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Partial Purification and kinetic study of purified Angiotensin-converting enzyme from Hyperthyroid Patients

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Abstract:

Hyperthyroidism is marked by low thyrotropin and elevated triiodothyronine and/or free thyroxine levels. Angiotensin-converting enzyme (ACE) plays a key role in regulating blood pressure by converting angiotensin I to angiotensin Objective: This study aimed to partially purify and study the kinetics of ACE from hyperthyroid patients, as well as quantify ACE levels in these individuals. Materials and Methods: Crude ACE was precipitated using ammonium sulfate saturation, followed by dialysis. Purification involved ion exchange and gel filtration chromatography. ACE levels were quantified using an ELISA kit. Results: HPLC analysis revealed ACE purity and a molecular weight of ~135.66 kDa. The purified enzyme showed an ideal pH of 7.0 and temperature of 35°C. Lineweaver-Burk plots were used to determine kinetic parameters, yielding Vmax = 2.144 µmol/min·mL and Km = 0.554 mM. ACE was purified 20-fold with a specific activity of 4.066 EU/mg. Compared to controls, ACE levels in hyperthyroid patients were significantly elevated ($P \leq 0.001$). Conclusion: The study successfully purified ACE from hyperthyroid patients and identified its optimal conditions and kinetic parameters, with significantly higher ACE levels observed in these individuals. The study also includes the receiver operating characteristics analysis of the discriminating capabilities for ACE biochemical parameters as diagnostic markers for hyperthyroid.

Keywords Angiotensin-converting, ACE, Purification, Hyperthyroidism.

التنقية الجزئية والدراسة الحركية للإنزيم المحول للأنجيوتنسين المنقى من مرضى فرط نشاط الغدة الدرقية

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البحث مستل من رسالة ماجستير الباحث الاول

مستخلص:

يتميز فرط نشاط الغدة الدرقية بانخفاض هرمون الغدة الدرقية وارتفاع مستويات ثلاثي يودوثيرونين و/ أو هرمون الغدة الدرقية الحرة. يلعب الإنزيم المحول للأنجيوتنسين (ACE) دورا رئيسيا في تنظيم ضغط الدم عن طريق تحويل الأنجيوتنسين الأول إلى أنجيوتنسين غير موضوعي: تهدف هذه الدراسة إلى تنقية ودراسة حركية الإنزيم المحول للأنجيوتنسين جزئيا من مرضى فرط نشاط الغدة الدرقية ، بالإضافة إلى تحديد مستويات الإنزيم المحول للأنجيوتنسين لدى هؤلاء الأفراد. المواد والطرق: تم ترسيب الإنزيم المحول للأنجيوتنسين الخام باستخدام تشبع كبريتات الأمونيوم، متبوعا بغسيل الكلى. تضمن التنقية التبادل الأيوني وكروماتوغرافيا ترشيح الجل. تم قياس مستويات الإنزيم المحول للأنجيوتنسين باستخدام مجموعة ELISA. النتائج: كشف تحليل HPLC عن نقاء ACE ووزن جزيئي يبلغ ~ 135.66 كيلو دالتون. أظهر الإنزيم المنقى درجة حموضة مثالية تبلغ 7.0 ودرجة حرارة 35 درجة مئوية. تم استخدام مخططات Lineweaver-Burk لتحديد المعلمات الحركية ، مما أدى إلى Vmax = 2.144 ميكرولتر / دقيقة · مل و Km = 0.554 mM. تم تنقية الإنزيم المحول للأنجيوتنسين 20 ضعفا بنشاط محدد قدره 4.066 يورو / ملغ. بالمقارنة مع الضوابط ، كانت مستويات الإنزيم المحول للأنجيوتنسين في مرضى فرط نشاط الغدة الدرقية مرتفعة بشكل ملحوظ ($P \leq 0.001$).

الكلمات المفتاحية : تحويل الأنجيوتنسين ، ACE ، التنقية ، فرط نشاط الغدة الدرقية .

Introduction

Hyperthyroidism is a disorder marked by the overproduction of thyroid hormones and is frequently linked to thyrotoxicosis, a situation wherein tissues are subjected to heightened doses of these hormones. Hyperthyroidism denotes the excessive secretion of hormones, characterized by diminished TSH levels alongside high T3 and/or T4 levels (1). Lung capillaries and renal endothelium both express the angiotensin-converting enzyme (ACE), commission number (3.4.15.1) (2). It is an acidic glycoprotein composed of a single polypeptide chain of molecular weight in the range of about 135.66 kDa (3). Furthermore, ACE is a glycosylated integral membrane protein with a high molecular mass (Mr~-150000) that is situated on the cell membrane's luminal surface (4). On other hand The Renin-Angiotensin System (RAS) is an intricate and ever-changing network of proteins, enzymes, and receptors that is primarily involved in maintaining the homeostasis of the cardiovascular system and other bodily fluids (5). In order to raise blood pressure, angiotensin-converting enzyme (ACE) breaks down the vasodilator bradykinin and converts the hormone angiotensin I into the active form angiotensin II (6).

