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RESEARCH ARTICLE

Purification and Characterization of TRACP from Sera of Patients with Chronic Renal Failure

Hathama R. Hasan[1], Sura A. Abdul Sattar[2*]

1. *University of Baghdad/College of Science/Department of Chemistry/Iraq.*

2. *Al-Mustansyriah University /College of Medicine /Department of Biochemistry/Iraq.*

***Corresponding Author Email:** surasci@uomustansiriyah.edu.iq*

Abstract

Background and Objective Tartrate Resistant Acid Phosphatase (TRACP) also known as type 5 acid phosphatase is a glycoprotein with basic pI , and occurring as two similar isoforms 5a and 5b with different carbohydrate content. This study aims to characterize both TRACP isoforms physico-chemically and spectrophotometrically Materials and Methods: Ion exchange chromatography was applied in order to purify TRACP isoforms (5a and 5b) from sera of control and patients with End Stage Renal Disease (ESRD). Results: The results indicated different specific activities of TRACP 5a and 5b and their proportional amounts in sera of patients with ESRD were different from that of the control group, they had approximate molecular weight of (36.8-39.3 KDa) and a basic isoelectric point equal to 9.6 in control and patients with ESRD. The optimal pH for 5b is relatively higher than for 5a,the heparin (23 KU/L) had an inhibitory effect only on the activity of TRACP 5a ,the iron content of isoform 5b was found to be one mole per one mole of enzyme, and UV spectrum of TRACP 5a was found to be different from that of TRACP 5b in both control and patients with ESRD. Conclusion: We conclude that there are differences in the characterization of both isoform TRACP 5a and 5b in sera of end stage renal disease.

Keywords: *TRACP, Purification, Isoform, Characterization.*

Introduction

Acid phosphatase is represented by a number of enzymes that can be differentiated according to structural and immunological properties, tissue distribution, subcellular location and other features [(1)]; these ACP isoenzymes share a similar catalytic activity toward phosphate in an acidic medium [(2)]. Together with similar enzymes isolated from animals, plants, and fungi, it belongs to the group of purple acid phosphatase.

Acid phosphatase isoenzymes can be fractionated by electrophoresis into six bands (numbered 0-5) based on their increasing electrophoretic mobility toward the cathode in acidic polyacrylamide gel [(2), (3)]. Isoenzyme 0 moves very slowly and stays at the application point in the gel while isoenzyme 5 moves fastest[(4)].TRACP have unique properties among these isoenzymes, which include resistance to inhibition by tartrate, active at acid pH with pI of (8.5-9.0) ,and occurring as two similar isoforms 5a and 5b with different carbohydrate content[(1)]. The active site of TRACP contains a binuclear iron center [(5)]. The iron center is believed, not only to be essential for its enzymatic activity, but also to be a structural constituent [(6)]. The enzyme can exist in two different forms: an enzymatically active pink form where the binuclear iron unit is the ferrous-ferric state, and an enzymatically inactive purple differic state. Mild reductive agents can change the inactive form into active form [(7)].

TRACP actually consists of two related structural forms: 5a and 5b [(8)].TRACP 5a exists as a monomer, whereas 5b is a proteolytically nicked disulfide-linked (heterodimer) [(9)]. They differ in their pH optimum, which is 5.0 for 5a and (5.5-6.0) for 5b[(10)], in their carbohydrate content, where 5a contains sialic acid while 5b does not[(11)],in heparin effect, where 5a is inhibited but this compound has no effect on 5b activity[(12)].

Several methods have been used to measure exclusively the amount and activity of serum

TRACP: kinetic methods, immunological methods, and chromatographic methods, including ion exchange and heparin column chromatography [13]. To obtain sufficient isoforms TRACP 5a and 5b for biochemical characterization, these isoforms were isolated from sera of control and patients with ESRD by sequential chromatography on DEAE-Sepharose and CM-Sepharose.

Materials and Methods

Patients and Samples

A total of 40 patients with chronic renal failure at end stage renal disease attending Al-Karama and Specialist Surgeries Hospitals in Baghdad city was included in this study. They were all undergoing hemodialysis treatment for (2-24) months at the time of the study. Patients diagnosed as having hepatitis were excluded. As a control, 37 age matches healthy individuals were included in the present study. Six milliters (ml) of venous blood were collected from the healthy donors and the patients (before hemodialysis).

Blood samples were centrifuged at (2000 g) for 10min after blood coagulation, serum thus separated into two test tubes in which one of them was mixed with 20% glacial acetic acid in proportion of 10µl : 1ml serum to stabilize acid phosphatase activity[14],[15]. All sera were stored at -20°C until being used.

Purification of Serum TRACP

Tartrate-resistant acid phosphatase was purified via two stages using Anionic Ion-Exchange Column Chromatography [16], [17], then Cationic Ion Exchange Column Chromatography [18].

Discontinuous Polyacrylamide Gel Electrophoresis

The presence of different TRACP isoformes in sera samples of control and patients groups was detected using discontinuous polyacrylamide gel electrophoresis according to Lam ET. al. [19] method with some modification, where Separating gel concentration was 6.7% instead of 7.7%, Ammonium persulfate concentration was 0.32g/100ml D.W instead of 0.28g/100ml D.W.

Gel Staining Methods

The gel was stained for ACP activity depending on method of Lam et. al.[19] with modification ,where Basic Fuchsine was used instead of Fast Garnet GBC as a stain. Also the gel was stained for Protein [20], and Glycoprotein Stain [21].

Physico-Chemical Characterization of TRACP

Molecular Weight Determination of TRACP

The approximate molecular weight of TRACP was determined using the relative mobility value (Rm) of the TRACP band on polyacrylamide gel electrophoresis [22].

Analytical Electro Focusing in Polyacrylamide Gel

Electro focusing is a technique used to determine the isoelectric points of the protein samples [23].

Effect of pH on TRACP Isoforms Activity

In order to test the pH effect on each TRACP isoform activity, the activity was carried out using the eluted CM-Sepharose fraction tubes (no.=18-29) at optimum reaction condition according to the microplate assay method of Lau et.al[24] ,with modification[25].

Heparin Effect on TRACP Isoforms Activity

Heparin effect on TRACP isoforms activity was studied at pH 6.0.The TRACP activity was carried out on fraction tubes (no. =18-29) eluted from CM-Sepharose column using heparin (23 KU/L).

Iron Content of TRACP 5b

The iron content of TRACP 5b was determined using flame atomic absorption spectrophotometer type GBC 933 plus at λ = 248.3nm.

Spectroscopic Studies on TRACP 5a and 5b

The spectra of TRACP isoforms, pH effect and heparin effect were studied using (U.V. Visible Reces Spectrophotometer UV-160).

Results

The first chromatographic stage on DEAE-Sepharose removed most of the contaminating proteins, including tartrate sensitive acid phosphatase. Tartrate resistant acid phosphatase was eluted from column by tris-HCl buffer solution(50mM,pH8.0) containing NaCl (100mM).Each eluted fraction was acidified, at once by the addition of 20% acetic acid to

preserve the phosphatase activity[14],[15]. Figure (1) and (2) shows TRACP elution profile from sera of control and patients with ESRD respectively. TRACP isoforms 5a and 5b were separated by CM-Sepharose mini column.

Typical findings, displayed in Figures (3) and (4), showed that serum TRACP activity is separated into two distinct activity peaks. In the control group the first peak (5a) contained 51.7% of the total TRACP activity, and the second peak (5b) contained 48.3% of the total TRACP activity while in patients with ESRD the first peak contained 28.7% of the total TRACP activity and the second peak contained 71.35% of the total TRACP activity. Chromatographic separation of TRACP 5a and 5b from pooled sera of patients with ESRD showed marked relative increase in 5b activity in comparison with that of the control group. Therefore the increased TRACP activity that had been observed in sera of ESRD patients was attributed to an absolute increase of circulating isoform5b. The yield and the fold of purification of each separated form from control and patients with ESRD were calculated, and the results are presented in Table (1 A, B) respectively.

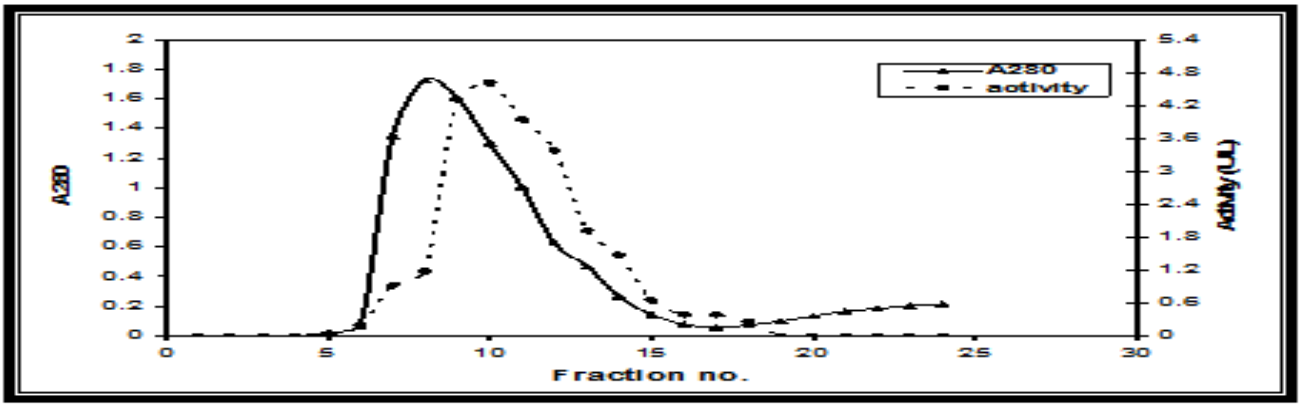

Figure 1: Separation of TRACP from sera of the control group using DEAE-Sepharose column chromatography

