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Eureka Journal of Physical and Chemical Research (EJPCR)

ISSN 2760-490X (Online) Volume 2, Issue 6, June 2026

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KINETIC STUDY AND PARTIAL PURIFICATION OF PHENYLALANINE HYDROXYLASE ENZYME IN THE URINE OF KIDNEY CANCER PATIENTS BY GEL FILTRATION

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Abstract

Kidney cancer is a malignant tumor that originates in the renal tubules and in the lining of the kidneys. Cancer begins to spread into the lymph nodes or adjacent organs and tissues depending on the nature of the tumor and its aggressive spread. The survival rate for kidney cancer drops to 70% and depends on several factors, including: age, stage, gender, tumor shape and location.

Phenylalanine hydroxylase (PAH) is a monooxygenase enzyme primarily found in the liver and, to a lesser extent, in the kidneys of mammals. It belongs to the class of oxidoreductases and the subclass of monooxygenases. PAH metabolizes dietary phenylalanine (Phe) into tyrosine in the liver.

Any defect or deficiency in PAH function leads to metabolic disturbance and the occurrence of gene mutations in PAH. PAH causes changes and imbalances in the body's metabolites, thus leading to a decrease in immune function with an increased risk of kidney cancer due to elevated Phe levels

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resulting from its toxic accumulation in the blood and urine. The kidneys contribute about 60% of the stimulation process of the PAH enzyme to maintain Phe concentrations within the body's physiological range.
PAH enzyme was partially purified in the urine of kidney cancer patients using precipitation methods via ammonium sulfate solution. Ion exchange and gel filtration techniques were used for purification. Two (symmetric) peaks of the purified enzyme were obtained. The molecular weight of the enzyme's highest activity peak was determined to be approximately (50.478 kDa). Kinetic studies were performed to determine the optimal conditions for PAH. The values were (Km=1.51 mM), (Vmax=2.4), ([S]=5 mM), (pH=7.5), (Temp.=25 C°C), and (Time=20 min).

Keywords: Gel filtration; Ion exchange; Kidney Cancer; Km; Molecular weight; Optimum conditions; Purification; Vmax.

1. Introduction

1.1. Kidney cancer

Kidney cancer (KC) is a malignant tumor that originates in the renal tubules and the lining of the kidney. KC is the fourteenth most common cancer, ranking ninth in men and fourteenth in women [1]. Men have twice the risk of developing KC and dying from it compared to women [2]. KC is sometimes called the "internist's tumor" because its complications often lead patients to consult an internist for the first time. KC is classified by morphology, histology and genetics into three main types of malignant tumors: chromophoretic renal cell carcinoma (5%), papillary renal cell carcinoma (10%) and clear cell renal cell carcinoma (90%) [1].

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Early diagnosis of kidney cancer (KC) is challenging. Eighty-seven percent of patients are diagnosed incidentally at stage 1A without any associated symptoms [3]. Treatment of KC in its early stages is surgical, and it becomes difficult in its advanced stages [4]. KC begins to spread into the lymph nodes or adjacent organs and tissues depending on the aggressive nature of the tumor. Survival rate in KC is related to age, stage, sex, tumor shape and location, reducing the survival rate by 70% [1].

The risk factors for KC are divided into two main categories: genetic determinants (5%) and modifiable lifestyle-related risk factors (40%) (obesity, smoking, exposure to ionizing radiation and trichloroethylene in a toxic environment, chronic kidney disease, diabetes, age and high blood pressure) and non-modifiable genetic risk factors for KC. See Figure (1) [2,5,6].

KC sometimes causes many complications, including: meta-neoplastic syndromes, wasting, hypercalcemia, hypertension, fever of unknown origin, anemia, elevated erythrocyte sedimentation rate, coagulopathy, Cushing's syndrome, liver dysfunction without metastases, amyloidosis, glucose metabolism disorder and galactorrhea [4]. Chemical, conventional, immunotherapeutic and targeted therapies affect patients' health as a result of increased kidney toxicity, which forces the doctor to reduce doses or stop treatment [3]. Benzene derivatives, welding fumes, drilling operations, and asbestos exposure are carcinogenic substances that are transported through the bloodstream and excreted in urine, and are associated with kidney and bladder cancer [5].