Materials and methods

This study involved 90 individuals, including women and men, aged between 20 and 70 years. Samples were obtained from the Smart Health Tower in Sulaymaniyah from November 2024 to December 2024. The samples were categorized into two groups: the first group (control) and the second group (patients with hyperthyroidism). 5–8 ml of whole blood was collected from each participant (patients and controls) using a medical syringe. The blood sample was placed in a gel tube for 10 minutes at ambient temperature to facilitate coagulation. The sample was subsequently centrifuged at 1006 xg for 15 minutes to isolate the serum; a portion of the serum was utilized for the assessment ACE using ELIZA methods. We used Tris _HCL, Ammonium sulfate Potassium hydroxide was bought from BDH company UK, and C Sephadex G100, DEAE cellulose were bought from Sigma-Aldrich. Human Angiotensin Converting Enzyme (ACE) ELISA Kit was bought from

Sun long Biotechnology Chinese.

Obtain of the Hyperthyroid Patients for purification: Fifteen individuals with hyperthyroidism.

Enzyme precipitation by ammonium sulfate

Mixing 10.75 g of salt with 14 ml of crude enzyme under cold circumstances while stirring continuously for 60 minutes and centrifuging at 2000 xg for 30 minutes at 4°C resulted in a precipitation of the enzymes with a 75% ammonium sulfate (NH4)2SO4 saturation. Total proteins, specific activity, and activity of every enzyme were computed following the dissolving of precipitates in a trace quantity of Tris-HCl (0.8g in 100ml of D.W) solution. Dialysis against a 10mM Tris-HCl buffer pH 8 was done 24 hours at 4°C by stirring for desalting. Following the measurement of each enzyme activity and protein content, specific activity was calculated (7).

Dialysis

It is used to remove residual ammonium sulfate after protein deposition. The dissolved protein is placed inside the diesel bag (Dialysis bag). This bag is then immersed in the Tris-HCl solution with a concentration of 10 mmol and pH 7.2 after you have been replaced by the regulated solution in 24-hour cycles. This process was done at 14 ° C.

Ion exchange chromatography method

The process of ion exchanges and the separation of molecules is facilitated by the chromatography technique, which is an essential tool in biochemistry. The method relies on the disparity between molecular weight and size (8). Using the fraction collector, components are extracted from the gel filtration process. To separate protein components, one can use a 280 nm absorbance reader or a pre-made protein assay kit. The DEAE-Sepharose column, which has the structure of Sepharose 4B-L-tyrosine-1-naphthylamine, was loaded with the serum after it had equilibrated with 25 mM Tris/HCl (pH 8.0)/10 mM. A solution of 25 mM Tris/HCl (pH 8.0)/10 mM CaCl2 was used to precipitate ACE from individuals at a flow rate of 0.5 mL/min (9).

Gel filtration chromatography method

This technique was used after pooling the fractions (20 in total) from the ion exchange chromatography run,

they were all run via molecular exclusion chromatography with Sephadex G-100 resin to yield 20 total fractions. Size exclusion chromatography is the common name for gel filtration chromatography (10) .

Total Protein determination

Biuret reaction is a colorimetric reaction where the outcome is indicated by a color change from blue violet. The cupric (Cu+2) ions of biuret reagent complex with nitrogen atoms of peptide bonds in protein when in alkaline medium to produce a violet-colored copper coordination complex. A purple coloration developing indicates peptide bonds present in the sample. The intensity of the color is directly proportional to the concentration of total protein in serum (11).

Molecular weight for ACE by Size-exclusion chromatography (HPLC-SEC) technique

This method uses the molecular weight of analytes to separate them (12). As a tool for the assessment of molecular proteins in the researched range, a simple, rapid-size-exclusion chromatography (SEC-HPLC) method has been created. This method could be applicable to many molecules in different research domains. By employing the SEC-HPLC technique, which stands for size exclusion chromatography.

