

# Purification of Urokinase Enzyme from Serum Blood of Patients with Pneumonia

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Article History:

Submitted: 13.04.2020

Revised: 17.05.2020

Accepted: 24.06.2020

## ABSTRACT

Among the most common lung diseases such as Tuberculosis, Chronic Obstructive Pulmonary, Asthma, Lung Cancer, Cystic fibrosis, etc., Pneumonia includes all lung diseases that affect the airway and other parts of the lung. Urokinase (UK) [EC 3.4.21.73] is strong plasminogen activator, also known urokinase type plasminogen activator (uPA), which is serine protein. that activates an inactive proenzyme plasminogen to an active plasmin via cleavage of Arg-Val bond. The active plasmin causes break down the fibrin polymers of blood clots. In this study, the UK enzyme was purified from serum blood of patients with pneumonia and the activity of the purified enzyme were measured. The purification step includes the precipitation by Ammonium sulfate salt saturation (60%) followed by isolating the enzyme using chromatography Gel filtration by sephadex G-100 and DEAE-Cellulose ion exchange to produce iso-enzyme. The apparent molecular weight of the isolated UK using SDS-PAGE. The

results show that the enzyme activity values in male patients is  $191.0 \pm 11.8$  (I.U.) while in female patients is  $158.1 \pm 9.59$  (I.U.). The enzyme activity in male control  $87.8 \pm 5.9$  (I.U.) and Female  $82.5 \pm 4.15$  (I.U.). Further, the kinetics of the purified enzyme suggest that the value of the reaction rate ( $K_m$ ) is 0.4 M. The enzymes shown an optimum pH at 8.8, and optimum temperature at 37 C°.

**Keywords:** Pneumonia, Urokinase enzyme, Purification.**Correspondence:**

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E-mail: [dr.asma.bio@gmail.com](mailto:dr.asma.bio@gmail.com)DOI: [10.31838/srp.2020.6.119](https://doi.org/10.31838/srp.2020.6.119)

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## INTRODUCTION

Urokinase (UK) (trade name Abbokinase) [EC 3.4.21.73] is strong plasminogen activator, which also known as urokinase-type plasminogen activator (uPA), is serine protease <sup>(1)</sup> that activates an inactive proenzyme plasminogen to an active plasmin via cleavage of Arg-Val bond. The active plasmin causes break down the fibrin polymers of blood clots as shown in Figure 1 <sup>(2)</sup>. It naturally exists in most animals' fluids and tissues <sup>(3)</sup> and can be produced by tumor and normal cells. The plasminogen (PA) is usually categorized into tissue plasminogen activator (t-PA) and urokinase-like activator <sup>(4)</sup>. Urokinase was initially isolated from human urine <sup>(5)</sup>, however, it has been shown that it also exists in other locations such as extracellular matrix and blood stream. The plasminogen is the primary substrate that is an inactive zymogen form of the serine protease plasmin. The activation of plasmin facilitates a proteolysis cascade that participates in thrombolysis or extracellular matrix degradation in an extent that depends on the physiological environment which eventually links the urokinase to the vascular diseases and cancer <sup>(6)</sup>. Further, plasminogen activator influences the fibrinolysis, chemotaxis, collagen degradation and cell spreading <sup>(7)</sup>, thus it has been clinically used as a thrombolytic agent in the treatment of severe venous thrombosis, myocardial infraction, pulmonary embolism, and dialysis cannulas <sup>(8)</sup>. The mechanism of the urokinase activator action is the cleaving of the proenzyme/zymogen to create an active enzyme plasmin. It is like the trypsin enzyme that stimulate the cleavage of the Arg-Val bond in the plasminogen structure via a first-order reaction and the produced active plasmin dissolves the fibrin polymers of blood clots <sup>(9)</sup>.