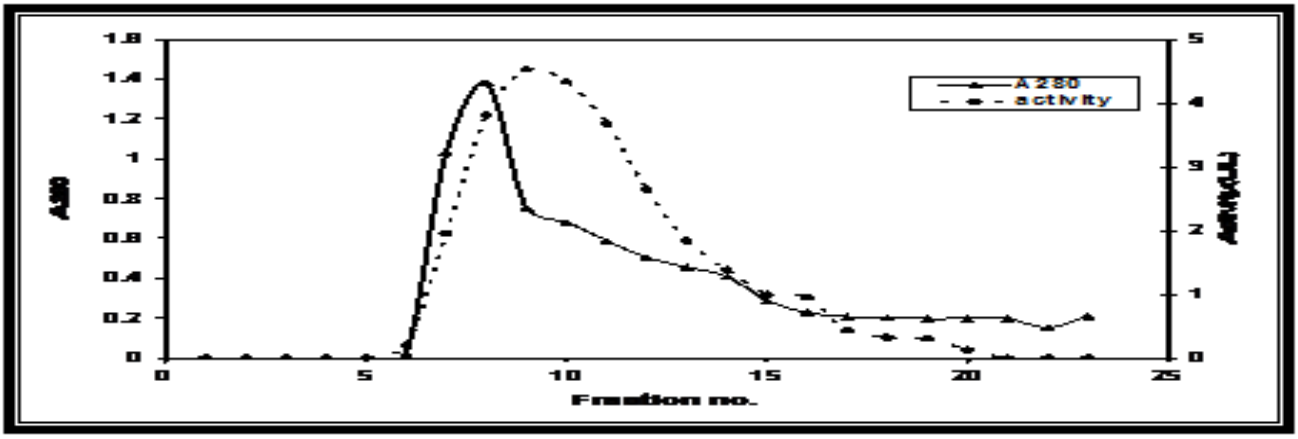

Figure 2: Separation of TRACP from sera of patients with ESRD using DEAE-Sepharose column chromatography

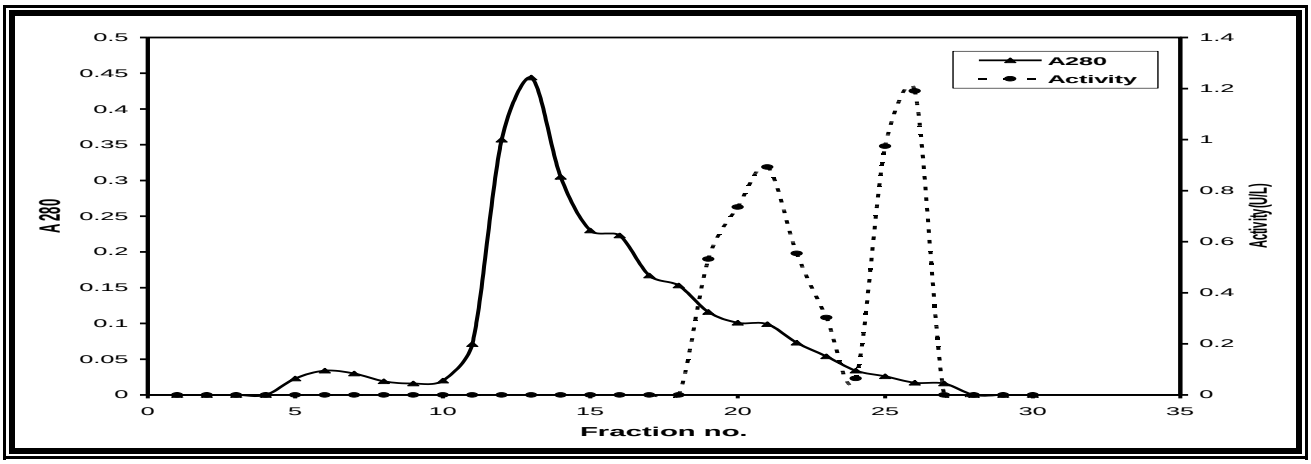

Figure 3: Separation of TRACP isoforms 5a and 5b from sera of the control group using CM-Sepharose column chromatography

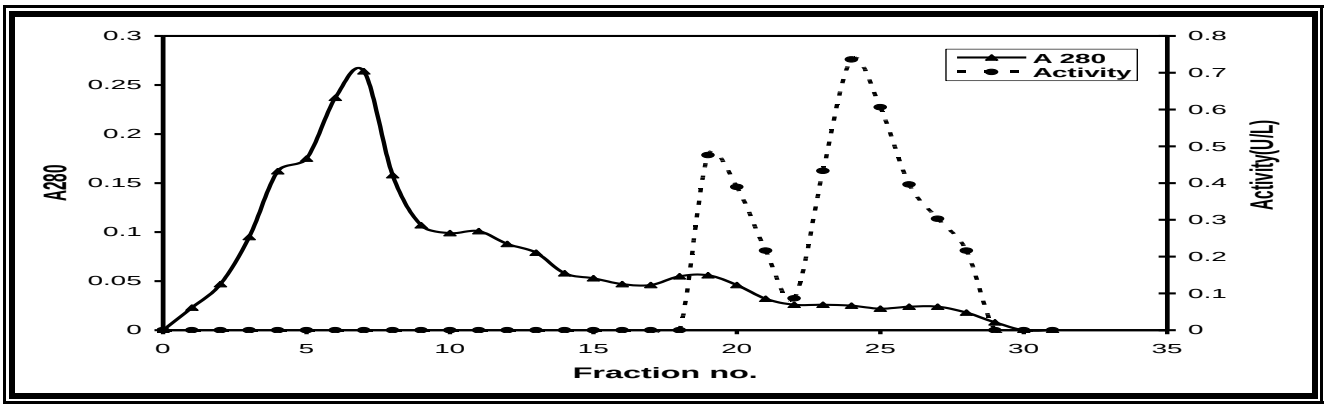

Figure 4: Separation of TRACP isoforms 5a and 5b from sera of patients with ESRD using CM-Sepharose column chromatography

Table 1: Purification of serum TRACP isoforms from control (I) and patients with ESRD (II)

I	Volume mL	Activity U/mL ×10³	Total Activity U×10³	Protein mg	Specific activity U/mg×10³	Yield %	Fold of purification
Crude	4	7.2	28.8	312	0.0923	100	1.0
DEAE-Sepharose	14	1.69	23.6	17.7	1.33	82	14.0
dialysis	2.5	3.49	8.73	17.6	0.49	30.3	5.37
CM-Sepharose	16	0.39	6.25	2.89	2.2	21.7	23.4
A	12	0.33	4.02	2.17	1.48	14.0	16.0
B	4	0.54	2.17	0.055	39.5	7.5	427
II							
Crude	4	13.13	52.5	284	0.184	100	1.0
DEAE-Sepharose	15	2.75	41.25	31.6	1.305	79	7.0
dialysis	3	5.37	16.11	31.6	0.51	31	3.0
CM-Sepharose	20	0.574	11.48	1.91	6	21.9	33
A	8	0.41	3.25	1.77	1.84	6.2	10
B	12	0.68	8.1	0.1	81	15.4	444

Electrophoresis Patterns of TRACP

In order to test the separation and purity of TRACP isoforms from sera of the control and patients with ESRD, these isoforms were analyzed by PAGE. The gel was stained for protein Figure (5, 6), glycoprotein Figure (7, 8), and enzyme activity Figure (9, 10).

Figure 5: Protein profile on polyacrylamide gel electrophoresis (6.7%) for serum crude and partially purified enzyme of the control. The samples used were 1: Crude pooled sera(control,2: Partially purified TRACP eluted from DEAE-Sepharose column,3: Partially purified TRACP 5a,4: Partially purified TRACP 5b.

Figure 6: Protein profile on polyacrylamide gel electrophoresis (6.7%) for serum crude and partially purified enzyme of patients with ESRD. The samples used were:1: Crude pooled sera (patients with ESRD),2: Partially purified TRACP eluted from DEAE-Sepharose column,3:Partially purified TRACP 5a,4: Partially purified TRACP 5b