Table 1: Operational conditions for finding molecular weight by HPLC

Col.Manufacturer	Col. Type	Col. Length	Col. Diameter	Col. Particle Size
Zrorbax PSM 60 S	BIMODAL	250 mm	4.6 mm	5 µm

Statistical Analysis

Statistical software package SPSS version 29 was employed to examine the data. Normality of distribution was established using Student's t-test. $P < 0.001$ is highly significant probability, $P < 0.05$ is significant probability, and $P > 0.05$ is non-significant probability. Diagnostic accuracy and receiver operating characteristic (ROC) curves were measured using MedCalc version 23.1.7 software.

Results and Discussion

Measurement of angiotensin-con-

verting enzyme level

The (mean ± SD) of ACE level (g/dl) of patients and control groups in serum were expressed in Table 2 and Fig.2. The results showed the ACE level in the patient hyperthyroidism group is high significant (P≤0.001) relative to the control group. The result showed there was no significant difference (P>0.05) in levels of ACE between M and F in patients and control. Also, there was no significant difference (P >0.05) in ACE between A1, A2 and A3 in control and patients groups.

Table 2: Mean ± standard deviation of ACE level

Groups		Control	Hyperthyroidism	P value
TOTAL		0.608 ± 0.309	1.581 ± 0.199	<0.001
AGE	A1(15-30) Year	0.556 ± 0.338	1.528 ± 0.163	<0.001
	A2(31-45) Year	0.652 ± 0.342	1.653 ±0.185	<0.001
	A3 >45 Year	0.569 ± 0.237	1.537 ± 0.227	<0.001
(ANOVA)P-value		>0.05	>0.05	
SEX	Female	0.566 ± 0.3	1.566 ± 0.217	<0.001
	Male	0.662 ± 0.325	1.62 ± 0.143	<0.001
P-value		>0.05	>0.05	

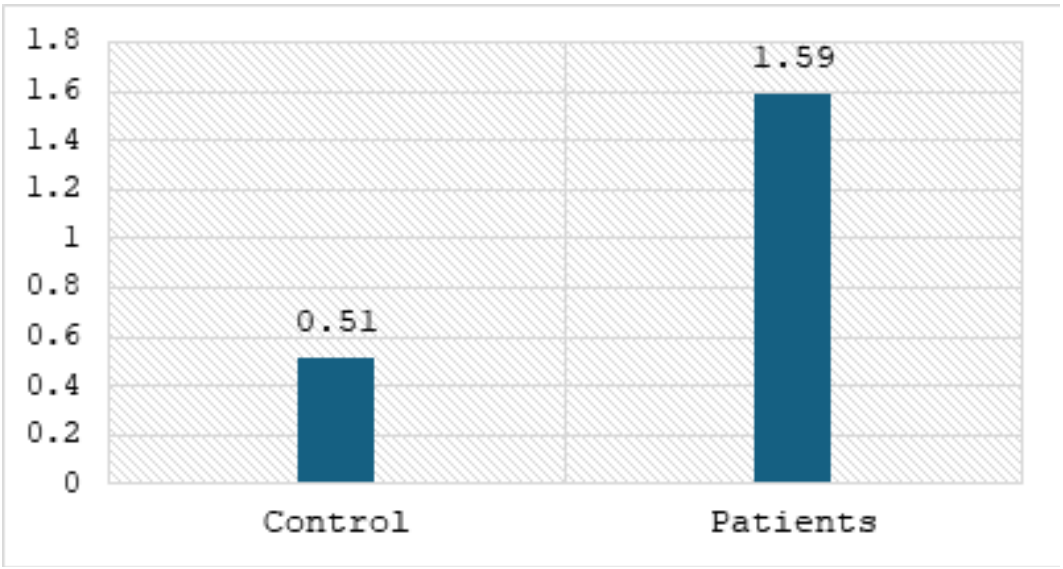

Fig.2 The Mean of ACE.

This result agrees with Shixian and Qingyang (13) and Mitchell C Buckley, A Subramaniam, S Crowther, S C Donnelly (14). Receiver operating characteristic (ROC) curves were employed to evaluate the efficacy of biochemical diagnostic tests for disease in this study and to ascertain the suitable cut-off or criteria values for various parameters. Sensitivity, specificity, and area under the curve (AUC) are frequently employed as summary metrics for diagnostic accuracy. Table 3 and Fig.2 illustrate the diagnostic validity criteria (sensitivity, specificity, criterion, and accuracy tests of ACE levels in the patient group relative to the control group.

Table 3: cut off values, sensitivity, and specificity for biochemical parameters by ROC analysis in patients and control groups.