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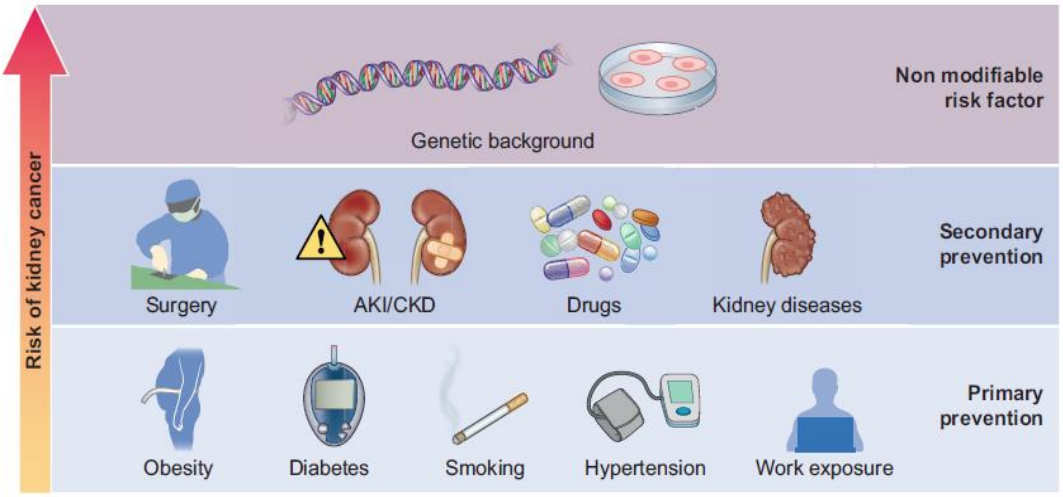

Figure (1) Risk factors in kidney cancer [2].

1.2. Phenylalanine hydroxylase

Phenylalanine hydroxylase (PAH) (EC 1.14.16.1) is an enzyme belonging to the Oxidoreductases class within the Monooxygenase subclass. PAH metabolizes food-ingested phenylalanine (Phe) and converts it to tyrosine in the liver [7]. Any malfunction in PAH function leads to a metabolic disturbance in the body, causing the accumulation of toxic Phe in the blood and urine [8]. PAH has a moderate affinity for Phe and acts as its regulatory domain at the amino terminus. PAH exhibits high substrate specificity due to its location in the liver. PAH is activated and regulates elevated Phe concentrations in mammalian blood [9]. The American College of Medical Genetics and Genomics recommends that the Phe level be (30-360 micromoles/L) [10].

A deficiency of the PAH enzyme in the blood leads to genetic mutations in PAH, causing elevated Phe levels [9]. Specific treatments for PAH deficiency,

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such as sapropterin HCl and Pegvaliase, are used to lower high Phe concentrations and prevent adverse complications [10].

1.3. The relationship between phenylalanine hydroxylase and kidney cancer

PAH is expressed in abundance in the liver and kidneys of mammals. Any defect or deficiency in PAH function leads to metabolic disturbance and the occurrence of gene mutations in PAH. PAH acts to alter and disrupt the body's metabolites, thus leading to a decrease in immune function with an increased risk of kidney cancer as a result of elevated PAH levels due to its toxic accumulation in the blood and urine [11]. The kidneys contribute about 60% of the PAH stimulation process to maintain PAH concentrations within the body's physiological range [12]. Low PAH levels with high Phe levels is an important risk factor for other metabolic disorders such as diabetes and obesity, and consequently tumor formation and development in KC [13].

1.4. Study objective

The study aimed to purify the enzyme Phenylalanine hydroxylase from kidney cancer patients in order to determine the molecular weight of the enzyme PAH and to understand its physical properties. The disease and the PAH enzyme were described, along with the relationship between them. Understanding the optimal conditions for the enzyme, including temperature, acidity, incubation time, and its tolerance in the tumor environment. Kinetic studies were also conducted to determine the rate of enzyme activity in chemical reactions with high accuracy, especially when knowing the values of the Michaelis-Menten constant Km and the value of the maximum velocity Vmax. To understand

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the uses of the enzyme in medical and diagnostic applications as treatments and as an indicator for detecting diseases.

2. Materials and Methods

2.1. Sample Collection

Urine samples were obtained at Kirkuk Teaching Hospital from patients with kidney cancer of both sexes. Patient ages ranged from 30 to 75 years. Their infection with KC was confirmed through diagnosis, tests and medical reports. Patient consent was obtained as ethical approval before urine samples were collected from them, as part of the job requirements.

2.2. Estimation of Phenylalanine Hydroxylase (PAH) Activity and Total Protein:

PAH activity was estimated using the Bublitze method [14]. Phenylalanine hydroxylase converts the substrate phenylalanine to thyrosine, a yellow-orange complex is formed. Absorbance was measured at 450 nm. Total protein concentration was estimated using the Lowry method [15].

