Studies of PSA, (^{125}I-Anti Total PSA Antibody), and Their Complexes

B-1: The UV Spectra of PSA and Its Antibody:-

1- Twenty µls of PSA (100 ng/ml) were diluted to 500µl with TED buffer pH 7.4.

2- The solution was placed in a 0.5 cuvette in sample beam and the absorption spectrum was immediately measured against the same buffer in reference beam in the range (200-350 nm).

3- The experiment was repeated by using 20 µls (100 µg) of ^{125}I-anti total PSA antibody.

B-2: The UV Spectra of the Complex of ^{125}I - Anti Total PSA Antibody Bound to (PSA-ACT) and (F-PSA)

The gel filtration experiment for benign and malignant uterus tumors gave three peaks profile. The fractions under each peak were pooled and the absorption spectrum was measured in the area (200-350 nm) using a 0.5 cm cuvette against TED buffer pH 7.4 in the reference beam.

C: Conventiol Polyacrylamide Gel Electrophoresis

Polyacrylamide Gel Electrophoresis (PAGE) for: benign and malignant uterus tumors homogenates, the complexes of ^{125}I-anti total PSA antibody with (PSA-ACT) in benign and malignant uterus tumors homogenates, and for standard PSA was performed using the protocol described by *LAEMMLI*[18].

Results & Discussions

A: Separation of PSA Immunoreactive Isoforms (F-PSA) and (PSA-ACT) Complexes of Benign and Malignant Uterus Tumors Homogenates Using Gel Filtration Chromatography

Figure (1 A&B) shows the results of gel chromatography that was used to separate the (^{125}I- anti total PSA antibody) bound to PSA complexed to protease inhibitor α_1-antichymotrypsim (PSA-ACT) from (^{125}I-anti total PSA antibody) bound to free PSA in homogenate tissues of patients with benign and malignant uterus tumors homogenates.
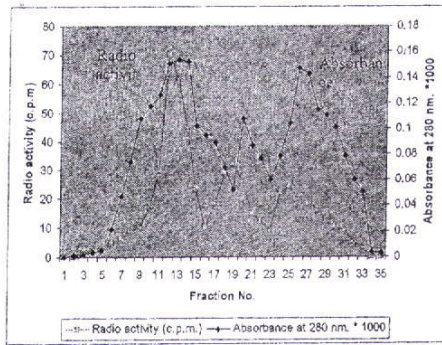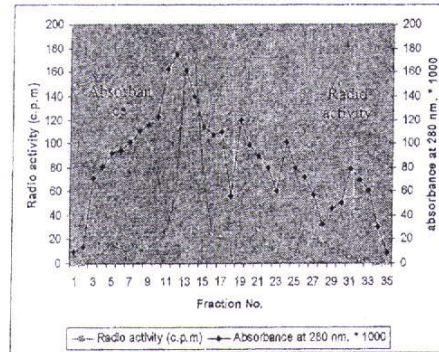
(A)   (B)

Figure (1): The elution profile of the (^{125}I - anti total PSA antibody) binding with PSA immunoreactive isoforms in:-

A: Benign uterus tissue homogenate

B: Malignant uterus tissue homogenate from sephadex G-200 Column (1×27 cm). The void volume= 7 ml and 1 ml fraction were collected.

The trials of this experiments revealed three peaks profile. The first peak represented the antibody to (PSA-ACT) complex, the second peak represents the antibody bound to F-PSA while the third peak represents the labeled Antibody.

The results show that the PSA complexed to ACT is the predominate fraction of PSA in both benign and malignant uterus tumors homogenates while the minor fraction is PSA not associated with ACT (F-PSA). Moreover, the high molecular weight of the first peak which was expected from its low retention volume may attributed to the PSA- ^{125}I--anti total PSA antibody polymerization and aggregation reaction which may indicate the bivalency or multivalency of PSA[19, 6].

Table (1) represents the ratio of (bound PSA/total PSA) or (bound PSA%), (PSA-ACT%), (F-PSA%) and (unbound PSA%) in benign and malignant uterus tumors homogenates.