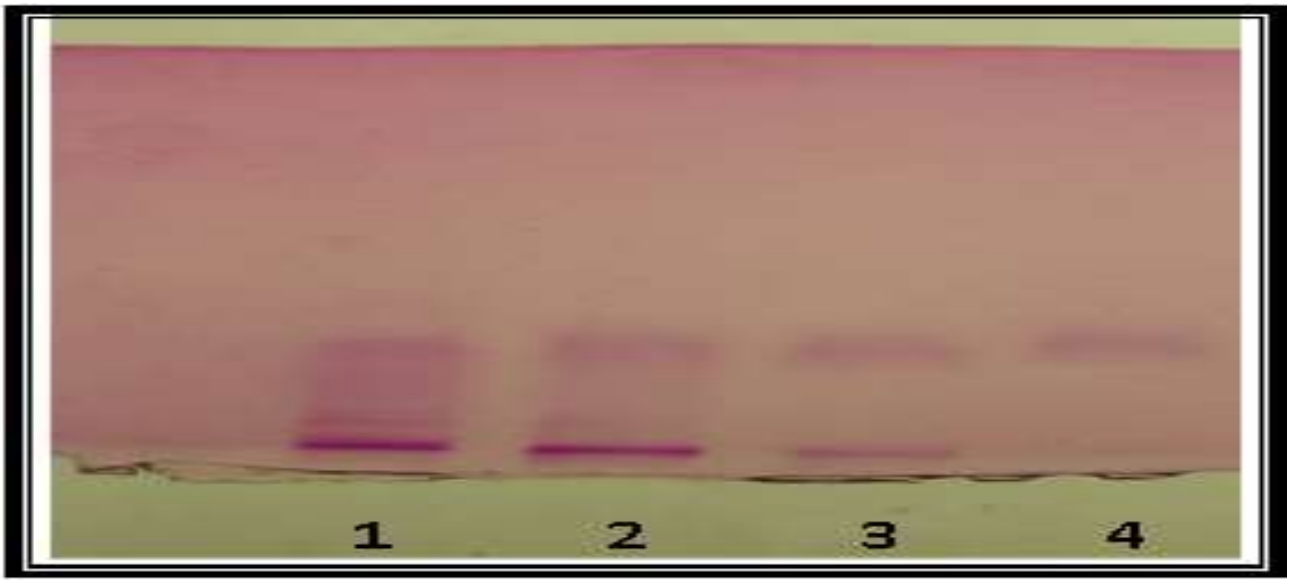

Figure 7: Glycoprotein profile on polyacrylamide gel electrophoresis (6.7%) for serum crude and partially purified enzyme of the control. The samples used were 1: Crude pooled sera (control), 2: Partially purified TRACP eluted from DEAE-Sepharose column, 3: Partially purified TRACP 5a, 4: Partially purified TRACP 5b

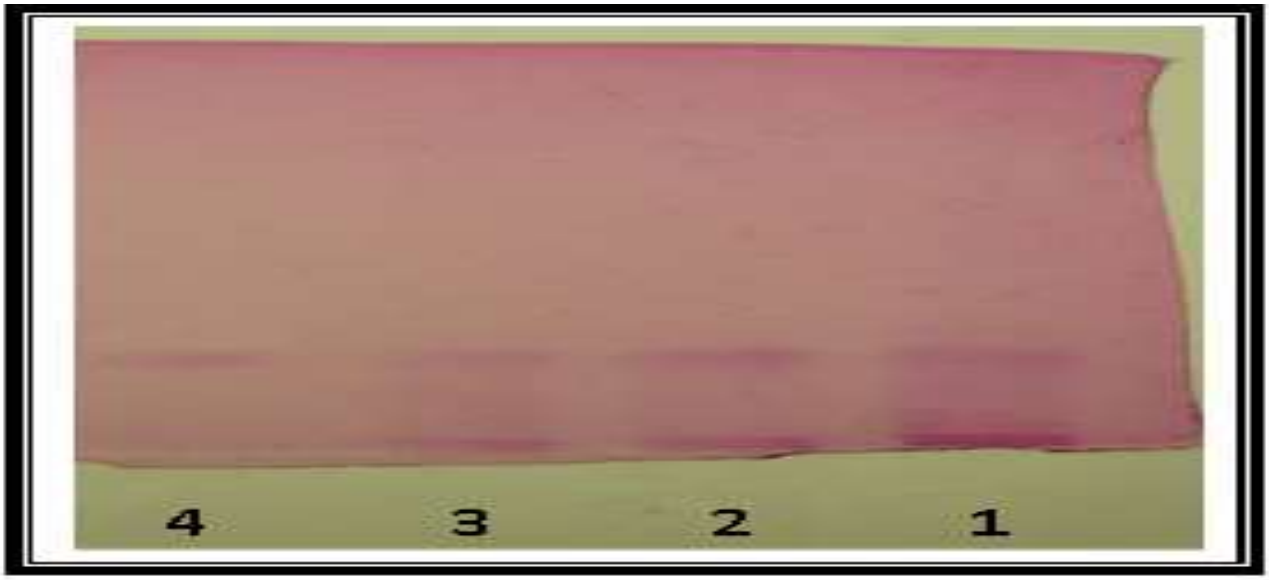

Figure 8: Glycoprotein profile on polyacrylamide gel electrophoresis (6.7%) for serum crude and partially purified of patients with ESRD. The samples used were1: Crude pooled sera ((patients with ESRD), 2: Partially purified TRACP obtained from DEAE-Sepharose column, 3: Partially purified TRACP 5a, 4: Partially purified TRACP 5b

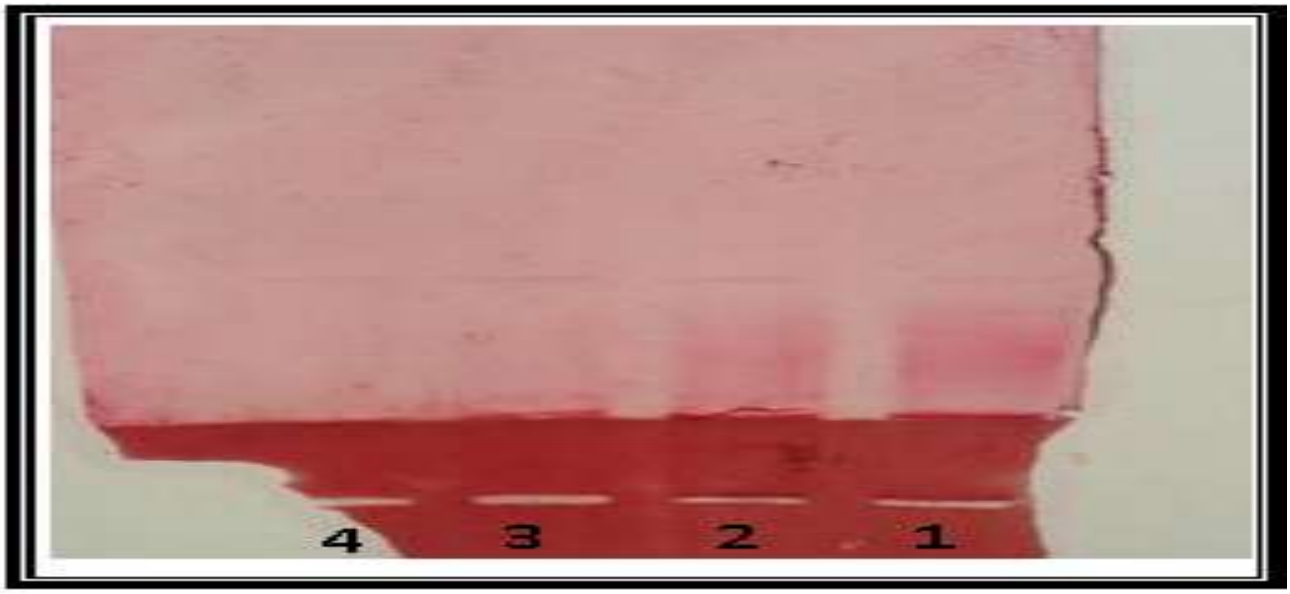

Figure 9: Enzymatic activity on polyacrylamide gel electrophoresis (6.7%) for serum crude and partially purified enzyme of the control. The samples used were1: Crude pooled sera (control), 2: Partially purified TRACP eluted from DEAE-Sepharose column, 3: Partially purified TRACP 5a, 4: Partially purified TRACP 5b

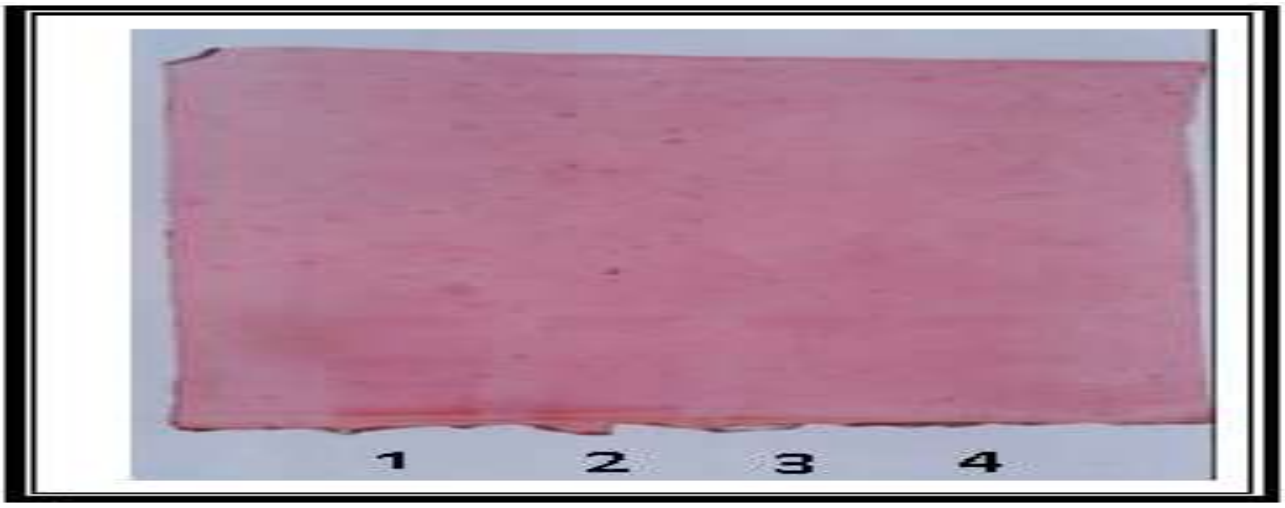

Figure 10: enzymatic activity on polyacrylamide gel electrophoresis (6.7%) for serum crude and partially purified enzyme of patients with ESRD. The samples used were1: Crude pooled sera (patients with ESRD), 2: Partially purified TRACP eluted from DEAE-Sepharose column, 3: Partially purified TRACP 5a,4: Partially purified TRACP 5b

Physico-Chemical Characterization of TRACP

Molecular Weight of TRACP Isoforms

Approximate molecular weight of partially purified TRACP isoforms was determined using the standard curve of molecular weight, obtained from electrophoretic analysis of standard proteins.