2.3. Steps for purifying phenylalanine hydroxylase enzyme from the urine of kidney cancer patients

2.3.1. The first precipitation step: A buffer solution of ammonium sulfate (solution A) was used at varying concentrations (ammonium sulfate, concentrated ammonia solution at a ratio of (1:20), pH=7.5) [16] to precipitate the proteins present in the raw urine.

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2.3.2. First gel filtration step: The gel separation column with dimensions (2cm x 30cm) was injected with (8 ml) of the concentrated sample with (Sephadex G-25) gel, to get rid of the remaining salts associated with PAH.

2.3.3. Step for preparing the protein sample: The concentrated sample obtained from the second step was placed in a membrane sorting bag and then immersed in a flask containing sucrose crystals, for a period ranging from (30-45 minutes).

2.3.4. Ion Exchange Step: An ion exchange column measuring (2 cm × 40 cm) was injected with (8 mL) of the concentrated sample with DEAEcellulose A50 resin at a temperature of (12°C) [17]. The flow rate for the exuded parts was set at 1 ml/min.

2.3.5. Re-precipitation step (second): (4 ml) of the concentrated sample from the previous step was taken, and (4 ml) of (solution A) was added to it. Then it was placed in a centrifuge at a speed of (10000 rpm) for (15 minutes). After that, the precipitate that was formed was taken and a quantity of solution A was added to it to bring the volume up to (9 ml).

2.3.6. Second gel filtration step: A separation column with dimensions of (2 cm × 100 cm) with a gel filter (Sephadex G-200) [18] was used. The concentrated protein sample resulting from the ion exchange step was gently placed in the column, and the flow rate was set at (1 ml/min). The effluxed fractions were collected. The PAH enzyme activity and total protein content of each fraction were measured.

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2.3.7. Study of PAH enzyme kinetics: The factors affecting the kinetics of PAH purified from the urine of kidney cancer patients and the optimal conditions for each factor were studied.

3. Results and Discussion

The results are shown in Table (1), Steps for the partial isolation of PAH enzyme from the urine of kidney cancer patients. The salts associated with the protein sample were removed by precipitation in steps 2 and 5 using an ammonium sulfate buffer solution. The specific activity of PAH increased, especially in step (5) of the precipitation, and became (5.87 U/mg), and the yield rate was (91.69%). In the third step, gel filtration technology with Sephadex G-25 gel was used to remove the remaining salts. PAH activity was observed to double and become (4.13 U/mL) compared to crude urine (2.89 U/mL), and the yield rate was (57.16%). In the fourth step, ion exchange technology was used on the protein sample, and an increase in PAH activity to (5.31 U/mL) and specific activity to (5.40 U/mg) was observed compared to crude urine, and the yield rate was (73.49%). In the sixth and final step, gel filtration was used with (Sephadex G-200) gel. Two peaks (A, B) were obtained as shown in Figure (2).

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Table (1) Steps for partial purification of the PAH enzyme in the urine of kidney cancer patients

No.	Purification steps	Volume (ml)	Total protein (mg)	Activity of PAH (U/mL)	Total activity (U*)	Specific activity (U/mg)	Fold	Yield %
1	raw urine	20	11.12	2.89	57.8	5.20	1	100
2	ammonium sulphate	10	8.01	3.25	32.5	4.06	0.78	56.23
3	Gel Filteration Sephadex G25	8	6.32	4.13	33.04	5.23	1.01	57.16
4	Ion Exchange DEAE-Cellulose A50	8	7.87	5.31	42.48	5.40	1.04	73.49
5	ammonium sulphate	4	5.08	7.45	29.8	5.87	1.45	91.69
6	Gel Filteration Sephadex G200							
	peak A	9	4.03	5.71	51.39	12.75	2.45	88.91
	peak B	9	3.02	6.32	56.88	18.83	3.62	98.41

