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Kinetic Studies of Na^+/K^+-ATPase in Tissue Aerobic Thyroid Patients

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Abstract:

Na^+/K^+-ATPase is a prevalent enzyme that maintains the Na^+ and K^+ gradients across the cell membrane by transporting three Na^+ out and two K^+ into the cell, the aim of this study is to provide detailed mechanistic insights, potentially with important effects on physiological regulation of active Na and K transport in tissues of Aerobic Thyroid Patient. Thyroid tissues were obtained from a 35 year old patients, the operation was carried out at the Al-Hadi Specialist Hospital in Samarra city, the sample was stored at -20°C until used. The purification protocol included Salt Precipitation, Ion Exchange Chromatography, Gel Filtration and Electrophoresis, a spectrophotometric method was used to determine the enzyme activity. kinetic parameters was also obtained for the enzyme. Partial purification of Na^+/K^+-ATPase revealed two isoenzymes (I ,II). The purity of separated isoenzymes were proved by SDS-PAGE electrophoresis. The kinetic characteristics of Na^+/K^+-ATPase showed that optimum substrate concentration about 1.5mM, K_m 1.052mM, and V_{max} 6.062, optimum temperature was 37 °C, optimum pH 7.4 and optimum time in 25 min. Na^+/K^+-ATPase purified from Thyroid tissue has distinct kinetic characteristic that reflects the importance of intracellular regulation of specific Na^+/K^+-ATPase pump which gives cells the ability to precisely coordinate to their physiological requirements .

Key words: Aerobic Thyroid Gland Tissue, Gel Filtration, Ion Exchange, Na^+/K^+-ATPase (Sodium/Potassium-Adenosine Triphosphatase).

Introduction:

Sodium Potassium Adenosine Tri phosphatase (Na^+/K^+-ATPase) a protein containing two large units of α and β types contain glucose molecules (1). The The α unit (110Kda) has 8-10 lobs (segments) crossing from the outside to inside of membrane (2) and contains sodium and potassium binding sites which are located within this enzyme as well as contains a site of energy(3) while β unit is a complex of glycoprotein that is necessary for the enzyme activity and if it removed, the enzyme will lose its activity (4). Na^+/K+-ATPase is an active transporter that uses ATP to 'pump' three sodium ions out of the cell and tow potassium ions into the cell (5).

Thyroids hormones play an important and vital role in regulating the activity of the sodium pump and controlling the ionic balance in the body fluids by influencing on efficiency of iodide pump, which increases the rate of iodine capture by thyroid cells(6). The thyroid cells have a basement membrane especially for the transport of iodine (7), effectively from extracellular fluid into the cell by active transport that called an Iodide pump (8).

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This step requires energy obtained from Adenosine triphosphate (ATP) molecule, where sodium is considered a necessary ion that are present in a great amount outside the cells in the blood (9), in addition, is a necessary element for fission and when absence or reducing it inside the cells may affected on the efficiency of ATP production (10,11).

Material and Methods:

A sample of thyroid tissues was obtained from a patients of 35 year old, the operation was carried out at the Al-Hadi Specialist Hospital in Samarra and the sample was stored at -20°C until used.

Preparation of tissue sample extract:

The tissue was extracted using a buffer solution that prepared from (250 mM Sucrose, 50 mM Tris-HCl, 1 mM EGTA pH 7.4) at ratio (1: 3) (12) and stored at -20°C until used.

The study included:

Determination of Na^+/ K^+-ATPase (EC 3.6.3.9) activity:

ATP is the substrate of the enzyme, Kirchgesser M. method was carried out to

determine Na^+/ K^+-ATPase activity from Aerobic Thyroid tissue (13).

The Bradford method was used to measure the amount of Na^+/K^+-ATPase protein (14).

Partial purification of Na^+/ K^+-ATPase from the tissue:

A precipitation: Na^+/ K^+-ATPase was extracted from the tissues of thyroid patients throught proteins precipitating by 60% ammonium sulphate, the precipitate melts by 5 ml of the Tris-HCL-0.125M solution at pH7.4. The Ammonium sulphate is removed from a solution via dialysis in (Tris-HCl 0.125M) at pH 7.4, with several alternatives batches for time to time overnight at 4° C. After that, the protein was concentrated by immersing it in sucrose for 30-45 min at 4° C.