Table (1):The ratio values of bound and unbound PSA in benign and malignant uterus tumors

Case	Bound %	PSA-ACT%	F-PSA%	Unbound%
Benign Uterus Tumors	77. 5	54. 79	22. 72	22. 49
Malignant Uterus Tumors	86.49	74. 79	11. 7	13. 51

The data in this table show that : the bound%, PSA-ACT%, and F-PSA% in benign groups are less than those in malignant groups, and the F-PSA% are less than the PSA-ACT% in both benign and malignant uterus tumors

homogenates (i. e: in both cases the PSA showed high complexation to ACT).

This results are in agreement with another study on prostatic tissues homogenate, which indicated that the F-PSA% was 26.73% for benign prostate tumor patients and 10.95% for prostate cancer patients[20], while the results are not consistent with other study which indicated that the only immunologically active type of PSA in cancerous prostatic tissue is free PSA[21].

Many previous studies on prostate tumors emphasized the clinical significance of PSA immnoreactive isoforms (F-PSA) and (PSA-ACT) in detection of prostatic cancer [22], it have been suggested that F-PSA% allows a better discrimination between benign and malignant tumors compared with t-PSA[23,24]. The results of this experiment indicated that the ratio of free to total PSA (and also the ratio of F-PSA to the PSA-ACT complex) may be used for differentiation between benign and malignant uterus tumors homogenates as well as the prostate tumors.

B: Spectral Studies of PSA, ([125]I- anti total PSA antibody), and Their Complexes

B-1: The U. V Spectra of PSA and Its Antibody:-
The U. V spectra of PSA and its antibody were measured to determine their maximum wavelengths, and the alteration in their U.V spectra as a result of the interaction with each other.

Figure (2 A&B) illustrates the U.V spectra of PSA and its antibody (^{125}I-anti total PSA antibody).
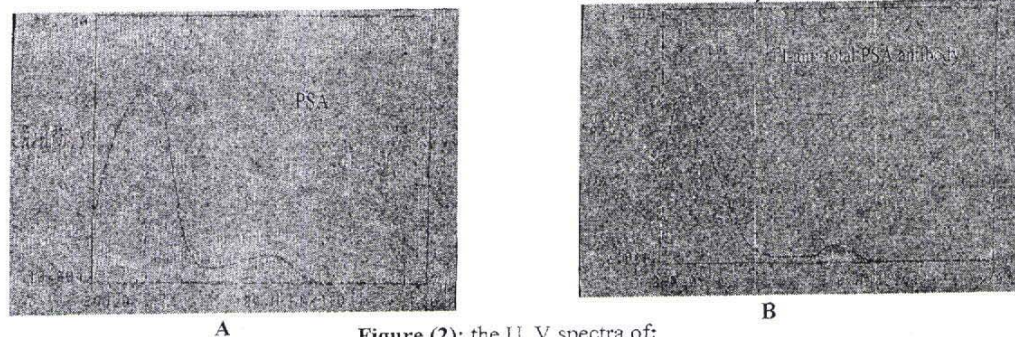
Figure (2): the U. V spectra of:
A: PSA
B: ([125]I- anti total PSA antibody)

The spectrum of PSA consisted of two maximum wavelengths, λ_{max1}, at 276.0 nm with an absorbance 0.47 and λ_{max2}, at 223. 4 nm with an absorbance 1.763. also the U. V spectrum of its antibody consisted of two wavelengths, λ_{max1} 278. 0 nm with an absorbance of 0. 244, and λ_{max2} 209. 8 nm with an absorbance of 0. 988.

The absorption at 276.0 and 278.0 nm is characteristic of the side chain chromophore of tyrosyl residues. While the absorption at 224.0 nm is due to the amide group in the poly peptide bond of PSA molecule with contribution of the histidyl residues [25,26].

It seems that each of Tyr. & Phe. residues in the PSA molecules is located in away, that part of it is on the surface of the protein molecules while the other part is buried.

As a result, PSA and its antibody has a characteristic spectrum and can be identified by their peaks.

B-2: The U. V Spectra of the Complex of ([125]I - anti Total PSA Antibody) Bound to (PSA-ACT) and (F-PSA):-

Table (2) shows the λ_{max} values of ([125]I - anti total PSA antibody) bound to (PSA-ACT) and F-PSA complexes in benign and malignant uterus tumors homogenates which were purified and obtained by gel filtration chromatography as described previously.

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Table (2): The λ_{max} values of the UV spectra of the (AbAg) complexes.