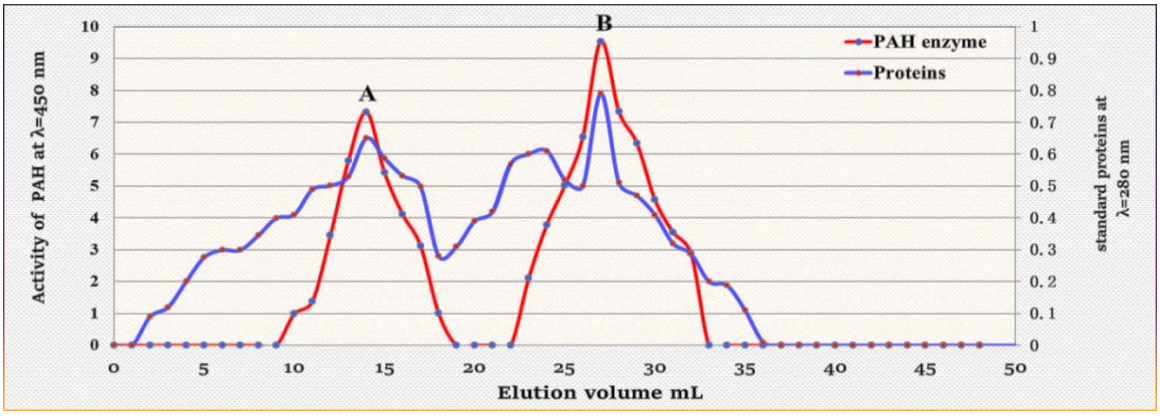

Figure (2) Gel filtration technique with (Sephadex G-200) for purifying PAH enzyme in the urine of kidney cancer patients

The PAH enzyme activity at peak (B) (6.32 U/mL) was higher than at peak (A) (5.71 U/mL). The specific activity at peak (B) (18.83 U/mg) was higher than at peak (A) (12.75 U/mg). The yield percentage also increased at peak

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(B) and was (98.41%) compared to peak (A) (88.91%). It was observed that the specific activity at peak (B) was significantly higher compared to the specific activity of the crude urine.

Peak (B) was used to estimate the approximate molecular weight of purified PAH, as well as to study the kinetics and optimal conditions of PAH. This study was consistent with the study by Kaufman and his colleague regarding the appearance of two (symmetrical) peaks of the PAH enzyme from the human liver [19]. While it was inconsistent with the study by both Stonier et al. [20] and Jallis et al. [21] who obtained a single homolog for the purified PAH enzyme. Which obtained one symmetrical of the purified PAH enzyme.

3.1. Determination of the molecular weight of phenylalanine hydroxylase

The molecular weight closest to the purified enzyme was determined using gel filtration chromatography [22]. Protein materials with known molecular weights (standard proteins) shown in Table (2) were gently poured into the separation column.

Table (2) Standard protein materials with known molecular weights

No.	Materials	Molecular Weight	Elution Volume	Log M.wt.
1	Blue dextrin	2000000	30.2	6.30
2	Bovine serum	67000	55.7	4.83
3	Amylase	58000	66.0	4.76
4	Egg albumin	45000	83.3	4.65
5	Pepsin	36000	103.2	4.56
6	Insulin	5750	123.3	3.76
7	Oxytocin Hormone	1051	145.5	3.02
8	Tryptophan	204	171.4	2.31
8	PAH	50.478	35.5	1.70

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The elution volumes were obtained and were (35.5 mL) at the highest activity of the PAH enzyme, as shown in Figure (3).

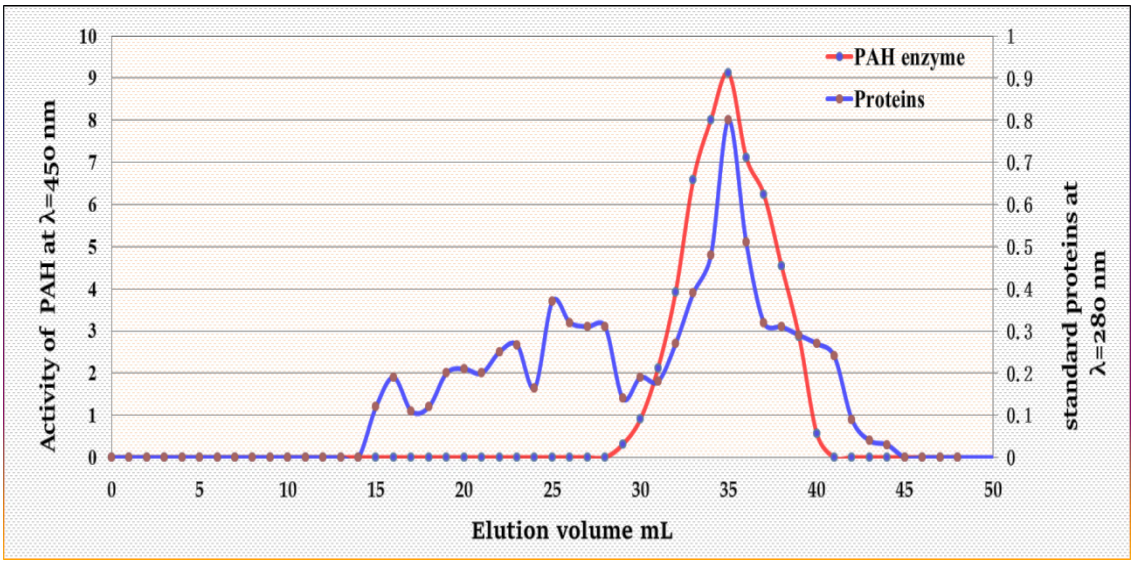

Figure (3) Gel chromatography of standard protein materials for molecular weight extraction of PAH enzyme