Table (2) shows the relative mobilities (Rm) of standard proteins and TRACP isoforms for control and patients with ESRD. The results indicated that in control TRACP 5a and 5b had approximate M. wt of 39.28 KDa and 37.6 KDa respectively. In patients with CRF TRACP 5a and 5b had approximate M. wt of 37.61 KDa and 36.81 KDa respectively.

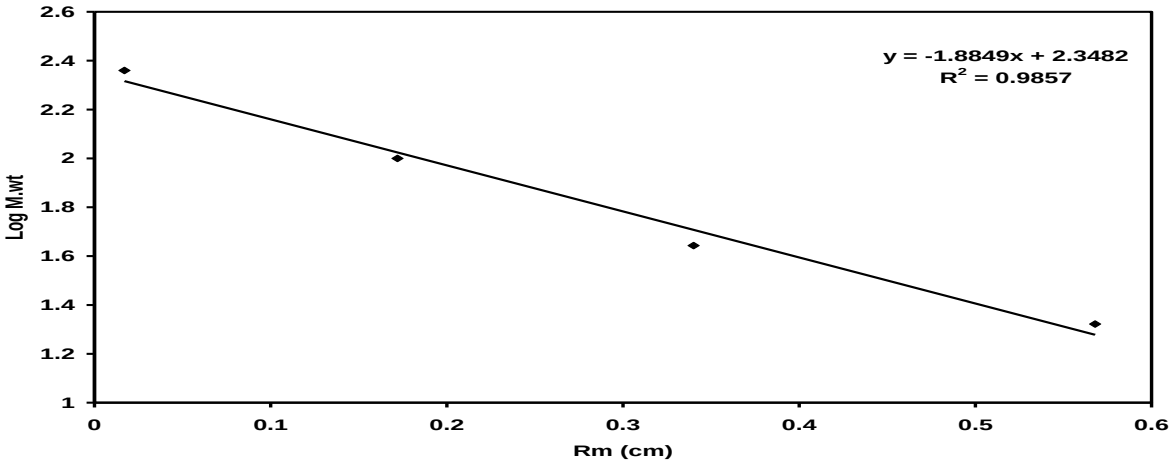

Figure 11: Standard curve of M.wt. determination

Table 2: Relative mobilities (Rm) for Standard proteins and TRACP isoforms

Protein	Rm	M.wt (KDa)
Catalase	0.017	232
Acid phosphatase	0.172	100
Ovalbumin	0.34	44
Trypsin	0.568	21
TRACP 5a (control)	0.40	39.28
TRACP 5b (control)	0.41	37.61
TRACP 5a (ESRD)	0.41	37.61
TRACP 5b (ESRD)	0.415	36.81

Isoelectropoint (pI) of TRACP

In order to scan the variation in pI values of proteins present in sera of control and patients with ESRD, isoelectrofocusing process was carried out in pH range (3.5-10.5). The result (Figure 12) indicates the presence of distinct variations in pI values of proteins in sera of patients with ESRD in comparison with that of the control group.

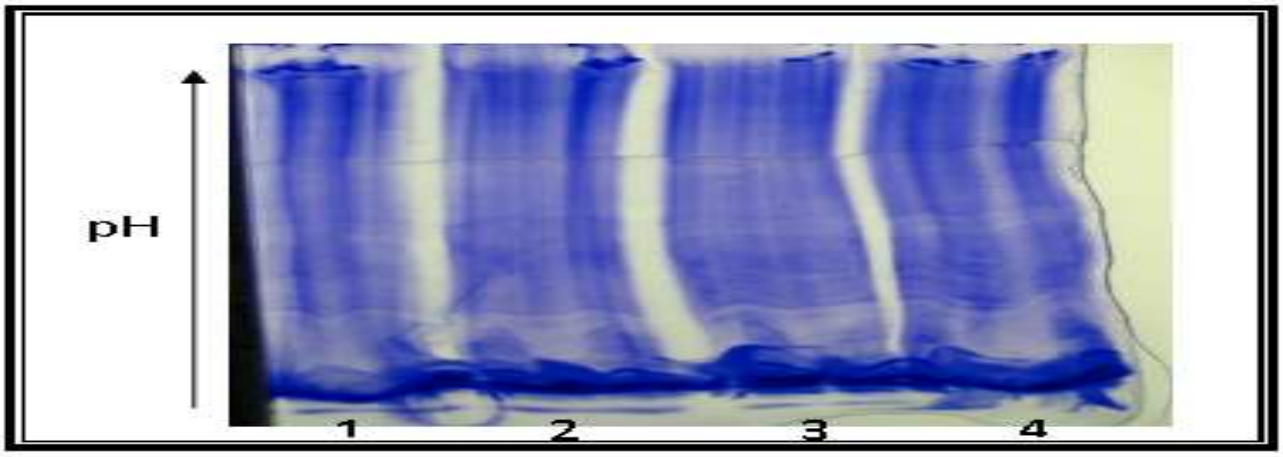

Figure 12: Protein profile on polyacrylamide gel electrofocusing 5% (pH 3.5-10.5). The samples used were:1,2 : Crude serum (control),3,4 :Crude serum (patients with ESRD)

IEF was so carried out in pH range (7.8-10.0) in order to determine the pI value of TRACP. The gels were stained for proteins and enzymatic activity, Figure (13) and (14). Based on the calibration curve (Figure 15), the results indicated that TRACP have a pI value approximately equal to 9.6.

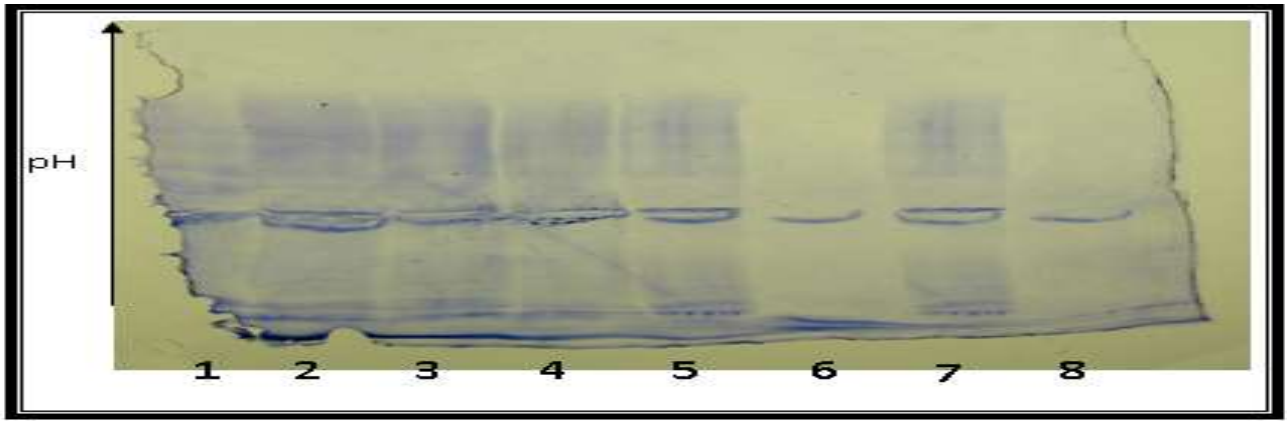

Figure 13: Protein profile on polyacrylamide gel electro focusing 5 %(PH 7.8-10). The samples used were:
- **Crude serum (control).**
- **Crude serum (patients with ESRD).**
- **Crude sera pooled (control).**
- **Crude sera pooled (patients with ESRD).**
- **Partially purified TRACP 5a (control).**
- **Partially purified TRACP 5b (control)**
- **Partially purified TRACP 5a (patients with ESRD).**
- **Partially purified TRACP 5b (patients with ESRD).**
-

Figure 14: Enzyme activity on polyacrylamide gel electro focusing 5% (pH 7.8-10). The samples used were:
- **Crude serum (control).**
- **Crude serum (patients with ESRD).**
- **Crude sera pooled (control).**
- **Crude sera pooled (patients with ESRD).**
- **Partially purified TRACP 5a (control).**
- **Partially purified TRACP 5b (control)**
- **Partially purified TRACP 5a (patients with ESRD).**
- **Partially purified TRACP 5b (patients with ESRD).**