Case	Anti total PSA antibody bound to (PSA-ACT) (λ_{max} nm)	Anti total PSA antibody bound to (F-PSA) (λ_{max} nm)
Benign Uterus Tumors	257. 0, 213. 5	261. 2, 218. 5
Malignant Uterus Tumors	258. 0, 212. 5	262. 5, 216. 0

From this table we can conclude that:-

- Each of PSA isoforms has characteristic spectrum that can be identified by the specific absorption coefficient (a_s) of its λ_{max}.
- The λ_{max} values of the spectrum of all complexes in both benign and malignant uterus tumors homogenates are different from those of the PSA and its antibody alone.
- The λ_{max} values of both benign and malignant uterus tumors homogenates are approximately the same. That may be due to the structure similarity between the complexes.

C: Conventiol Polyacrylamide Gel Electrophoresis:-

Electrophoresis on polyacrylamide gel was carried out on benign and malignant uterus tumors homogenates, the complexes of ^{125}I –anti total PSA antibody with (PSA-ACT) which resulted from the gel filtration experiment of benign and malignant uterus tumors, and standard PSA, as described previously and shown in figure (3).
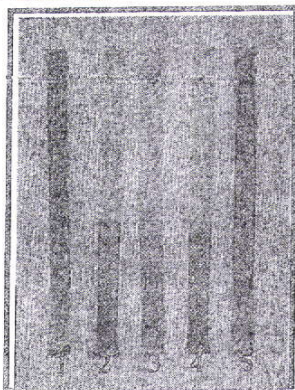

Figure (3): Conventional PAGE profile for uterus tumors (from left to right)
1. crude benign tissue homogenate. 2. benign complex. 3. standard PSA. 4. malignant complex. 5. crude malignant tissue homogenate.

It is obvious from this figure that:

- The standard PSA have five bands, that because the PSA is not a simple molecule, existing in the serum in five isoforms [27].
- There are several protein bands in crude homogenates in comparable with that of the standard PSA, that because the tissue homogenates contained many proteins in addition of PSA and there are bands in the region of the PSA of both benign and malignant uterus tumors .
- The malignant homogenates contain proteins more than that of benign homogenates.
- The complexes migrate faster than that of the standard PSA in spite of that the complexes have larger molecular weight, that may be due to the charge of the complexes at the used pH.
- There are a similarity in the bands of complexes of both benign and malignant tumors in spite of that the concentration of proteins in benign tumors are less than that of malignant tumors, that can be deals with that the molecular weight of the antigen in both cases are the same.

Conclusions

- PSA appears to be present in two immunoreactive isoforms (F-PSA and PSA-ACT) in both benign and malignant uterus tumors homogenates.
- The ratios of PSA isoforms in benign patients differ from those of malignant patients,this indicates that these ratios in benign and malignant uterus tumors tissues may be used as a biochemical markers for uterus tumors prognosis.
- The spectroscopic study revealed that each of PSA, anti-PSA antibody and their complex has a characteristic spectrum.

- Electrophoresis on polyacrylamide gel show that there are bands in the region of the PSA of both benign and malignant uterus tumors.

Acknowledgment

References

1- Belanger A., Halbeek H.V., and Graves H.C., *Molecular and carbohydrate structure of prostate specific antigen:studies for establishment of an international PSA standard*, The prostate, **1995**, 27, 187-97.

2- Ambruster D. A.; PSA: *Biochemistry, Analytical methods, and clinical applications*, Clin. Chem., **1993**, 39(2):181-5.

3- Wu J. T., *Assay for (PSA): problems and possible solutions*, J. Clinc. Lab. Analysis, **1994**, 8, 51-62.

4- Tewari P. C., Willians J. S., Blasé A.B., and Skoloff R.L., *Analytical characterization of several fluid PSA differ from those of serum PSA* ;Clin. Chem., **1998**, 44(1):191-3.

5- Zhang W.M., Leinonen J.,Kalkkinen N.,Dowell B., and Stenman UT., *Purification and characterization of different molecular forms of PSA in human seminal fluid.*, Clinc. Chem., **1995**, 41: 1567-73.

6- Lilja H.V., Christensson A. Dahlen V., Maitikanine MT., Nilsson O.,Pettersson K.,and Lovgren T., *PSA in human serum occurs predominantly in complex with α2-antichymotrypsin.*, Clin. Chem., **1991**, 37:1618-25.