The linear equation (y = -0.0897x + 4.8874) was obtained from the calibration curve in Figure (4). The molecular weight of the purified enzyme was estimated by substituting the value of the elution volume (35.5 mL) from Figure (3) into the linear equation. The molecular weight of the purified PAH enzyme was estimated to be (50.478 kDa). This study is consistent with several studies conducted on the same PAH enzyme, such as those by Kaufman and Fisher [19], Flatmark and Stevens [23], and Yamashita et al. [24].

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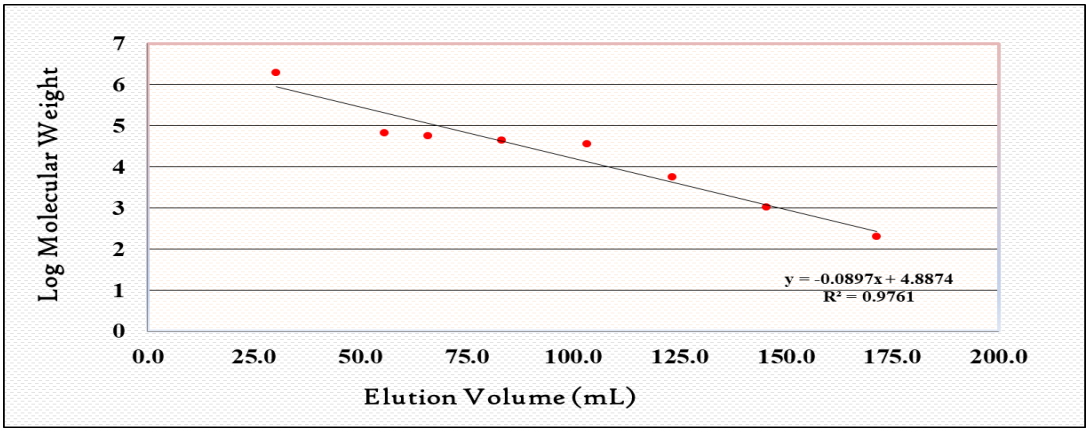

Figure (4) Calibration curve for standard proteins of known molecular weights of the PAH enzyme

3.2. Kinetic study, influencing factors, and optimal conditions for phenylalanine hydroxylase PAH enzyme

3.2.1. Studying the impact of the substrate phenylalanine on the rate of the enzymatic reaction.

Various concentrations of the substrate (phenylalanine) were prepared, ranging from (0.5 to 3.5 Mm). The relationship between PAH enzyme activity and substrate concentration was plotted, as shown in Figure (5). An increase in enzyme activity was observed as the substrate concentration increased, and PAH activity increased until it reached the maximum velocity (Vmax), at which point the velocity remained constant despite the increase in substrate concentration. The optimum substrate concentration at the highest PAH enzyme activity was (5 mM).

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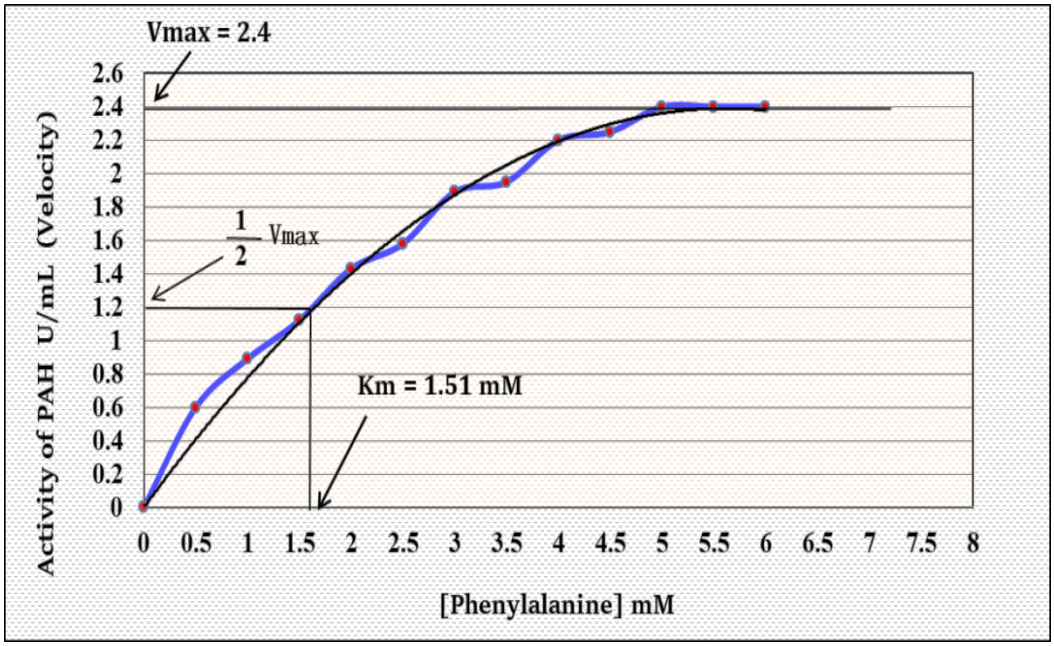