The urokinase structure is divided to three main domains which are the C-terminal catalytic domain, the N-terminal domain homologous to the epidermal growth factor and kringle domain <sup>(10)(11)</sup>. The N-terminal domain is in charge of for the interaction of the urokinase with the u-PAR. The

protease domain of urokinase contain the active site of the enzyme such as serine protease amino acid triad His204, Asp255 and Ser356. the kringle domain include a sequence interacts with plasminogen activator inhibitors-1 (PAI-1). The domains have rigid structures supported by disulfide bonds, three exists in the N-terminal domain, three bonds are located in the kringle domain and six bonds exists in the proteolytic domain. The disulfide bridges in the catalytic domain are responsible for maintaining the amidolytic and fibrinolytic activities of the plasminogen<sup>(12-15)</sup>

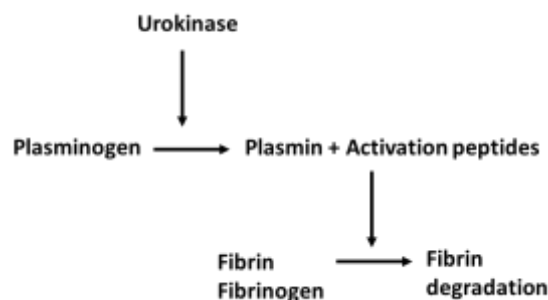


Figure 1: A schematic diagram illustrates the uPA in fibrinolytic system for blood clot dissolution.

## METHODOLOGY

### 2.1 Samples

Samples were collected from patients with pneumonia diseases, after diagnosed by the specialized doctor, in Balad general Hospital. 50 samples were collected from both males and females patients with ages range between 15-84 years.

### 2.2 Determination of urokinase activity with Chromogenic-2444

The urokinase activity is determined by its amidolytic effect on the chromogenic substrate Chromogenic substrate S-2444 (pyro-Glu-Gly-Arg-pNA). The rate at which p-

nitroaniline (pNA) is released is measured photometrically at 405 nm and a temperature of 37 °C<sup>(16)(17)</sup>.

### 2.3 Purification of UK from serum blood of patient with pneumonia

#### 2.3.1. Precipitation by ammonium sulfate

The proteins were precipitated using gradual concentrations of ammonium sulfate up to 60%, whereas ammonium sulfate was added to 40 ml of the blood sample for 45-60 minutes. The serum is then placed in the ice with a continuous stirring at 4 °C. The sample is then centrifuged for 20 minutes at 6000 rpm and the precipitate is dissolved in a buffer prepared by dissolving a 6.1 g of Tris Base and 2.2 g of NaCl in 80 ml of distilled water. The PH is maintained at 8.8 by adding 1 M HCl and the solution is kept in 2-8 °C temperature environment. 10 ml of the Aprotinin is then added and the activity and protein concentration are measured.

#### 2.3.2. The concentration of the dissolved protein

The dissolved protein was kept in a dialysis bag and merged in a container of in a sucrose particles whereas the salt were removed.

#### 2.3.3. Gel filtration

This technique depends on the different in the molecular weight. It was used to purify the urokinase enzyme separated by the ion exchange chromatography. The filtration step was done in Sephadex G-100 column.

#### 2.3.4. Ion exchange chromatography

The ion exchange chromatography technique was utilized to purify the urokinase enzyme from the concentrated proteinic part. The ion exchange chromatography was done in a DEAE-Cellulose A50 column in 25 °C.

#### 2.3.5. Electrophoresis

The separation gel in the electrophoresis step was prepared by Laemmli method with some modifications, whereas the separation was done on a acryl amide gel in the presence of sodium dodecyl sulfate (SDS).

### 2.4. The kinetics of the urokinase enzyme

#### 2.4.1. The influence of the concentration of Chromogenic S-2444

The influence of the Chromogenic S-2444 concentration on the urokinase enzyme activity were investigated by choosing 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 M to determine the optimum Chromogenic S-2444 concentration that gives the higher enzyme activity.

#### 2.4.2. Determining the activity rate (Km)

The urokinase enzyme activity rate (Km) were determined by utilizing Lineweaver-Burk method that connects the reciprocal of the activity and the concentration of the Chromogenic S-2444 (1/v vs. 1/[S]).

#### 2.4.3. The effect of PH

The influence of PH on the buffer solution prepared by dissolving 6.1 g of Tris Base and 2.2 g of NaCl in 800 ml of the distilled water. The PH was then set at 8.8 by adding HCl solution with a concentration of 1 M and the volume was completed to 1000 ml. The solution was preserved at 2-8 °C and 10 ml of Aprotinin were added. The enzyme activity was measured in the presence of solutions with PH values of 7.7, 8.3, 8.8, 9.0 and 9.5 as well as the Chromogenic S-2444 substrate. The optimum PH was determined by plotting the reaction speed and the PH values.