Cut Off	Specificity	Sensitivity	Accuracy	AUC
1.057<	90	93.33	0.9333	0.993

The appropriate cut-off values for ACE across all groups were determined from ROC curves; based on these findings, the test was deemed positive if the result exceeded the threshold value (criterion or Cut Off values). The data demonstrated a strong correlation between ACE and hyperthyroidism. The ROC curve results indicate an AUC value of 0.993 for ACE, which serves as a strong indicator of the disease. Elevated ACE levels are associated with hyperthyroidism.

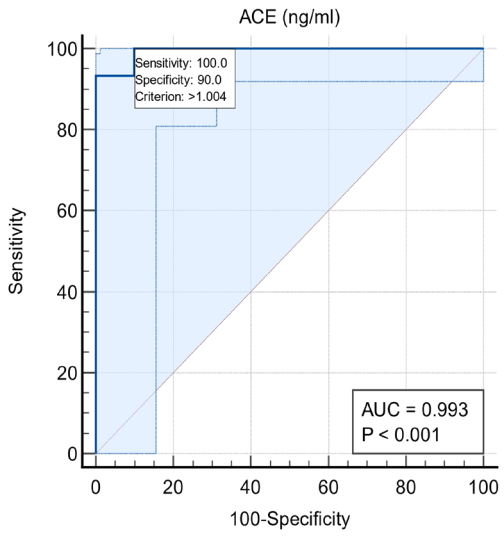

Fig.3 The ROC curve for ACE enzyme in patients compared to control.

Partial Purification for Angiotensin-converting enzyme

Salting out

As can be seen from Table 4, the proteins of most of the samples were precipitated at a 80% level of saturation during partial purification. This increased the specific activity of ACE from 0.258IU/mg in the crude extract to 0.303IU/mg after the first purification step. Consequently, the purification fold of ACE increased from 1 to 1.17 after precipitation. This is made possible through the correct use of ammonium sulfate, where it enables concentration of enzymes and proteins by removing proteins and enzymes from small interfering molecules and precipitating immune components without interfering with enzyme activity negatively. Protein solubility relies on the solution ionic strength. Proteins at the primary stage of enzyme purification are commonly concentrated by reducing water content and therefore achieving greater purity. Salting-out occurs when the deposition of salt neutralizes protein surface charges, reducing solubility and promoting precipitation. The process increases purification efficiency as well as removing major impurities in the enzyme extract. Furthermore, the incremental addition of salts allows controlled precipitation, minimizing the negative impact on enzyme activity as well as increasing the overall yield of purification (15).

Dialysis

The results of ACE enzyme purification, as presented in Table 4, indicate that dialysis led to an increase in the specific activity of the enzyme, reaching 0.381IU/mg, with a corresponding rise in the purification fold to 1.5 at the initial stage of enzyme separation. However, dialysis can also reduce ACE activity, which may be attributed to enzyme instability or the removal of essential cofactors to which the enzyme exhibits a high affinity. Additionally, dialysis facilitates the removal of trace contaminants by extensive dilution in a large volume of water. While this effect is less critical in crude preparations, it becomes increasingly significant in the later stages of purification(16). This step also agreed with the Previous study (17).

Table 4: Steps of ACE purification from human Serum

Steps of Purifications	Elute volume (ml)	Activity IU/ml	Total Activity IU	Protein Conc. (mg/ml)	Specific activity (IU/mg)	Yield %	Folds	Total protein (mg)
Crude	15	3.927	58.905	15.217	0.258	100	1	228.255
Ammonium Sulphate	7	3.148	22.036	10.39	0.3029	37.4	1.174	72.73
Filtrate	12	1.11	13.32	7.119	0.1559	22.6	1	85.428
Dialysis	10	3.02	30.2	7.921	0.3812	51.2	1.47	79.21
Ione-Exchange Chromatography	4	2.69	10.76	5.0956	0.5279	18.2	2.046	20.3824
Gel filtration	3	1.659	4.977	0.408	4.066	8.4	16	1.224

Ione-Exchange chromatography

This chromatographic technique is used to fractionate, separate, and isolate proteins, enzymes, hormones, antibiotics, and nucleic acids in solution. This technique was utilized here to separate and isolate enzymes. This was achieved by precipitating ammonium sulphate and isolating it on a DE-AE-cellulose.