Figure (5) Impact of phenylalanine substrate concentration on the activity of purified PAH enzyme

A Lineover-Berke diagram was plotted between the PAH enzyme activity (1/V) and the substrate concentration (1/[S]). The maximum velocity value was obtained as (Vmax = 2.4 U/mL), and the Mikaess-Menten constant value was (Km = 1.51 mM), as shown in Figure (6). The Km value is also obtained from Figure (5) when the substrate concentration (phenylalanine) is half the maximum velocity (Vmax) of the PAH enzyme activity.

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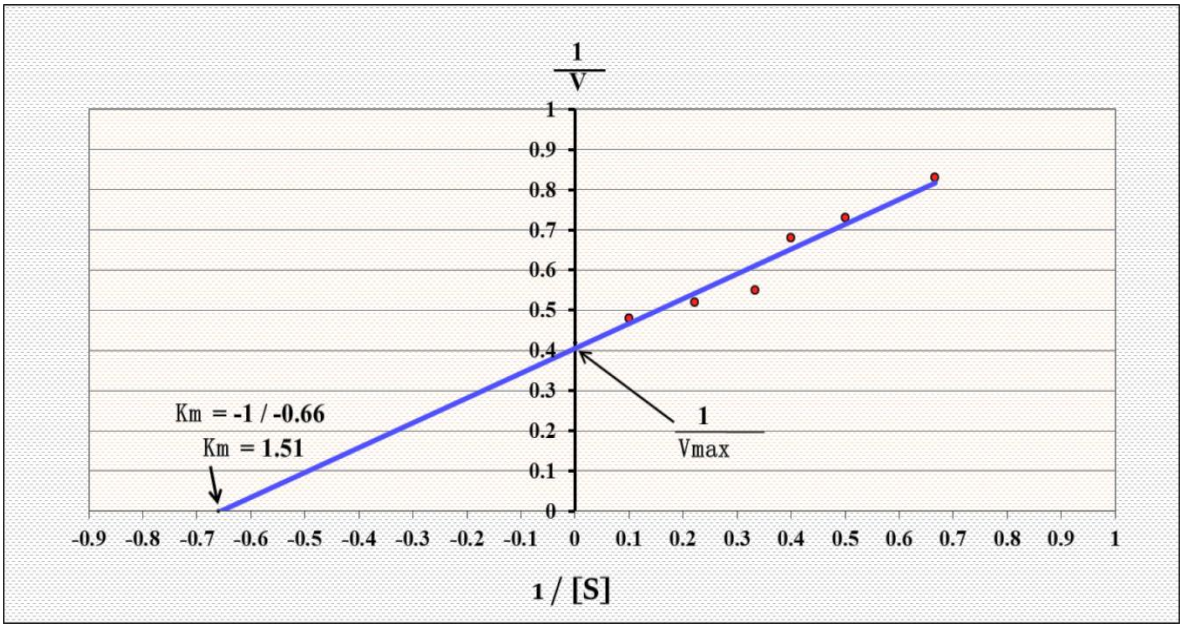

Figure (6) Lineover-Berke plot of purified PAH enzyme activity to find Vmax and Km values

3.2.2. Studying the impact of purified PAH enzyme concentration on the rate of the enzymatic reaction.

Several concentrations of purified PAH enzyme were prepared (0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6 mM), and the activity was measured at each concentration. It was observed that the reaction rate increased with increasing concentration of purified PAH, as shown in Figure (7).