#### 2.4.4. Determining the optimum temperature

The urokinase enzyme activity was measured at 25, 30, 35, 37, 40 and 45 °C in the presence of PH solution of 8.8 and the Chromogenic S-2444 substrate and the optimum temperature was determined by plotting the relation between the reaction speed and the temperature.

## RESULTS AND DISCUSSION

### 3.1. The estimation of urokinase activity in serum

The urokinase enzyme activity in the serum taken from the patients with pneumonia as well as healthy was measured by determining the UK activity with Chromogenic S-2444 by utilizing Friberger method. Table 3.1. Shows the enzyme activity rate in the patients and healthy blood samples. It shows that there is a significant difference in the enzyme activity between the samples of patients and healthy people. The activity is higher in the patients with pneumonia which is consistent with the findings of Ping-Kun Tsai and co-workers, Wrotek and co-workers, Wittenhagen and co-workers and Citlenbik and co-worker<sup>(18-21)</sup>.

**Table 1:** The enzyme activity in the samples taken from male and female patients with pneumonia as well as healthy males and females.

Sample origin	No. of samples	Activity (I.U./L)	P value
Male Patients	22	191.0 ± 11.8	P ≤ 0.001
Male Control	40	87.8 ± 5.9	
Female Patient	28	158.1 ± 9.59	P ≤ 0.001
Female Control	22	82.5 ± 4.15	

3.2. The purification of urokinase enzyme from pneumonia patients' blood

The proteins were precipitated at the early stages of the enzymes purification to remove high percentage of the associated water and guarantee a higher purity. Ammonium sulfate is most common used salt due to the high solubility in water. The precipitation by salts occurs due to the protein charges neutralization which decrease the protein solubility

due to the salting out effect <sup>(22)</sup>. In the study, the urokinase enzyme were separated and purified from the patients' blood by several steps, whereas 60% of ammonium sulfate was used to precipitate the enzyme and a purity degree of 1.16 times were achieved. Further purification step was done by the ion exchange chromatography whereas a single iso-enzyme was produced with a purity of 2.11 and purifications times of 54 as shown in Figure 2.

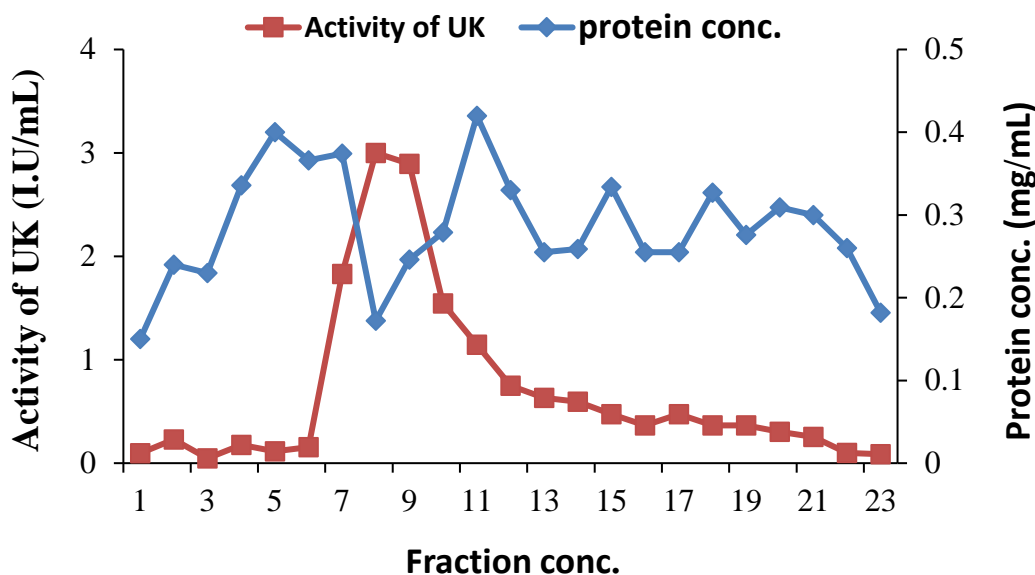


Figure 2: The enzyme purification by the ion exchange chromatography.