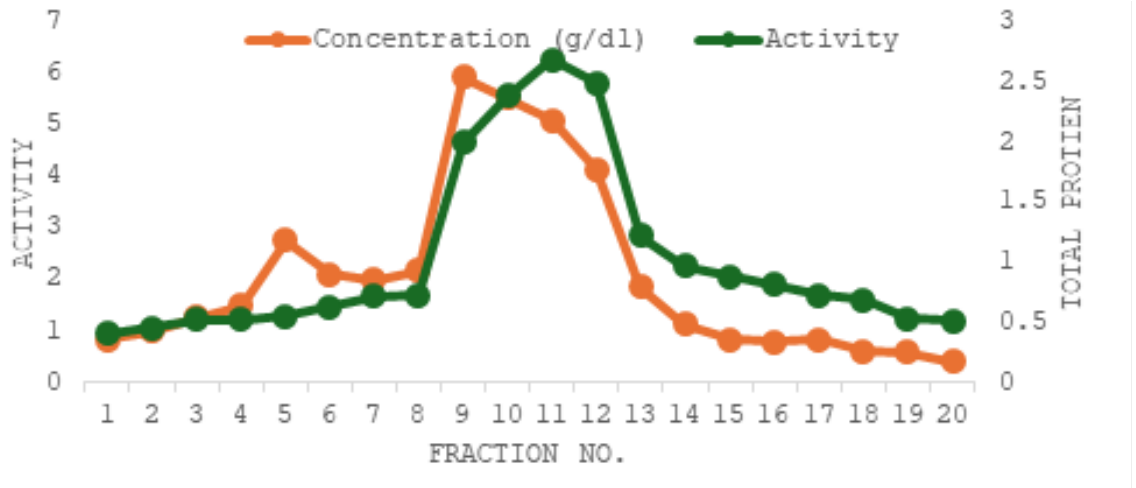

Fig.4 Ion _ Exchange of ACE.

The elution profile of the ACE enzyme, as illustrated in Fig.4 demonstrates that the enzyme was recovered as a single, symmetrical peak, indicating successful purification. As shown in Table 4, the enzymatic activity of the purified ACE reached 5.0956IU/L, while the specific activity was significantly enhanced to 0.528 IU/mg, representing a 2-fold increase in purification. Additionally, the enzyme yield was 18.266%, highlighting the efficiency of the purification process. The current study findings coordinate with the results of studies (18).

Gel filtration chromatography

This technique was employed in this study to separate and purify ACE. The purification process was carried out using ammonium sulfate precipitation, followed by further purification through a Sephadex G-100 column. The results of this process are illustrated in Fig.5. This Figure indicates that the enzyme was eluted in a symmetrical single peak with the fraction Fig.5 ACE. As indicated in Table 4, purified activity of ACE was up to (0.408IU/L), and specific activity of ACE was enhanced up to (4.066IU/mg) by (16) folds and enzyme yields (8.449%). Results showed that the specific activity and the purification fold increased in gel filtration to [4.066 U/mg, 16-fold]. These steps also agree with the Previous studies, (19–21).

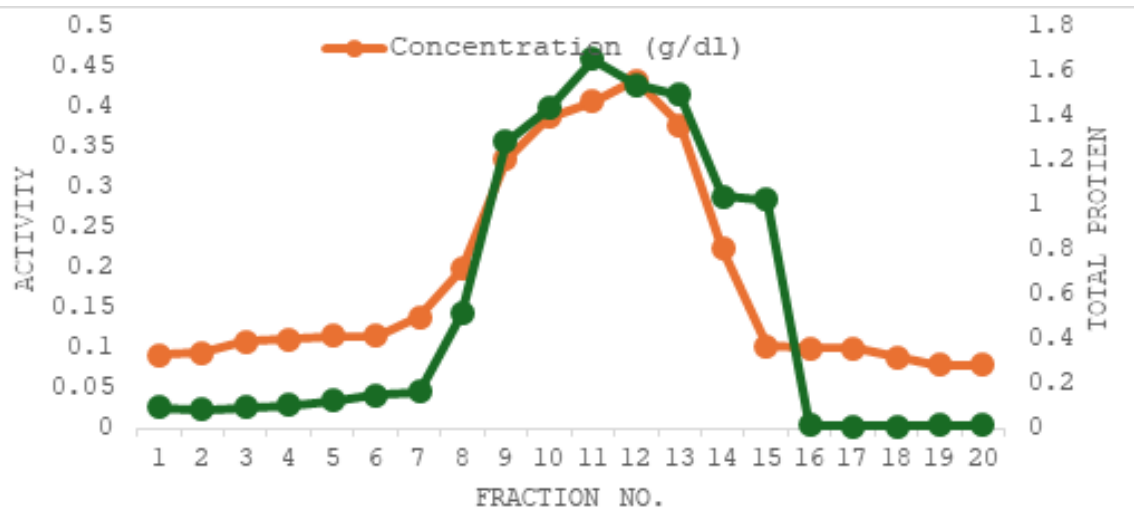

Fig.5 Gel Filtration of ACE.