Partial purification of Na $^+$/K^+-ATPase from the tissue by Ion Exchange Chromatography:

This procedure was carried out by using DEAE-Cellulose column(20 x 1.5 cm) with a flow rate of (60ml / hour), the eluting buffer was Tris-HCL (0.125M) (pH7.4) that contained progressive concentrations of NaCl /saline (0 , 50, 75, 100 mM), and the extracting parts of the column were collected at a size 5ml. The absorbance was measured at 280nm (13) and the concentration of protein was estimated by Bradford method (14). Samples were kept at -20°C until used.

Partial purification of Na^+/ K^+-ATPase from the tissue by Gel Filtration:

The column of the Sepharose-6B (60 x 1.5 cm) has a flow rate (60ml / hour) was used to purify Na^+/ K^+-ATPase from the fractions separated by the ion exchange , the collected volume was 5ml. The absorption of the extracting parts measured at 280nm. The contents of the tubes that showed the highest absorbance were collected and the enzyme activity was determined according to Kirchgesser M. method (13) also the protein concentration was determined by using Bradford method (14). The samples were stodged at -20°C until used.

Sodium Dodecyl Sulfate – Polyacryl Amide Gel Electrophoresis (SDS-PAGE):

Sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE) was used to identified Na^+/ K^+-ATPase isofomes (15).

Kinetic studies of the Na^+/K^+-ATPase:

Kinetic parameters of the purified enzyme were determined as following (13):

Substrate concentration:

Different oncentrations of ATP (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2 mM) were used to estimate the optimum concentration of the substrate.

Optimum pH:

Different pH solutions of (6, 7, 7.4, 8.3, 9, 9.5) were used with 1.5mM substrate at 37°C.

Optimum Temperature:

The reaction was carried out at different temperatures (4, 10, 25, 30, 37, 42, 54) and substrate concentration of 1.5mM and pH7.4 .

Time Interval:

The effect of the time on the enzyme activity was studied using substrate concentration of 1.5mM ATP and at multiple time intervals (0, 5,10,15,20,25,30,35,40,45 min) At 37°C and pH 7.4.

Results:

Partial purification of Na^+/K^+-ATPase from Aerobic Thyroid Tissues:

Na^+/ K^+-ATPase from thyroid tissues was purified by multiple steps as shown in Table (1). The first steps was protein precipitated using 60% ammonium sulphate in a purity of 0.018. DEAE-Cellulose Ion exchange chromatography was used to purify the enzymes depending on the difference of isozymes net charge, two Isozyme was obtained as shown in Fig (1) with a purity of 0.32 and yiled of 9.1% of isozyme- I at and isozyme- II in 0.55purity and yiled of 6.3%.The 3rd purification stage was gel filtration was performed using Sepharose-6B which gives a purifction fold of 1.42 with 42% yiled , as shown in Fig (2) .

Table1. purification of Na+/K+ -ATPase

Steps	Total Activity	Specific activity	(Fold)	Yeild %
Crude	0.0221	1.7	1	100
Salt perception	0.0109	1.847	1.08	49.3
Dialysis	0.0079	1.975	1.16	35.8
Isoenzyme - I	0.0019	0.543	0.32	9.1
Isoenzyme -II	0.0014	0.933	0.55	6.3
Gel Filtration	0.0094	2.41	1.42	42.5

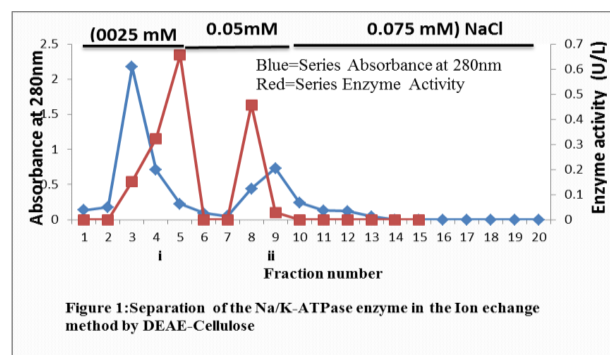

Figure 1:Separation of the Na/K-ATPase enzyme in the Ion echange method by DEAE-Cellulose