Similarly, the gel filtration results in pure urokinase isoenzyme with a purity of 4.42 and number of purification times of 20 times as shown in Figure 3. These observations are consistent with the studies of Stump and co-workers, Luay as illustrated in Table 2 <sup>(23-24)</sup>

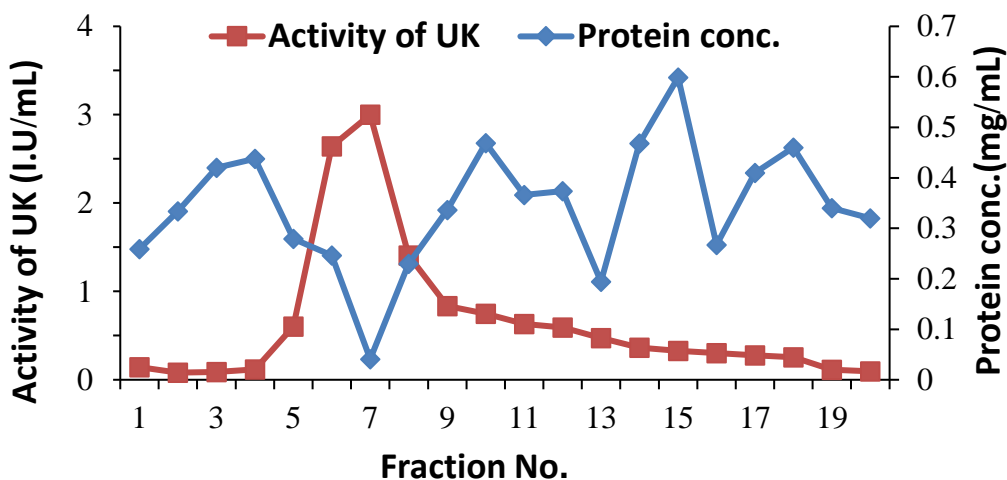


Figure 3: The urokinase purification by gel filtration chromatography.

Table 2: The urokinase purification steps from the pneumonia blood samples.

Step	Eiute (ml)	Activity (LU/ml)	Total activity (LU)	Protein conc. (mg/ml)	Total protein (mg)	Specific activity (LU/ml)	Purification Fold)(	Yield %
Crude Blood	40	0.188	7.52	0.331	13.24	0.567	1	100
Ammonium Sulphate	30	0.155	4.65	0.235	7.05	0.659	1.16	61

Ion exchange DEAE-Cellulose Isoenzyme- I	20	0.206	4.12	0.172	3.44	1.197	2.11	54
Sephadex G- 100	15	0.103	1.54	0.041	0.615	2.51	4.42	20

The results from the electrophoresis step, that has done on SDS-PAGA gel with a concentration of 10% using CBB R250, shows a unified urokinase with low molecular weight which is about 35 KDa (Lane B) as shown in Figure 4. The standard molecular weights are 97.4 KDa for Phosphorylase

b, 66 KFa for BSA, 48 KDa for Ovalbumin, 25 KDa for Carbonic anhydrase, 20 KDa for Trypsinogen and 14.3 KDa for Lysozyme (Lane A) which agrees with the finding of Wun and co-workers<sup>(25)</sup> and Yasuharu Itagaki and co-workers<sup>(26)</sup>

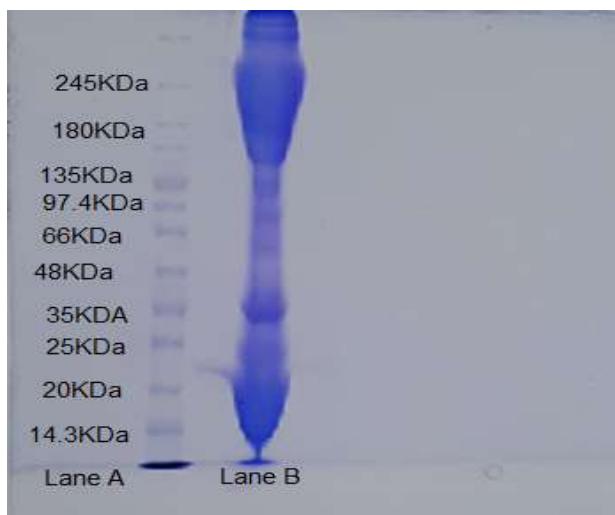


Figure 4: The results of the electrophoresis of the separated enzyme.