Determination of molecular weight of enzyme by high performance liquid chromatography _ size exclusion chromatography

The chromatogram in Fig.6 showed appeared peak for ACE with a molecular weight 75.66 Kda, this value is close to the molecular weight reported in other literatures (22,23).

Peak No.	Ret Time	Height	Area	Conc
1	10.223	138986.141	2388110.000	100.0000

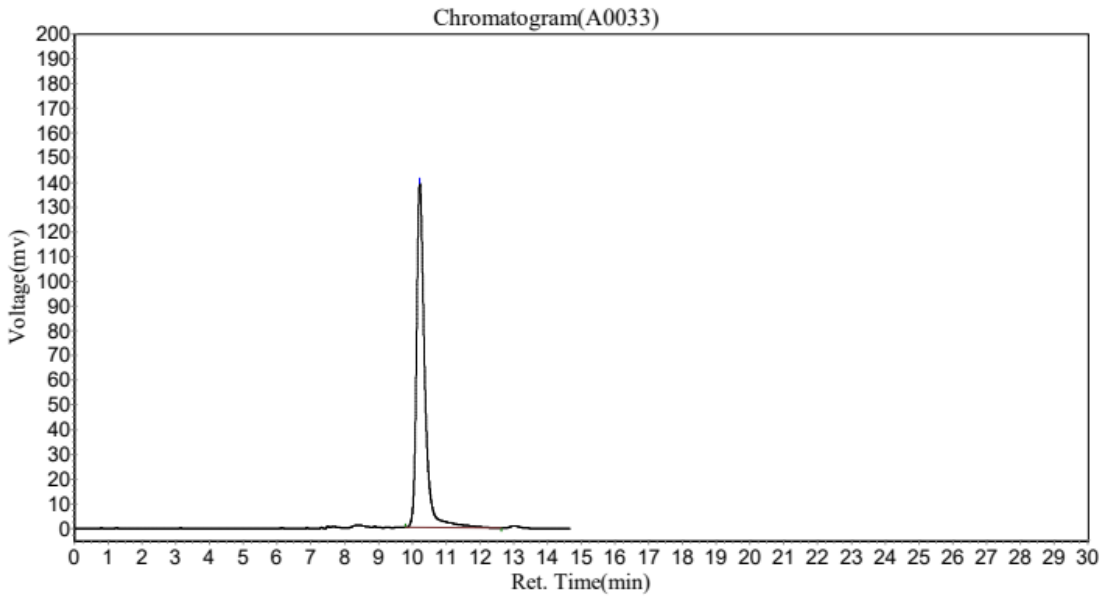

Fig.6 HPLC Chromatogram of ACE showing a single peak at approximately 10-11 minutes retention time.

Kinetic Study of optimum conditions for ACE Partially Purified

Effect of substrate concentration on enzyme activity with finding Km: The activity of the ACE was measured in the presence of different concentrations of angiotensin on ACE (2.5,2,1.5,1,0.5,0.1 mM) as substrate. It was found the maximum activity of ACE in the concentration (1.5 mmol/L) of angiotensin. Which is (1.669 IU/L) shown in Table 5, Fig7 showed increasing enzyme interaction velocity with an increase in concentration of substrate.

Table 5: The effect of substance concentration on the activity of ACE.

Conc.[S] mmol/L	Activity IU/L	1/s	1/v	KM	Vmax
2.5	1.645	0.4	0.6078	0.554	2.144
2	1.66	0.5	0.602537		
1.5	1.669	0.66	0.599181		
1	1.411	1	0.708512		
0.5	1.001	2	0.998594		

A number of methods exist for solving the Michaelis-Menten equation, which yields the value of the enzyme's constant Michaelis-Menten (Km). Due to its simplicity, lack of complicated computations, and effectiveness in proving experimental accuracy, the Line weaver-Berke approach is regarded as the most accurate and effective.