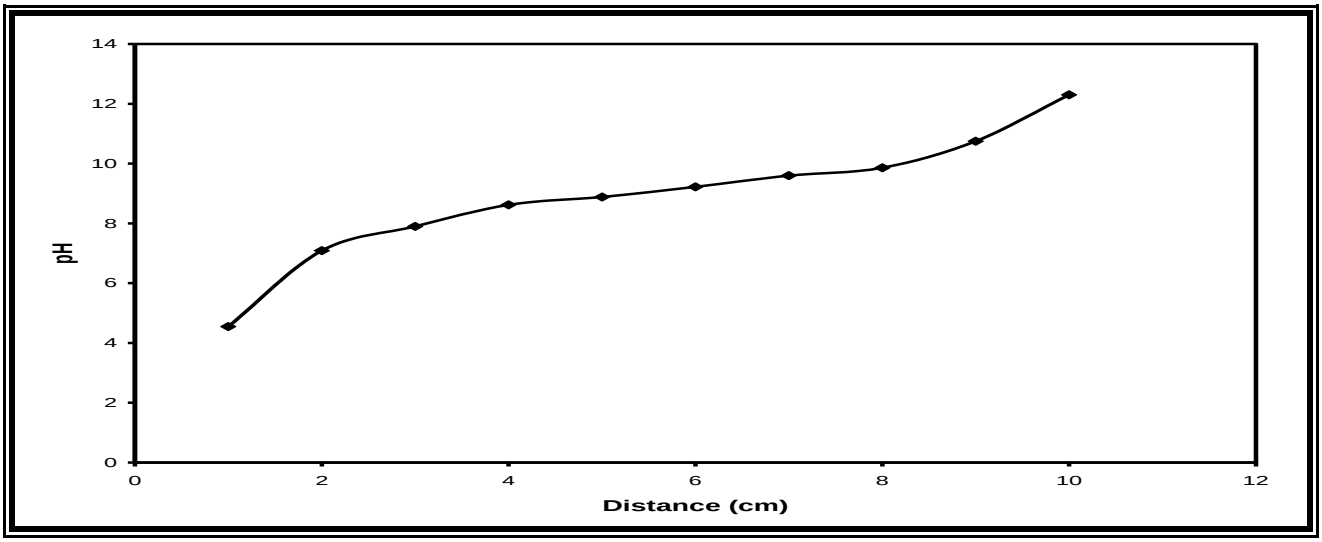

Figure 15: Calibration curve of pI determination using ampholine pH (7.9-10.0)

PH Effect on TRACP Isoforms

TRACP activity of the two peaks (5a and 5b) eluted from CM-Sepharose was measured at three different pH (5.0, 5.5, and 6.0).Figure (16) and (17) shows the pH effect on TRACP 5a and 5b activity in the sera of control and patients with ESRD respectively. As shown form these figures, with increases the pH, peak activity of TRACP 5b increased, and peak activity of TRACP 5a decreased, this finding show that the optimal pH among the studied pH for isoform 5b is relatively higher than that for isoform 5a.

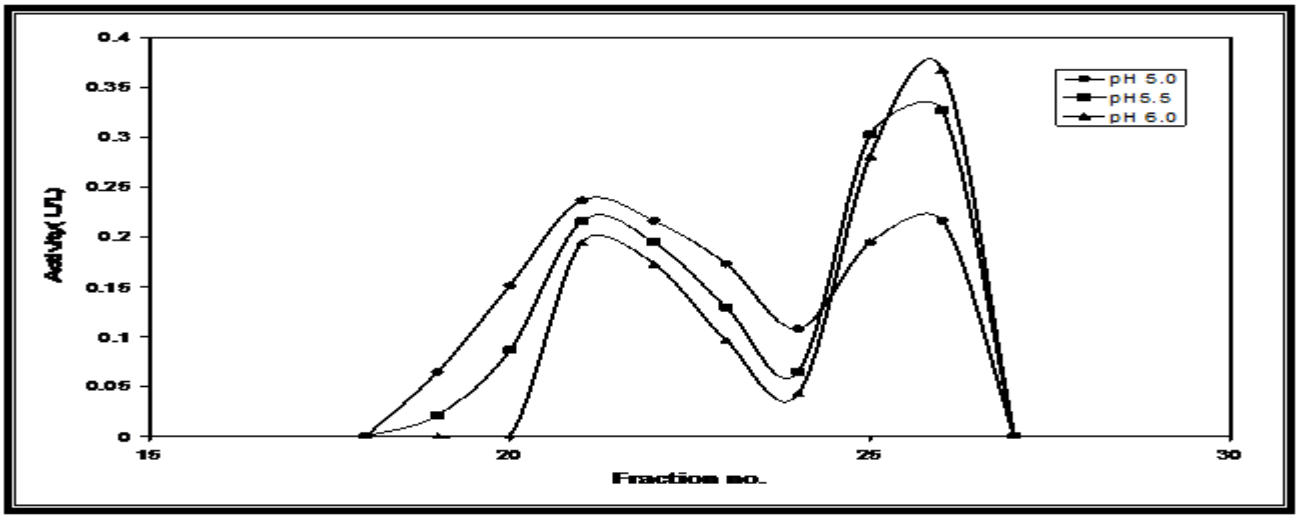

Figure 16: pH effect on TRACP activities separated from sera of control group

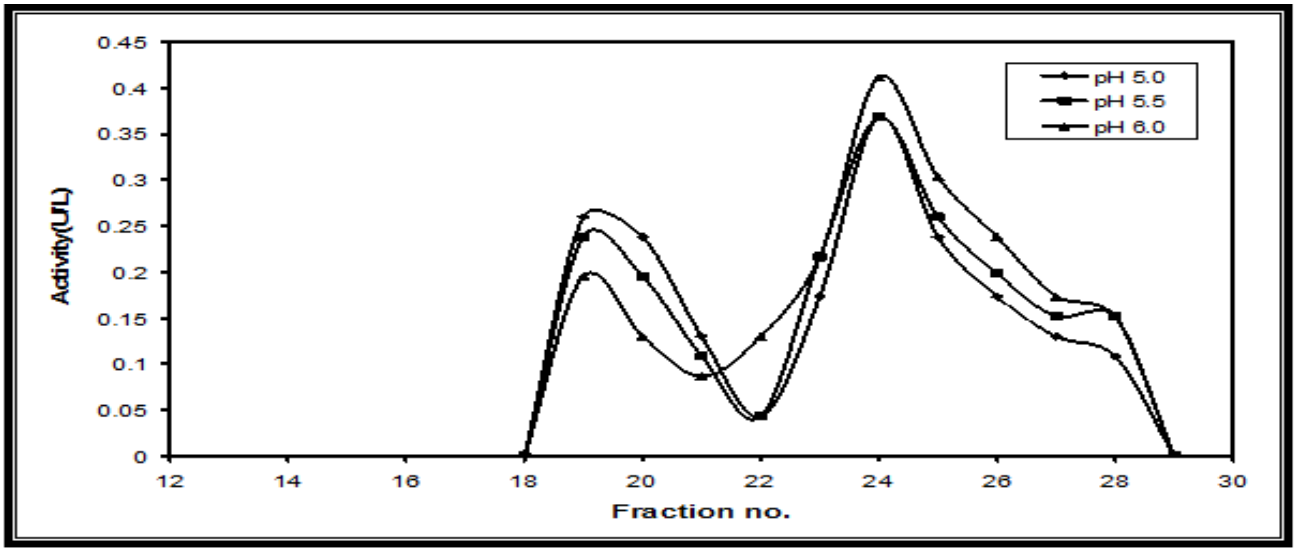

Figure 17: pH effect on TRACP activities separated from sera of patients with ESRD

Heparin Effect on TRACP 5a and 5b

TRACP activity of the two peaks(5a and 5b) eluted from CM-sepharose was measured in the presence of heparin at pH 6.0.The result indicated that the activity peak of 5a isolated from sera of control group was inhibited 90.5% in the presence of heparin, while no effect of heparin on the activity peak of 5b was observed Figure (18). In sera of patients with ESRD ,the result Figure (19) indicated that the activity peak of 5a was 74% inhibited in the presence of heparin, while no effect of heparin on the activity peak of 5b was observed.

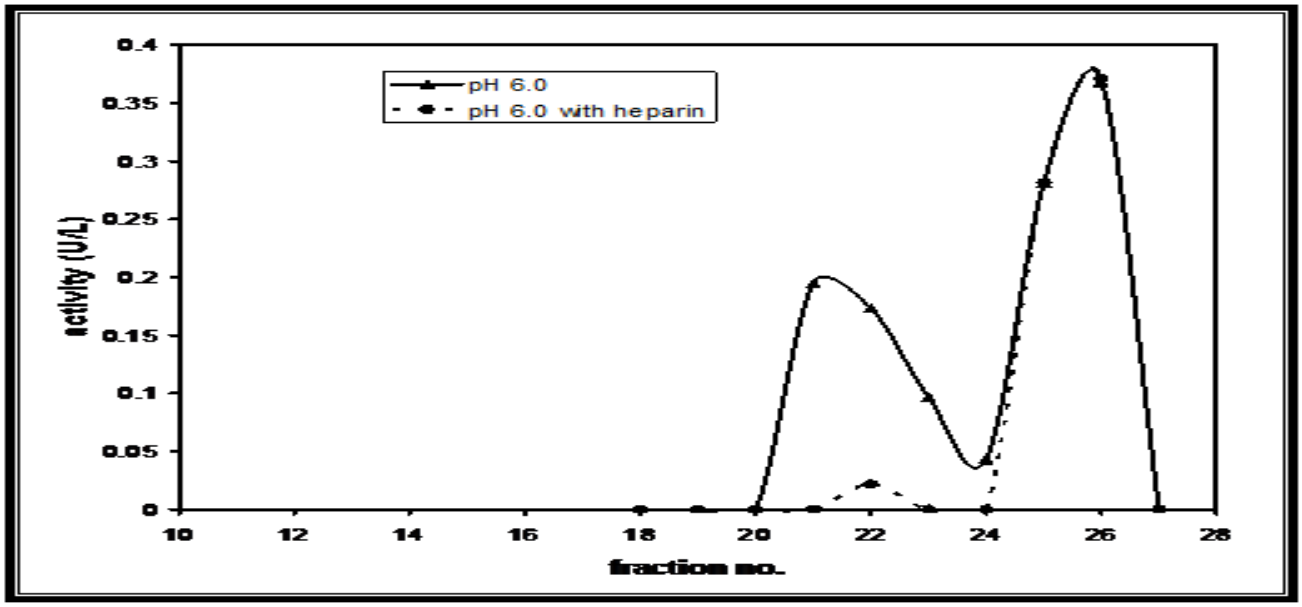

Figure 18: Heparin effect on TRACP activity at pH 6.0 separated from control group