The governed results show that the increase in the concentration of Chromogenic S-2444 substrate results in an increase in the urokinase activity to reach a maximum at a concentration of 0.4 M. The activity then decreased at

higher concentrations as shown in Figure 5. The data in Figure 5 were used to determine the reaction constant (Km) using Lineweaver-Burk method as stated in Figure 6. The calculated Km of the enzyme is 3.2.

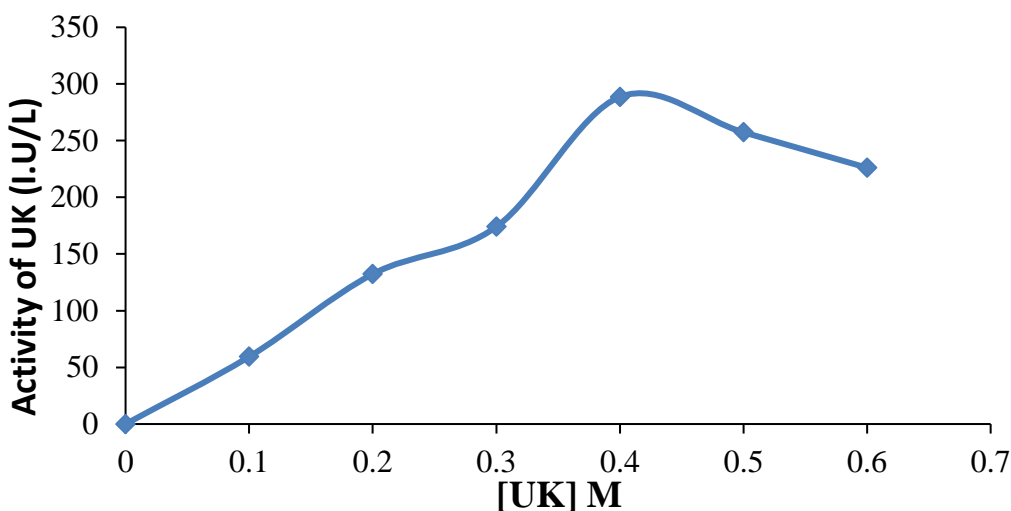


Figure 5: The activity of the urokinase as a function of the Chromogenic S-2444 concentration.

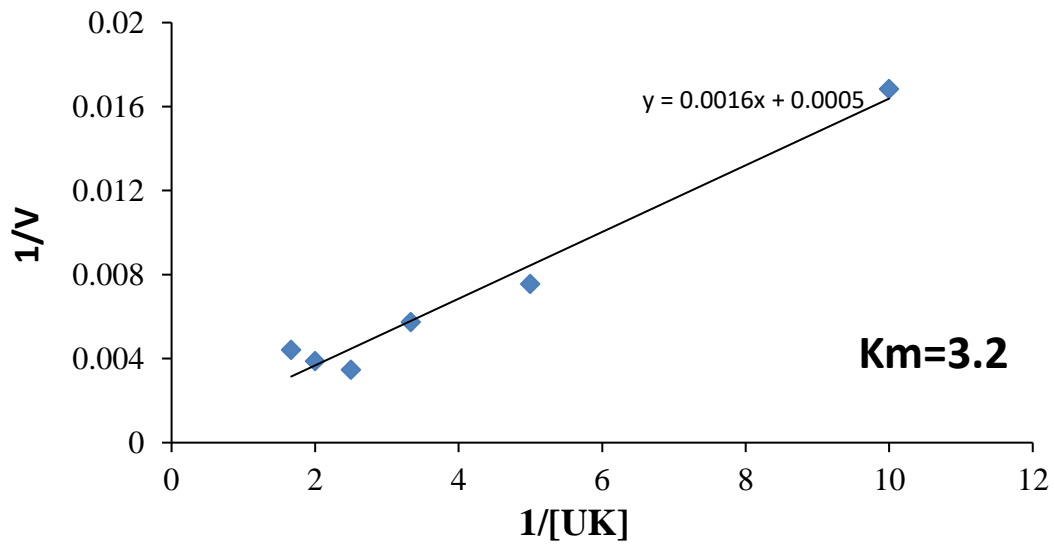


Figure 6: Lineweaver-Burk plot to calculate  $K_m$  from the pneumonia patients' blood samples.

Further, the study showed that the PH influences the enzyme activity profoundly, whereas the activity reaches a maximum at a PH of 8.8 followed by a decline at higher PH values as shown in Figure 7.