7- Zhang W.M., Finne P., Leinon J., Vesalainen S., Nordling S., Rannikko S., and Stenman VH., *Characterization and Immunological determination of the complex between PSA and α2-macroglobuline*,Clin. Chem., **1998**, 44:2471-9.

8- Deutscher M. P., *Methods in Enzymology Vol. 182, Guide to Protien Purification*, New York, Academic Press, Inc., **1990**, P. 317.

9- Johnstone A., Thorpe R., *Immunochemistry in Practice*, 3rd. ed., BlachWell Science, UK, **1996**, P. 167.

10- Noldus J.Chen Z., and Stamey T.A., *Isolation and Characterization of free PSA form in sera of men with Prostatic Cancer*, J. Urol., Oct.; **1997**, 158(4):1606-9.

11- Peter J., Uverzagt C.,and Hoesel W., *Analysis of free PSA after chemical release from the complex with α1-antichymotrypsin (PSA-ACT)*, Clin. Chem., Apr., **2000**, 46(4):474-82.

12- Wu J.T., Zhang P., Wang T., Wilson L., and Astill M., *Evaluation of F-PSA isoforms, PSA complex formation, and specificity of anti-PSA antibody by HPLC and PAGE immunobloting techniques*; J. Clin. Lab. Anal. ; **1995**, 9(1):1-14.

13- Isono T., Tanaka T.,Kageyama S.,and Yoshiki T., *Structural diversity of cancer-releated and non-cancer-releated prostate specific antigen*, Clin. Chem., Dec., **2002**, 48(12):2187-94.

14- Zhang W. M., *Finne P.,Leinonen J., and Stenman UH., Characterization and determination of the complex between PSA and α1in benign and malignant prostatic diseases*, **Scand J. Clin. Lab. Invest.** *2000* Suppl., 233:51-8.

15- Borchert G.H., Melegos D.N., Tomlinson G.,Giai M., Roagna R., Ponzon L., and Diamandis E.P., *Molecular forms of PSA in the serum of patients with benign and malignant Breast Diseases*, Br. J. Cancer, **1997**, 76(8):1087-94.

16- Scheraga H. A., *Protein Structure*, New York, Acadimic Press, Chap. 6, **1961**,p. 175.

17- Williams C. A. ,and Chanse M.W., *Methods in Immunology and Immunochemistry*, New York, Acadimic Press, Vol. 2,Chap. 10,**1968**, p.163.

18- Laemmli U. K., *Cleavge of Structural Proteins during the assembly of the head of the bacteriophage*, Nature, **1970**, 227:680-5.

19- Russel J.W., Quentin NM., and Nancy NP., *Fundamentals of immunology for students of medicine and related sciences*, USA ,Lea and Febiger-Philadelphia, **1982**, P. 122.

20- Al-Gurnawi, *Physical and Chemical Properties of PSA in some Prostate Diseases*, M Sc. Thesis, College of Science, The University of Baghdad, **1999**, P. 79.

21- Issa A. M. ,*Biochemical Studies on PSA in Prostate Cancer*, Ph D. Thesis, College of Science, The University of Baghdad, **2002**, P.98.

22- Allord W. J.,Zhov Z.,and Yeung KK., *Novel immunoassay for the measurement of complexed PSA in serum*, Clin. Chem., **1998**, 44(6):1216-23.

23- Prestigiacomo A., and Stany T. A., *Clinical usefulness of free and complexed PSA*, Scand J. Clin. Lab. Invest., **1995**, 55 (suppl. 221):32-4.

24- Lilija H., *Regulation of enzymatic activity of PSA and its reactions with extracellular protease inhibitors in prostate cancer*, Scand J. Clin. Lab. Invest., **1995**, 55 (Suppl. 220):47-56.

25- Leach S. J., *Physical Principles and Techniques of protein chemistry*, New York Acadimic Press, Part A, Chap. 3, **1962**, P.102.

26- Freifrider D., *Physical Biochemistry, Application to biochemistry and molecular biology*, San Fransico, W. H. Freeman and company, **1982**, P.667.

27- Ward A. M,Catto JWF.,and Hamdy FC., *Prostate specific antigen: biology, biochemistry, and available commercial assays*, Ann. Clin. Biochem., **2001**, 38:633-651.