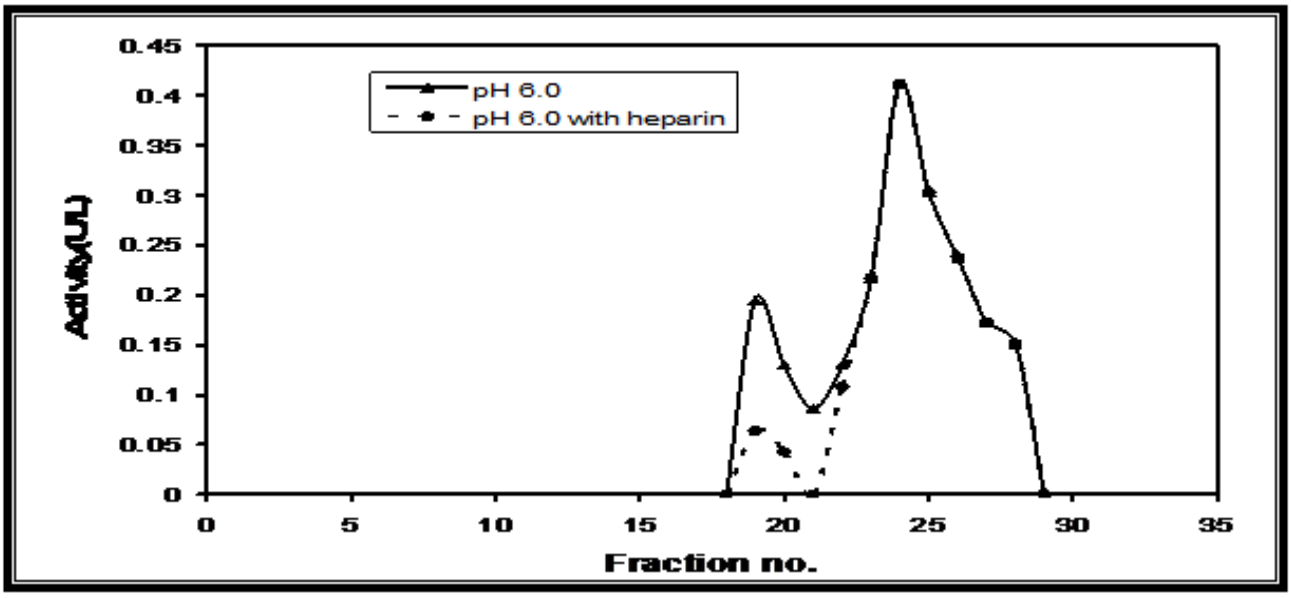

Figure 19: Heparin effect on TRACP activity at pH 6.0 separated from sera of patients with ESRD

Iron Content of TRACP 5b

The iron content of the partially purified TRACP 5b was determined using flame atomic absorption spectroscopy. The results indicated that 1:1 is the molar ratio of iron to enzyme for both control and patients with ESRD.

Spectroscopic Studies on TRACP 5a and5 b

The UV-spectra of 5a and 5b TRACP were measured at pH 5.5 within wave length covering the range of (200-380) nm. Figure (20) and (21) show the UV-spectra of TRACP 5a and 5b separated from control group. As shown from these figures, the isolated peak 5a has absorbance at 220.8 nm while peak 5b has absorbance at 210.6 nm. Based on the absorption spectra of standard tyrosine and standard histidine Figures (22) and (23),the absorption of peak 5a at 220.8 belongs to

tyrosyl or histidyl residues, while the absorption of peak 5b at 210.6 nm is due to the transitions of the peptide backbone of the protein. Figure (24) and (25) show the UV-spectra of TRACP 5a and 5b separated from sera of patients with ESRD. These figures indicate that the isolated peak 5a has absorbance at 218.4 nm while peak 5b has absorbance at 210.4 nm. The absorption of peak 5a at 218.4 belongs to tyrosyl or histidyl residues, while the absorption at 210.4 nm is due to the transitions of peptide backbone.