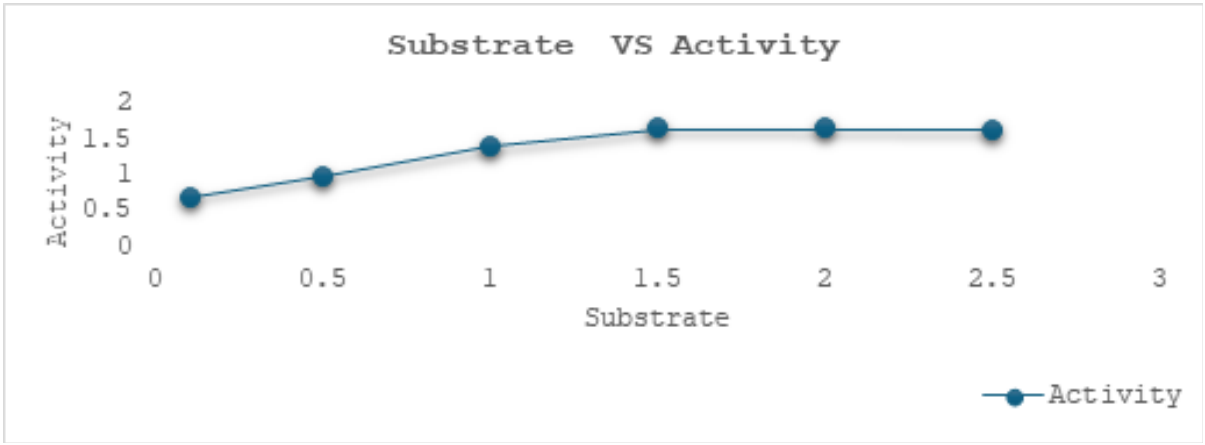

Fig.7 Impact of basal substance concentration on purified ACE activity

A linear relationship was found when the enzyme's Km and Vmax values were calculated using Line weaver-Burk plots; these values are 0.554 mM and 2.144 IU/L, respectively. The ACE enzyme was determined to have these values, as illustrated in Fig.8. A graph was created showing the inverse of the substrate concentration (1/S). Against the inverse of reaction rate (1/V) using the given equation: Vi = Vmax [S] / Km + [S] (24).

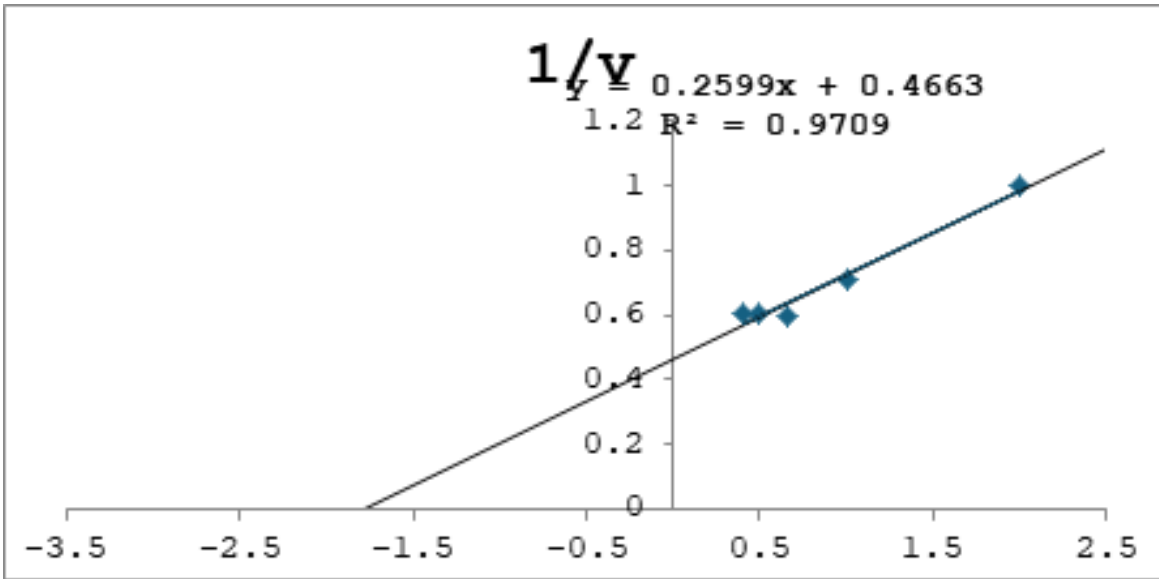

Fig.8 Line Weaver–Burk plot for partially purified of ACE.