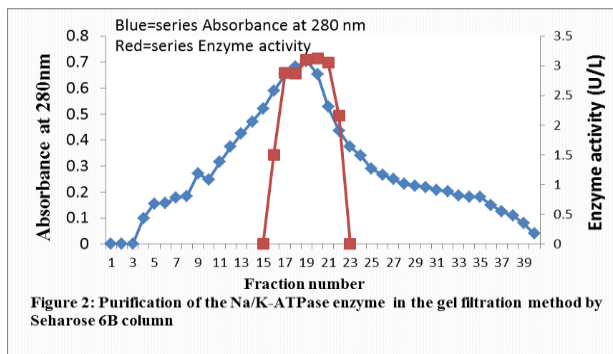

Figure 2: Purification of the Na/K-ATPase enzyme in the gel filtration method by Seharose 6B column

Gel Electrostatic separation method was used on SDS-PAGE gel at 10% and CBB R250 dye which indicates that Na+/K+-ATPase has two bands as shown in Fig(3).

L	1	2	3	4	5	6	7	8	L

M. wt
150
100
75
50
35
25
15
10

A

L	1	2	3	4	5	6	7	8	L

M.wt
150
100
75
50
35
25
15
10

A B

B

Figure 3. Electrical transfer of the purified Na^{+}/K^{+}-ATPase (A) and (B) Na^{+}/ K^{+}-ATPase. *(L) Standard proteins.

The kinetic parameters of Na^{+}/ K^{+}-ATPase:-
Optimal substrate and Km:

The effect of different substrate concentration on Na^{+}/K^{+}-ATPase activity what partially purified. The results showed the activity increased as the substrate concentration was increased till reaches maximum activity at 1.5mM, then the activity begins to decrease as shown in Fig (4). The Michaelis -Menten constant was 1.052mM and Vmax was 6.062Mm.L^{-1}.min^{-1} as shown in Fig (4, 5,6,7,8,9)n and Tab (2).