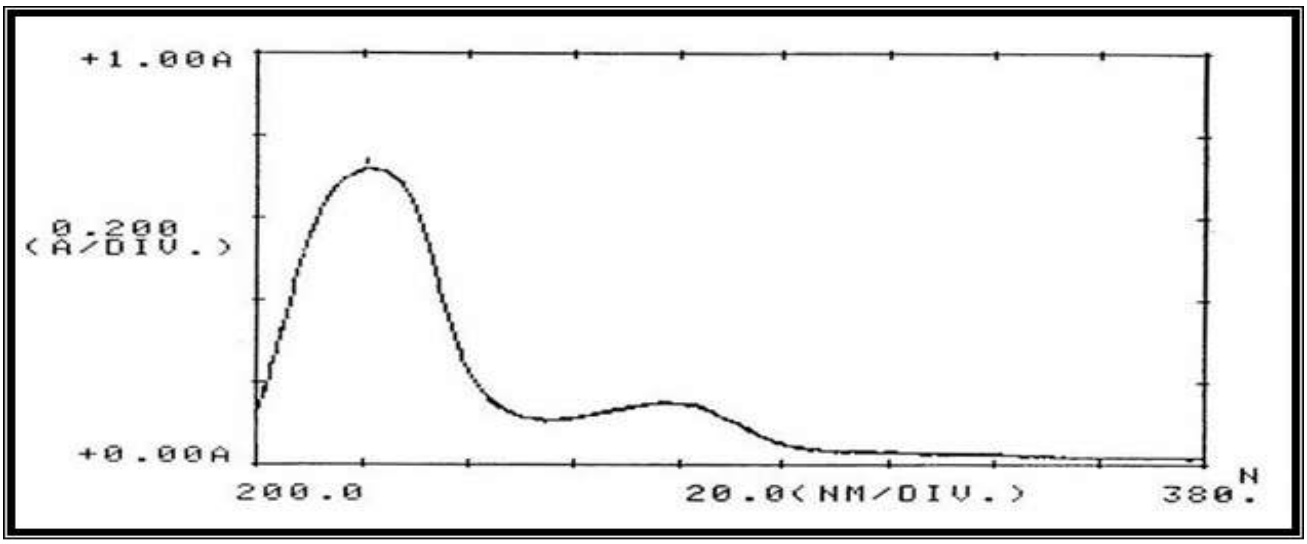

Figure 20: UV-Spectrum of TRACP5a in acetate buffer pH 5.5 of control group

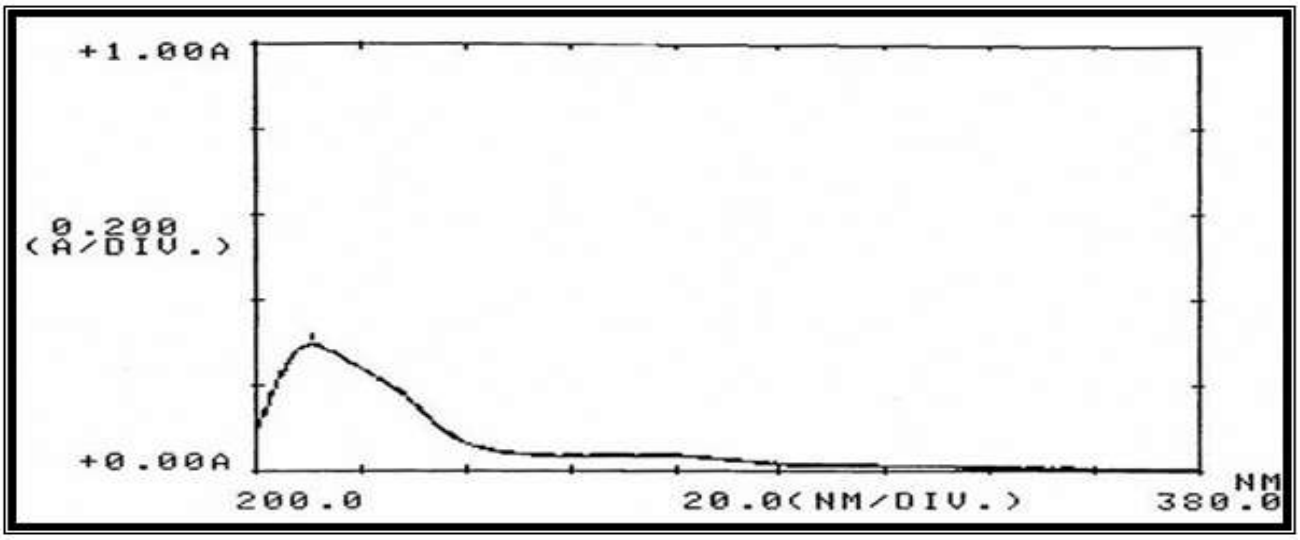

Figure 21: UV-Spectrum of TRACP5b in acetate buffer pH 5.5 of control group

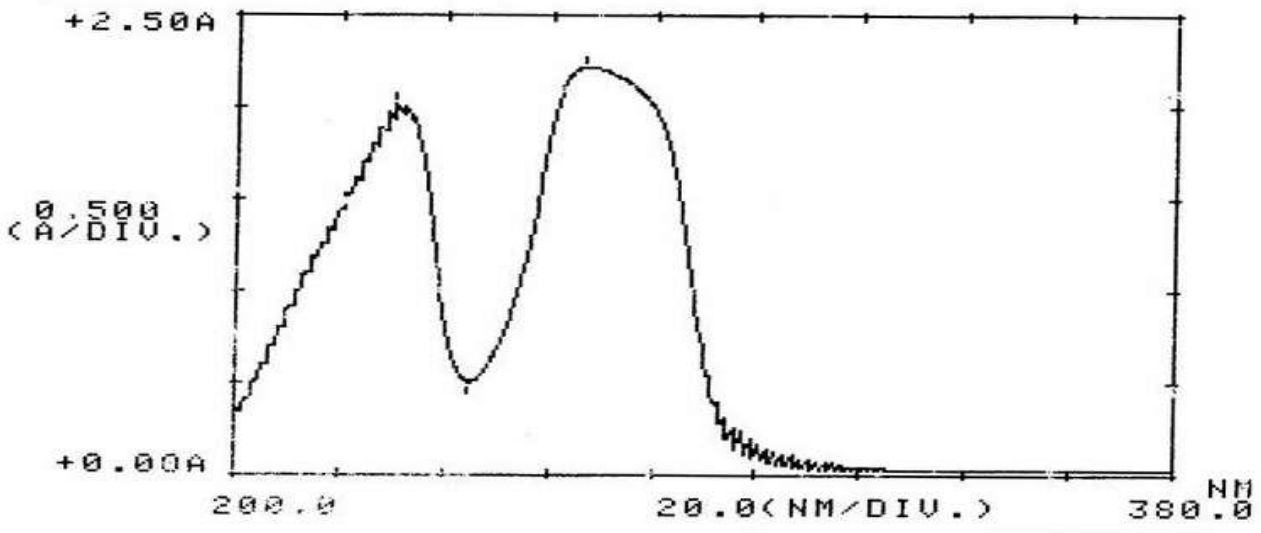

Figure 22: UV. Spectrum of tyrosine in acetate buffer pH 5.5

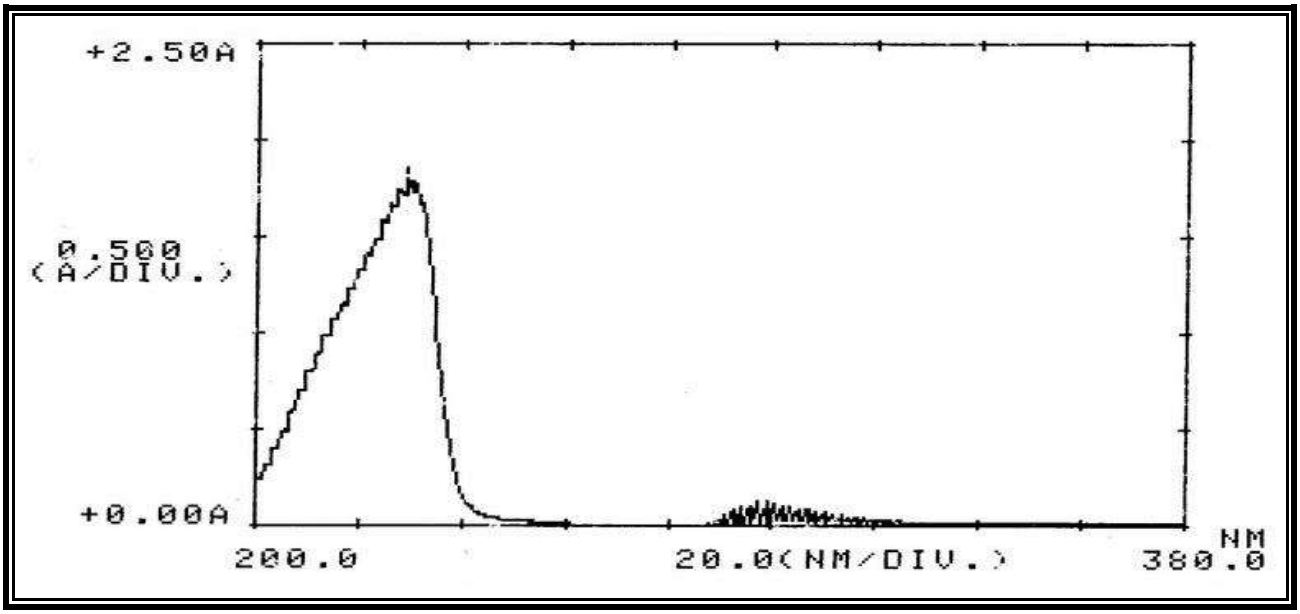

Figure 23: UV. Spectrum of histidine in acetate buffer pH 5.5

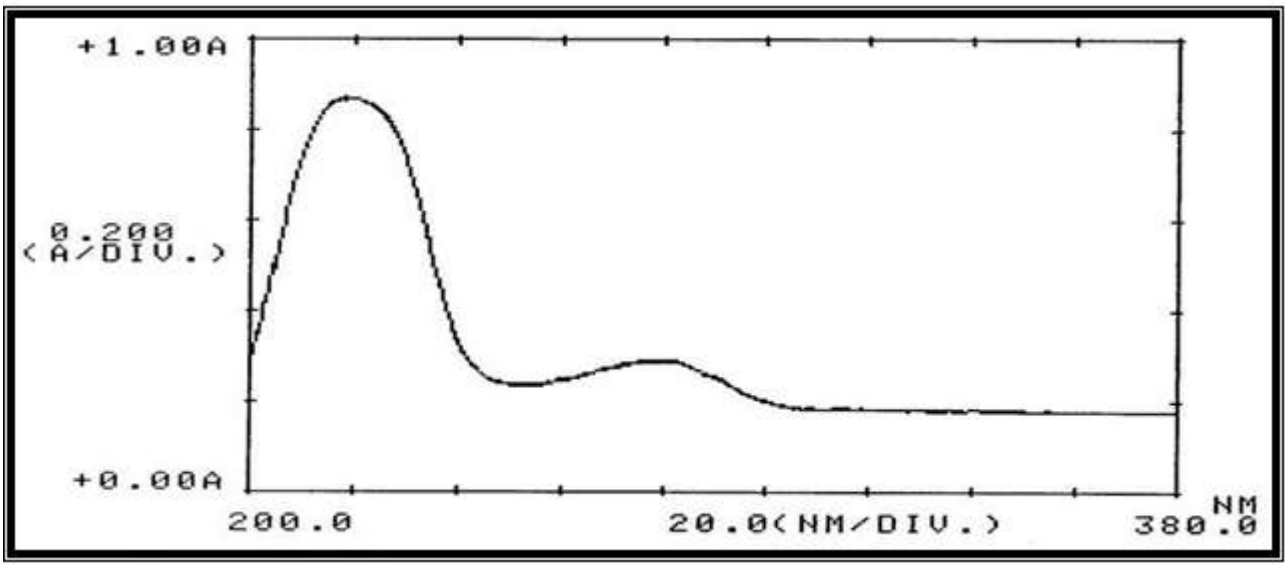

Figure 24: UV. Spectrum of TRACP5a in acetate buffer pH 5.5 of patients with ESRD

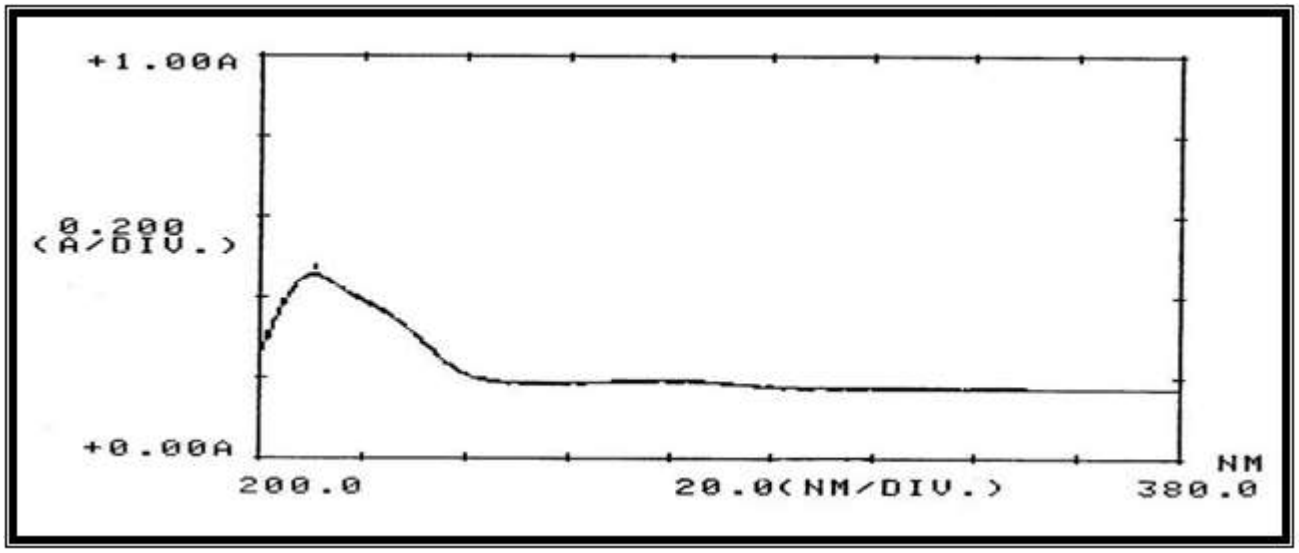

Figure 25: UV. Spectrum of TRACP5b in acetate buffer pH 5.5 of patients with ESRD

PH Effect on TRACP 5a and 5b Spectra

Three different pH (5.0, 5.5, and 6.0) were used in this experiment to examine the effect of pH on the TRACP 5a and 5b spectra. The spectrums of standard amino acid (tyrosine, tryptophan, phenyl alanine and hisidine) were also carried out in the same pH buffers

in order to interpret the results in this experiment. The results are shown in Table (3). Blue shift in the absorption of 5a for both control and patients with ESRD were observed with an increase of pH, while no difference in the absorption of 5b for control and patients with ESRD with increase of pH was observed.