Effect of temperature on ACE activity

The ideal temperature for ACE enzyme activity was established by testing at various temperatures (25, 30, 35, 40, 45, and 50) °C. The ideal temperature was 35°C, which aligns with prior studies indicating that the optimum temperature for the ACE enzyme extracted from human plasma ranges between 35–40°C (25). In another work, ACE was purified from rabbit lungs and the optimum temperature was 37 °C (26). Also, the optimum temperature for ACE purified from sheep lung were found 40 °C (27). The data demonstrates that increasing the temperature led to an initial elevation in ACE activity, which was subsequently followed by a decline in ACE activity, as illustrated in Fig.9 The pace of a certain reaction is usually acknowledged to grow with rising temperature until it attains the ideal temperature, beyond which it progressively declines. The enzyme molecule has experienced denaturation or damage, resulting in this outcome. At increased temperatures, the interaction among active amino acids diminishes due to the augmented kinetic energy of molecules. Consequently, the enzyme's efficacy diminishes. The drop may be attributed to the alteration in the structural configuration of enzymes. Elevated temperatures influence the ionization of

groups present on the surfaces of both the enzyme and its substrate. Enzymes, as intricate protein molecules, possess a conformation that directly influences their capacity to facilitate chemical reactions (28) .

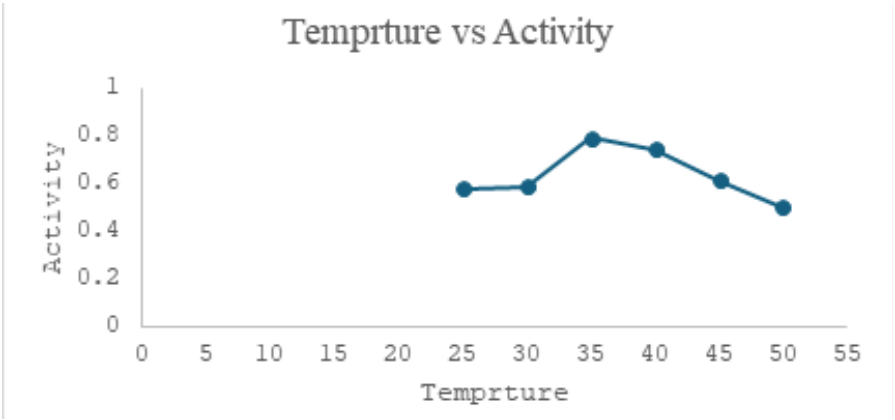

Fig.9 Effect of temperature on purified enzyme activities.

Effect of pH on enzyme activity

A study was performed to examine the diverse impacts of pH on the activity of the ACE enzyme. Various pH values have been examined to identify the ideal pH for maximal ACE enzyme activity (pH 4, 5, 6, 7, 8, and 9), as illustrated in Fig.10 The peak enzyme activity was seen at a pH of 7 in the pure ACE sample. Previous investigations indicated that the optimal pH value of the ACE enzyme isolated from human plasma was determined to be 7.4 (25). In another work, ACE was purified from rabbit lung and the optimum pH was 8.0–8.3 (26). Also, optimum pH for ACE purified from sheep lungs were found to be pH 7.4 (27).

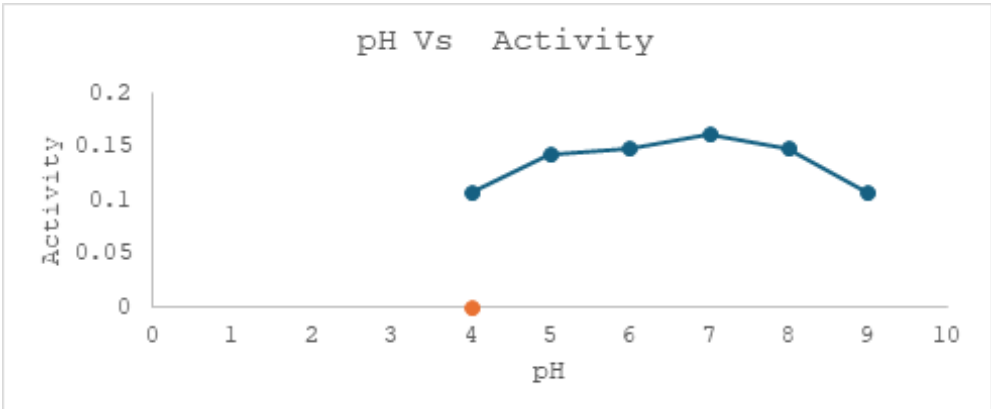

Fig.10 Effect PH on the activity of purified enzyme.

Conclusion

The study successfully purified ACE from hyperthyroid patients and identified its optimal conditions and kinetic parameters, with significantly higher ACE levels observed in these individuals.

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