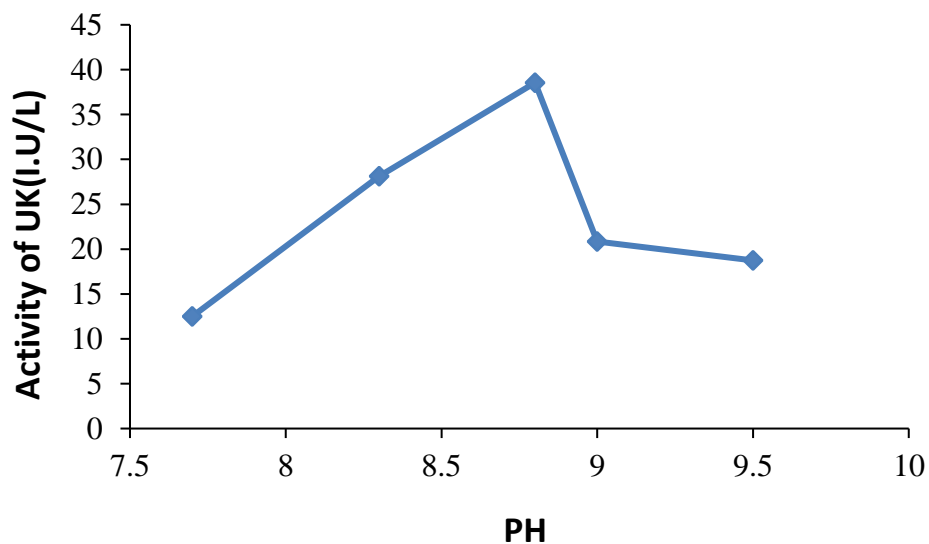


Figure 7: The relation between the PH values and the urokinase activity.

Finally, the study showed that the activity of the urokinase increase linearly as temperature increases from 25 °C to 37 °C, then it decreases as the temperature increase above 37 °C as shown in Figure 8.

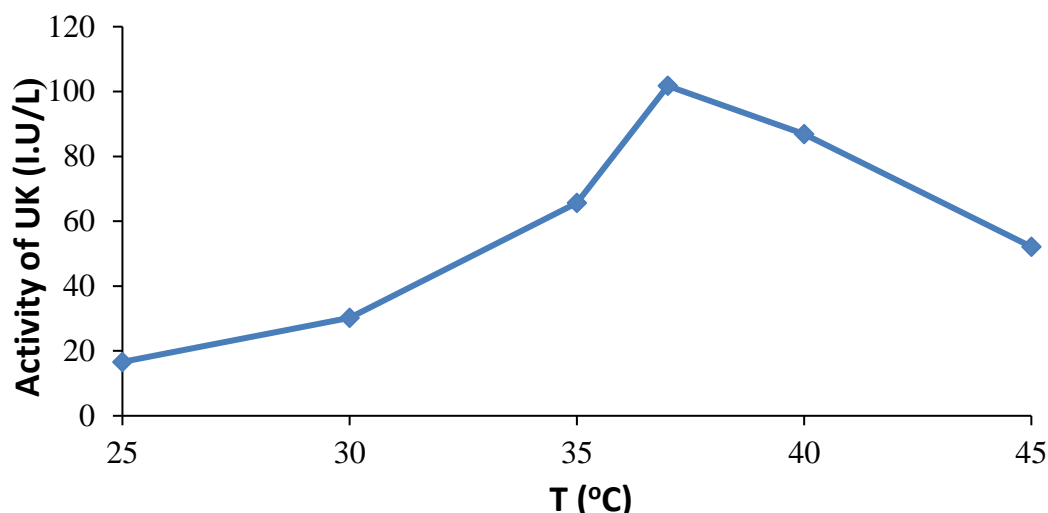


Figure 8: The activity of the urokinase separated from pneumonia patients as a function of the temperature.

## CONCLUSION

In this study, urokinase enzyme was purified from Serum Blood of Patients with Pneumonia and the kinetics studies of the purified enzyme. The enzyme showed higher activity in the patients with pneumonia compared to the control samples. The  $K_m$  value was 0.4 M. The enzymes shown an optimum pH at 8.8, and optimum temperature at 37 °C.

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