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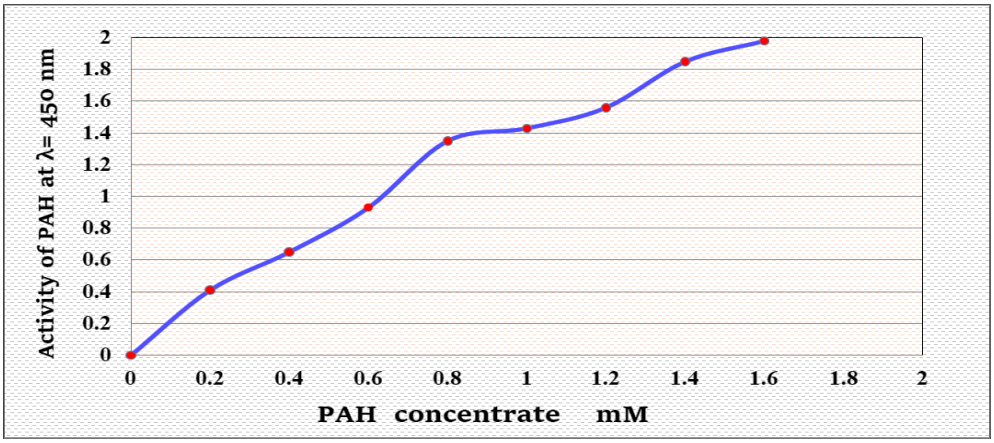

Figure (7) Impact of purified PAH enzyme concentration on the rate of the enzymatic reaction

3.2.3. Studying the impact of pH on the rate of the enzymatic reaction of purified PAH.

Several solutions with different pH values were prepared, as shown in Figure (8). PAH activity was measured at all pH values, and the optimal pH was (7.5).

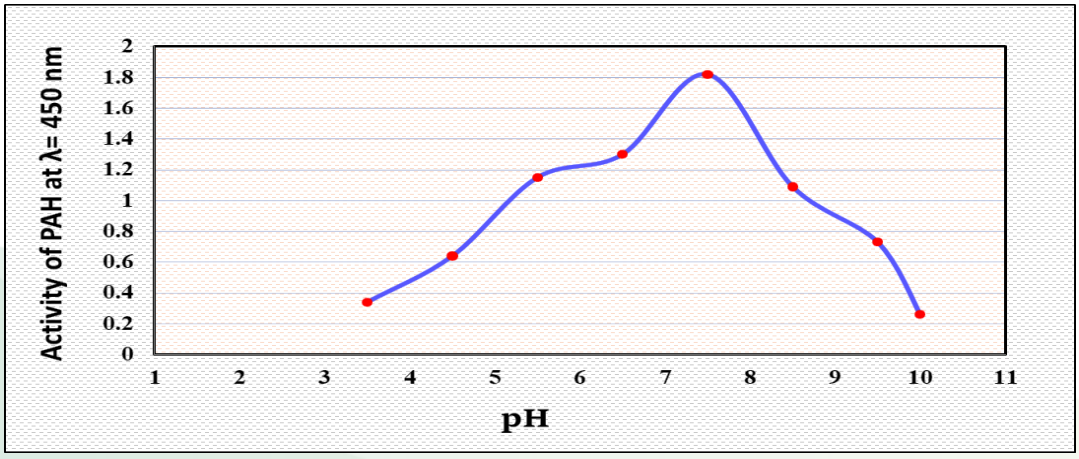

Figure (8) Optimal pH for purified PAH enzyme activity

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3.2.4. Studying the impact of temperature on the activity of purified PAH enzyme.

The activity of purified PAH enzyme was measured at different temperatures. The optimal temperature was (25C°), as shown in Figure (9).