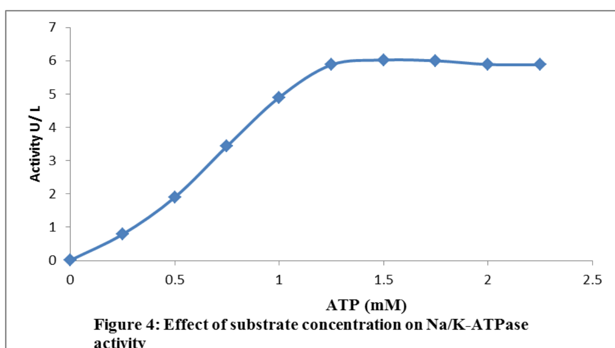

Figure 4: Effect of substrate concentration on Na/K-ATPase activity

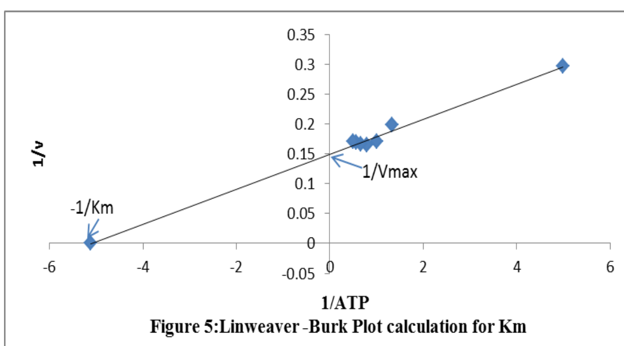

Figure 5:Linweaver -Burk Plot calculation for Km

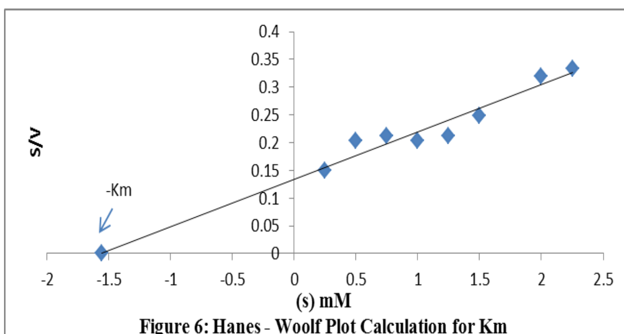

Figure 6: Hanes - Woolf Plot Calculation for Km

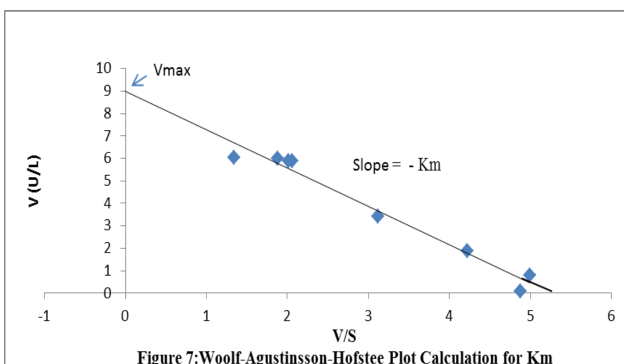

Figure 7:Woolf-Agustinsson-Hofstee Plot Calculation for Km

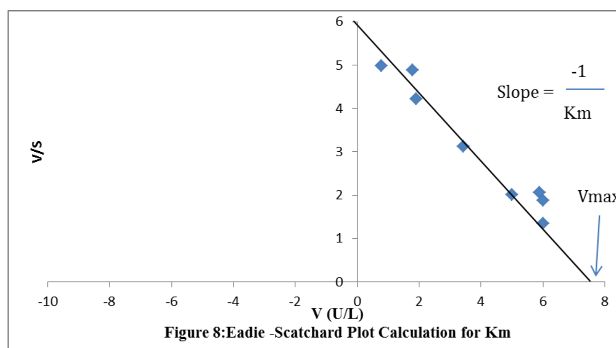

Figure 8:Eadie -Scatchard Plot Calculation for Km

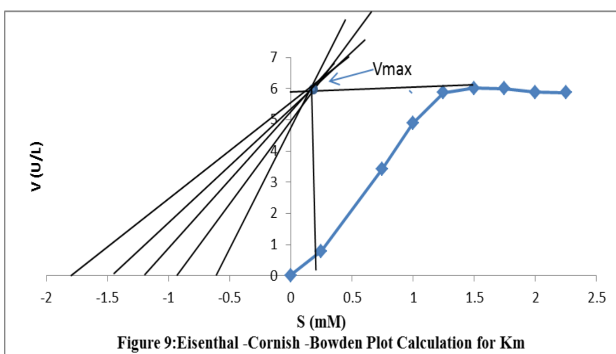

Figure 9:Eisenthal -Cornish -Bowden Plot Calculation for Km

Table 2. Kinetic parameters of Na^{+}/K^{+}-ATPase

methods	K_m(mM)	V_{max}(mM/l.min)
Linweaver-Burk plot	0.19	6.6
Hanes – Woolf plot	1.59	1.14
Woolf – Agustinsson - Hofstee plot	1.66	9
Eadie–Scatchard – plot	1.45	7.98
Eisenthal–Cornish – Bowden plot	0.4	5.95
Average	1.052	6.062

pH effect :

The effect of pH on Na^{+}/ K^{+}-ATPase actives purified from tissues .The results showed an increase in the activity as pH increased until reaching the maximum activity at pH 7.4 ,then the activity decreased at pH higher than 7.4 as showed in Fig (10).