Table 3: The effect of pH on λ_{max} (nm) of TRACP 5a and 5b for control and patients with ESRD and some standard amino acid in acetate buffer

	pH 5.0	pH 5.5	pH 6.0
TRACP 5a (control)	227.2	220.8	217.8
TRACP 5b (control)	210.8	210.6	209.6
TRACP 5a (ESRD)	219.0	218.4	216.2
TRACP 5b (ESRD)	210.6	210.4	209.4
Tyrosine	268,228	266,230	258
Tryptophan	300	300,247	300,246
Phenyl alanine	251,220	251,220	251
Histidine	278.2	228.8	228

Heparin Effect on TRACP 5a and 5b Spectra

Heparin effect on TRACP 5a and 5b spectra at pH 6.0 was examined. The results presented in Table (4), show the presence of red shift in the absorption of 5a for both control and patients with ESRD at pH 6.0 when compared with the absorption without heparin at pH 6.0.A slight red shift in the absorption of 5b for both control and patients with ESRD at pH 6.0 in comparison with the absorption without heparin at pH 6.0 was observed

Table 4: Heparin effect at pH 6.0 on λmax of TRACP 5a and 5b spectra for control and patients with ESRD

	λ_{max} at pH 6.0	λ_{max} at pH 6.0 in the presence of heparin
TRACP 5a (control)	217.8	226.2
TRACP 5b (control)	209.6	210.2
TRACP 5a (ESRD)	216.2	225.6
TRACP 5b (ESRD)	209.4	211.4

Discussion

An increased serum acid phosphatase activity has been repeatedly demonstrated in patients with bone disease. The enzyme among all ACP forms responsible for elevated activity is that resistant to tartrate [12].This tartrate resistant acid phosphatase is called type-5 acid phosphatase based on its electrophoretic mobility in nondenaturating PAGE [18]. Several studies of serum type 5 TRACP revealed that it was composed of two antigenically related isoforms 5a and 5b. In condition of increased bone resorption, only serum isoform 5b is increased, indicating its derivation from osteoclasts [12][26].

To analyze their properties independently, isoforms 5a and 5b were isolated from sera of control and patients with ESRD (group A) who had increased TRACP activity. In the present study the result (Table 1) was in agreement with Janckila *et.al.* (2001) finding [25], who demonstrated that TRACP 5a and 5b in sera of control, Rheumatoid arthritis, ESRD have different specific activities, and their proportional amounts, differed in disease specific ways. TRACP activity is often increased in patients with ESRD undergoing hemodialysis [27].

This is presumed to the result from increased bone resorption. In the present study chromatographic and electrophoretic results are strong evidence that increased TRACP in serum of ESRD is in fact attributed to the increase in 5b activity. The purification procedure, described in this work demonstrated that there was a big loss in enzyme activity during dialysis step which lead to decrease of specific activity of the enzyme. This activity loss may be due to loosing of activator during the dialysis process [28].

Brehme et. *al.* have reported that one mechanism of TRACP instability in serum is complex formation with α_2-macroglobulin (α_2M), a nonspecific proteinase inhibitor. α_2M is known to bind a variety of proteins in serum by covalent and noncovalent mechanisms [29]. Previously tartrate resistant acid phosphatases have been purified from spleen, placenta, lung, cord blood, bone, and osteoclastoma[29].

These enzymes share a number of properties, including molecular weight values of (30-

40)KDa [30], pH optimal (5-6) [2],[25] and pI value of 8.5-9.0[1],[30],[31]. The results in this study indicated that TRACP 5a and 5b had approximate molecular weight values of (36.8-39.3) KDa with basic isoelectric point value approximately equal to 9.6 in both control and patients with ESRD. Partially purified TRACP 5a and 5b eluted from CM-Sepharose column were used to study the effect of pH and heparin on their activities.

The results in Figures (16), (17), (18), and (19) demonstrated that TRACP 5b has a higher pH optimum than TRACP 5a. Previously lam *et.al.*, have reported that the optimal activity of TRACP 5a in adult serum was at pH 5, and of 5b was between pH (5.5-6.0) [18].

While Janckila *et. al.* have reported that the optimal pH of TRACP 5a in sera of patients with Rheumatoid arthritis was at 5.2 and 5.8 for TRACP 5b.[32]. While Nakanishi *et.al.* reported that the optimal reaction pH for TRACP 5a in healthy sera was 5.8, whereas TRACP 5b showed maximum activity at pH 6.6 [13]. TRACP 5a and 5b have different carbohydrate content, where 5a contain sialic acid while 5b lacks sialic acid [33].
The carboxylate group of sialic acid is deprotenated at physiological pH (pKa of 2.6) and confers the net negative charge that dominate its physiochemical properties [34].On the other hand it has been reported that histidyl residue is present in its active site [35].

So the difference in the optimal pH of both isoforms may be due to the conformation changes that result from deprotonation of the imidazole group of histidine at pH 6.0. Previously Janckila *et. al*. have shown that heparin (23 KU/L) had an inhibition effect (about 50%) on serum TRACP 5a of patients with chronic myelomonocytic leukemia at pH 5.5 or 6.1[33], while Nakanishi *et.al.* have reported that at pH 6.6, a peak of TRACP 5b activity was observed in the presence of heparin (23 KU/L) in the reaction mixture, whereas the TRACP 5a activity was near zero, when heparin was present in the reaction mixture [13].

Madder observed a maximal inhibitory effect of heparin at the concentration of (50 KIU/L), but this was accompanied by maximal turbidity in the reaction mixture. This turbidity presumably occurred because of the globulin-precipitating effect of heparin, which increase as the amounts of heparin added increase [36]. In the present study heparin was used at (23 KU/L) as recommended by Janckila *et.al*. The inhibition effect was observed on the activity peak of TRACP 5a only.

The inhibition ratio was approximately 95% in control and 73% in patients with ESRD. This difference in inhibition ratio between control and patients with ESRD was attributable to conformation changes of enzyme due to the increase of the anions in sera of patients with ESRD [37]. On the other hand no effect of heparin on the activity peak of TRACP 5b was observed. This is due to lack TRACP 5b of sialic acid that presence in TRACP 5a which lead to conformation change and presence of basic amino residue on the surface of enzyme.

The iron content was previously reported for beef spleen TRACP as two irons per 39000 molecular weight [38], and for the enzyme of pig allantoic fluid [39].On the other hand Campbell *et.al*. reported that the enzyme purified from beef spleen contains one mole of iron per one mole of the enzyme [40]. While Hayman *et.al.* reported that the enzyme purified from human osteoclastomas contains 4.8 mole of iron per mole of the enzyme [28]. In the present study the results indicated that the serum TRACP contains one mole of iron per one mole of enzyme.

The differences in the characterization of both isoform TRACP 5a and 5b that present in this study were confirmed by UV-spectroscopic studies where the spectra of both isoforms were different from each other. UV spectra of proteins are usually divided into the near and far UV regions.

The near UV in the context means 250-300, which is also described as the aromatic region, the spectra in this region is also to the transitions of disulphide bonds (cystines) that contribute to the total absorption intensity. The far UV < 250 nm is dominated by transitions of the peptide backbone of the protein, but transitions from some side chains also contribute in this region, and if the protein α helical content is low, this may give rise to erroneous protein structure determinations [41].

The lowest energy transition of the peptide chromophore is an n→π* transition analogous to that in ketones, and the next transition is π→π*. The n→π* transition occurs at about 210-230nm (depending mainly upon the extent of hydrogen bonding of the oxygen lone pairs) and its electric character is polarized more or less along the carbonyl bond [41]. The π→π* transition is dominated by the carbonyl π bond and is also effected by the involvement of the amide nitrogen in the π orbitals; its electric dipole transition moment is polarized somewhere near the line between oxygen and nitrogen and it is centered at 190 nm. In an α helix, the electric dipole coupling of the π→π* transitions on neighboring residues results in a long wavelength component of this transition at ≈208nm [41].

The absorption spectrum of a chromphore is primarily determined by the chemical structure of the molecule. However, a large number of environmental factors produce detectable changes in λ_{max} andε. Environmental factors consist of pH, the polarity of the solvent or neighboring molecules, and the relative orientation of neighboring chromophores. It is precisely these environmental effects that provide the basis for the use of absorption spectroscopy in characterizing macromolecules [42]. Thus spectra of free amino acids such as tyrosin, tryptophan, phenylalanine, and histidine were carried out in this study at the same condition of pH and polarity of the solvent, to support the interpretation of results. The results of pH effect on spectra of both TRACP isoforms 5a and 5b indicated that there was blue shift in the absorption of isoform TRACP 5a in the control and patients with ESRD with the increase of pH, while no difference was observed in absorption of TRACP5b purified from control and patients with ESRD, which demonstrated that the chromophore of TRACP 5a is on the surface of protein, while the chromophore of TRACP 5b must be buried in a non polar region of the protein [42].

Red shift in the absorption of TRACP5a and less more in absorption of TRACP5b were observed in the presence of heparin. This shift is due to π→π* transition which occurs at a longer wavelength region [41], as a result of the increase the polarity of the solvent in the presence of heparin, which is a linear anionic polyelectrolyte with negatively charged sulfate and carboxyl groups[43].

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