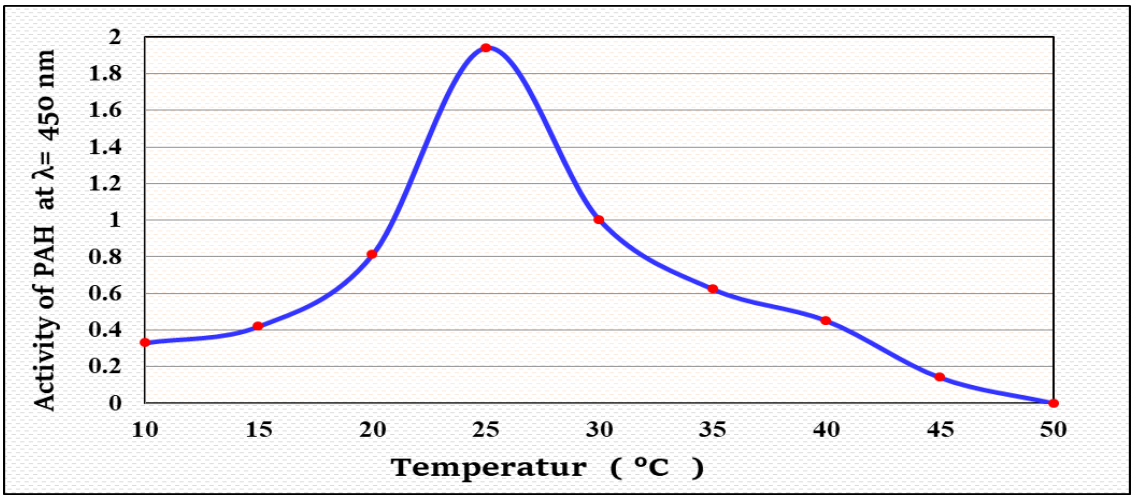

Figure (9) impact of temperature on the activity of purified PAH enzyme

3.2.5. Studying the impact of time period on the activity of purified PAH enzyme.

The activity of the PAH enzyme was measured for different time periods ranging from (5-30 minutes) to determine the optimal period. According to the results, the optimal time was (20 minutes), as shown in Figure (10).

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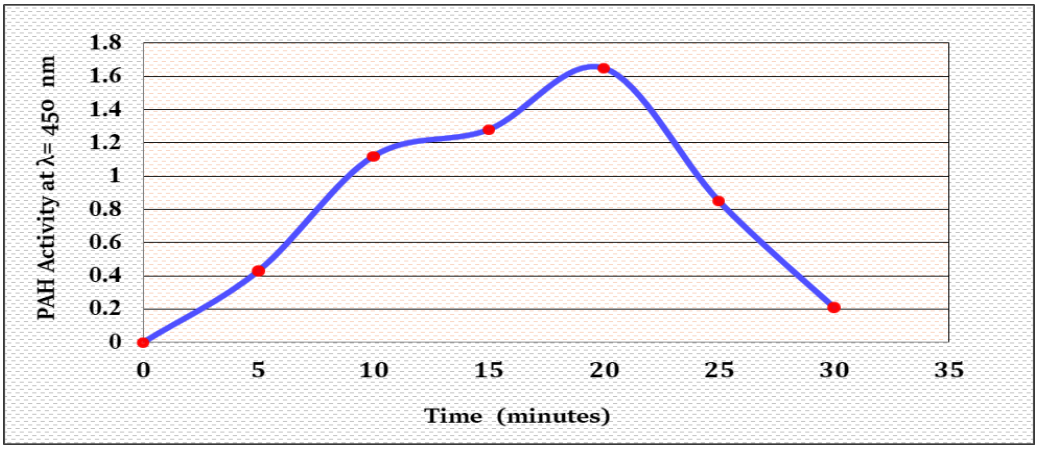

Figure (10) Optimal time period for the activity of purified PAH enzyme

Below is Table (3) showing the optimal values for the variables that affect the activity of purified PAH.

Table (3) Optimal conditions at the highest activity of purified PAH enzyme

[S] mM	Km (Mm)	Vmax U/mL	pH	Tempertur (Celsius)	Time (minutes)
5	1.51	2.4	7.5	25	20

4. Conclusion

We conclude that kidney cancer is a malignant disease affecting the renal tubules and the renal cortex. Men have twice the risk of developing kidney cancer and dying from it compared to women. The kidneys contribute approximately 60% of the PAH enzyme activation process to maintain Phe concentrations within the body's physiological range. Any defect or deficiency in PAH function leads to metabolic disturbance and the occurrence

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of gene mutations in PAH, causing a toxic increase in Phe levels due to its accumulation in the blood and urine.

The purified PAH enzyme exhibited two peaks (A and B). The specific activity of PAH at peak B was higher than at peak A. The yield rate was higher at peak (B) compared to peak (A) and was (98.41%, 88.91%) respectively. The specific activity of bundle (B) increased and was (18.83 U/mg) compared to the raw urine (5.20 U/mg). Gel filtration of the purified PAH activity of peak B was used to estimate the molecular weight, which was (50.478 KDa). The optimal conditions for PAH operation were: ([S]=5mM), (Km=1.51mM), (Vmax=2.4 U/mL), (pH=7.5), (Temperature =25 C°), (Time=20 min).

5. Ethical Approval

This study received approval from the Medical Ethics Committee at Azadi Teaching Hospital, Kirkuk, Iraq. Patient consent was also obtained when conducting the research.

6. Consent for publication

The researchers have no objection to publication.

7. Funding Statement

No funding

8. Acknowledgments

Thanks and appreciation to everyone who supported us in completing the research.

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9. Conflicts of Interest

The author declare that there are no conflicts of interest regarding the publication of this manuscript.

10. Data availability

Stydy data will be available upon request to the corresponding

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