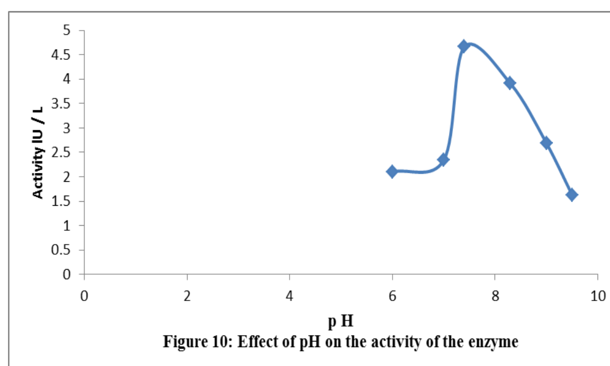

Figure 10: Effect of pH on the activity of the enzyme

Time Effect:

The effect of time on Na^{+}/K^{+}-ATPase activity was done through incubating of the enzyme with substrate in a pH 7.4 and at 37 ° C. for

(45,40,35,30, 25, 20, 15, 10, 5, 0 minutes).The results showed an increase in the activity as the incubation period increased till it reaches maximum activity 25 minutes and after that the activity declined as observed in Fig (11)

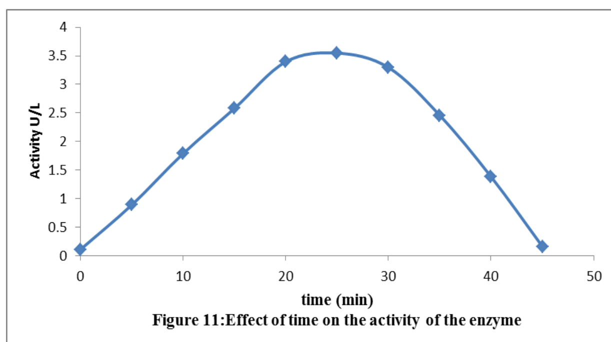

Figure 11:Effect of time on the activity of the enzyme

Temperature effect:

The results showed that maximum activity of enzyme was observed at 37 ° C as observed in Fig (12).

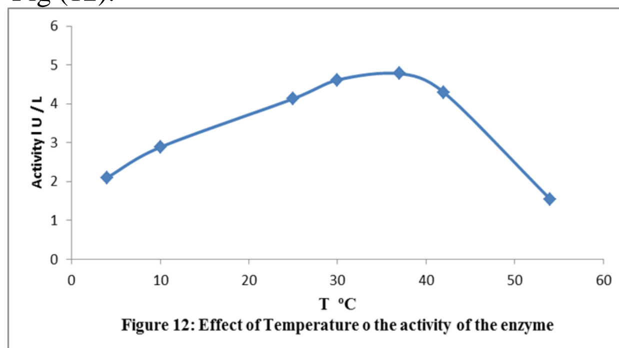

Figure 12: Effect of Temperature o the activity of the enzyme

Discussion:

Ammonium sulphat precipitating usually performed in the first step of purification in order to remove the extra proteins other than Na^+-K^+-ATPase the basic principle of this procedure depends on salting out phenomenon that the reaction of protein-charges with salt, which leads to reduced protein solubility and precipitating occurs (16). The purity of separated isozymes were achieved by (SDS-PAGE) which revealed two bands as shown in Fig (3), indicating that the purity of the enzyme reached to homogeneous state (17 ,18), the electrical chromatography was achieved by migrating the protein in pH 8.8 within an electrical field . the extracellular positive charges generated by the function of Na^+/K^+-ATPase left the cell with lack in positive ions (19). The kinetic studies of Na^+/K^+-ATPase indicates that the optimum substrate concentration was (1.5mM) which is a hyperbolic shape and therefore its undergo the Michaelis-Menten equation as shown in Fig (4), this results in agreement with other studies (21, 22, 23). For Na^+/K^+-ATPase purified from Aerobic Thyroid tissues, the optimum pH was (7.4) this can be explained by pH effects on the activity due to the difference in the nature of enzymes and also to the enzymatic composition of poly-ionic groups which carried by the amino acids (23), at low pH ,enzymes are not capable of bonding with substrate because they have no correct conformation or ionic states while at height pH it becomes very sensitive to H^+ concentration thus unable enzyme-substrate complex (ES) that might leads to denaturation and hence loss of activity (24, 26), our result in agree with other results (26, 27). At 25 min the enzyme reach optimum activity and as the time increase the activity will decrease, this may be due to the thermodynamic nature of $Na^{+/}$K-ATPase which leads to break bonds as the time increases (28) also the enzyme has optimum activity at 37°C this results are similar with other studies (26,29) Because Enzymes have an optimal temperature (equal, slightly higher or lower), enzymes activity increases as the temperatures increases until reach to the optimum temperature of reaction, then is gradually reduced due to denaturation (28).The kinetic finding of this studay indicates that showed that Km of Na^+/K^+-ATPase purified from Aerobic Thyroid tissues was 1.052mM and the Vmax was 6.062mM.L^{-1}.min^{-1}as shown in table 2,while another study indicates that Km of the enzyme from sheep kidney was 0.19 ± 0.04 mM and from rabbit kidney was mM 4.0 this can be explaine to the difference of tissue sources(30,31).

Conclusion:

The kinetic characteristics of Na^+/K^+-ATPase has distinct properties and varies with different sources. In addition, the regulation conditions of the pumps gives cells the ability to coordinate Na-K-ATPase activity precisely to their physiological requirements in tissues of Aerobic Thyroid Patient.

Conflicts of Interest: None.

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دراسة حركية لانزيم صوديوم/بوتاسيوم اتبيز Na^+/K^+-ATPase المنقى من نسيج مرضى الدرقية الهوائية

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الخلاصة:

انزيم صوديوم/بوتاسيوم اتبيز Na^+/K^+-ATPase عبارة عن بروتين يعمل على تنظيم تدرجات الصوديزم Na^+ والبوتاسيوم K^+ عبر غشاء الخلية من خلال ضخ ثلاث أيونات صوديوم إلى الخارج وأيونين بوتاسيوم إلى داخل الخلية ، تهدف الدراسة إلى تقديم رؤيا ميكانيكية مفصلة للانزيم يحتمل أن تكون لها آثار مهمة على التنظيم الفسيولوجي للنقل الفعال لايوني Na^+ و K^+ في نسيج مرضى الغدة الدرقية الهوائية . تم الحصول على عينة النسيج الدرقي من مرضى بعمر35 سنةحيثوأجريت العملية في مستشفى الهادي التخصصي الأهلي-سامراء تم حفظ العينات بدرجة($-20^{\circ}C$) لحين الأستعمال. تم عزل وتنقية الأنزيم بواسطة الترسيب الملحي ، التبادل الأيوني ،والترشيح الهلامي والترحيل الكهربائي . استخدمت تقنية مطيافية المرئية لتقدير فعالية الأنزيم .كذلك شملت الدراسة تعيين الخواص الحركية للأنزيم .تم الحصول على متناظرين (I , II) وتم التأكد من نقاوة الفصل بواسطة الترحيل الهلامي على هلام الاكريل امايد (SDS-PAGE) حيث ظهرت حزمتين . بينت النتائج الحركية ان التركيز الأمثل للمادة الأساس (1.5mM) وبلغت قيم ثابت Km عند (1.973mM) أما قيمةVmaxفقد بلغت ($5.95Mm.L^{-1}.min^{-1}$) أما درجة الحرارة المثلى فقد كانت عند ($37^{\circ}C$) ، الدالة الحامضية المثلى عند 7.4 ، ولوحظ اقصى فعالية للأنزيم عند زمن(5 min) . أن انزيم Na^+/K^+-ATPase المنقى من نسيج الغدة الدرقية الهوائية له خصائص حركية محددة تعكس اهمية التنظيم الداخلي للانزيم والتي تعطي الخلية قابليتها التنظيمية الدقيقة التي تتفق مع الملامح الفسيولوجية لنسيج الغدة الهوائية.

الكلمات المفتاحية: انزيم صوديوم –بوتاسيوم أدينوسين ثلاثي الفوسفات، التبادل الأيوني، الترشيح الهلامي, الغدة الدرقية الهوائية.