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# Partial Separation and some Kinetic Studies of Protenase Enzyme from Human Plasma

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## ABSTRACT

This study includes an isolation and partial purification the protenases from human plasma in city center of Mosul. Three proteinous components had been isolated by gel filtration technique from the precipitate produced by saturation ammonium sulfate. It was found that the peak (A) had a high activity of protenases using sephadex G-75. The apparent molecular weight of the isolated protenases the peak (A) using gel filtration was (75000± 1000 Da).

Maximum activity for protenases was obtained using (0.32) mM of casein as substrates, phosphate buffer (50 mmol) at PH (7.5) for (10) minutes in incubation at (45) °C. Using Line Weaver-burk plot were the maximum velocity (0.796) U/ml and Michaelis constant (0.035) mmol. EDTA and thiourea inhibition on the protenase activity, while magnesium sulfate and calcium chloride shown increase activity.

**Keywords:** protenase, Separation, Human plasma

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# فصل جزئي ودراسة بعض الصفات الحركية لإنزيم البروتينيز من بلازما الإنسان

## الملخص

تضمنت الدراسة عزل وتنقية جزيئية لإنزيم البروتينيز من البلازما لشخص سليم يعيش في مركز مدينة الموصل. إذ تم فصل ثلاث حزم بروتينية بتقنية الترشيح الهلامي للراسب ألبروتيني الناتج من الترسيب بكبريتات الامونيوم المشبع لبلازما الدم وأظهرت الحزمة الأولى ( القمةA ) فعالية عالية للإنزيم.

قدر الوزن الجزيئي التقريبي للإنزيم باستخدام تقنية الترشيح الهلامي باستخدام سيفيديكس نوع (G-75) ووجد انه ( 75000± 1000 Da ) تقريبا. تم إيجاد الظروف المثالية لفعالية إنزيم البروتينيز وكانت كالآتي: استخدام (0.32) ملي مول من الكازئين بوصفها مادة أساس في المحلول المنظم الفوسفيت (50) ملي مول عند اس هيدروجيني (7.5) لمدة ( 10) دقائق في درجة حرارة (45 ) مئوية وباستخدام رسم لاينو يفر-برك تم إيجاد قيمة السرعة القصوى وكانت مساوية لي (0.796) وحدة/مل وثابت مكيلس (0.035) ملي

مول. كذلك وجد تثبيط لفعالية الإنزيم بوجود اثيلين ثنائي الامين رباعي حامض الخليك والثايويوريا بينما وجد ارتفاع لفعالية الإنزيم بوجود كبريتات المغنسيوم و كلوريد الكالسيوم.

**الكلمات الدالة:** إنزيم بروتينيز، فصل، بلازما إنسان.

## INTRODUCTION

Protenase refers to a group of enzymes whose catalytic function is to hydrolyze (breakdown) peptide bonds of proteins. They are also called proteolytic enzymes or proteinases or peptidase (Barrett *et al.,* 2003 ; Rawlings *et al.,* 2010).

These enzyme can be classified according to their catalytic site into four major classes: Serine protenase, cyctein protenases, aspartic protenase and metallo protenases (Grzonka *et al.,* 2001 ; Barrett *et al.,* 2003). Protenases are essential for the synthesis of all proteins, controlling protein composition, size, shape, turnover and ultimate destruction( Rawlings *et al.,* 2010). Their actions are exquisitely selective, each protenase being responsible for splitting very specific sequences of amino acids under a preferred set of environmental conditions (Naidu and lakshmi, 2005 ; Renier and Hoorn, 2008).

Protenase inhibitors are being developed to treat parasitic, fungal, and viral infections; inflammatory, immunological, and respiratory conditions; cardiovascular and neurodegenerative disorders including Alzheimer's disease, and cancers (Barrett *et al.,* 2003). Human protenases have also been identified as important prognostic indicators of diseases( Hooper, 2002 ; Mitchell *et al.,* 2007).

Microbial protenases play an important role in biotechnological processes accounting for approximately 59% of the total enzyme used (Lucia and Tomás, 2010). Protenases are very important group of industrial enzyme in many foods, clinical and tanning industry and in the manufacture of biological detergent (Grzonka *et al.*,2001 ; Shumi *et al*., 2004).

### Aim of study

There is no previous biochemical study of protenases, thus we suggest to operate this research for isolation, purification and characterization of purified protenases from the plasma of healthy man. And using it in biological processes for example in biotechnological processes of industrial enzyme.

## MATERIALS AND METHODS

**Sample :** A human fresh plasma (25ml) was obtained from one normal male person age (31 year) living in Mosul city.

### Enzyme assay

The proteolytic activity was determined by casein as a substrate digestion method (Naidu and lakshmi, 2005).

$$\text{Casein} + H_2O \xrightarrow{\text{protenase}} \text{amino acid}$$

Condition T =37° C , pH = 7.5 , A= 660 nm

## Procedure

1- pipeth the following reagent into suitable tubes

| Reagent | test | blank |
|---|---|---|
| Casein 0.65% (w/v)in 50 mM Phosphate buffer, pH 7.5 | 5.0 ml | 5.0 ml |

Equilibrate to 37°C then add (1.0 ml ) plasma. Mix and incubated at 37°C for exactly 10 minutes then add

| | | |
|---|---|---|
| 110 mM TCA | 5.l ml | 5.0 ml |
| plasma | --------- | 1.0 ml |

Mix and incubate at 37°C for about 30 min filler and used the filtrate in color development then piptte the following reagent

| ----- | test | blank |
|---|---|---|
| Test filter | 2.0 | ------ |
| Blank filter | ---- | 2.0 ml |
| 500 mM Sodium Carbonate | 5.0 ml | 5.0 ml |
| Folin reagent | 1.0 ml | 1.0 ml |

Mix and incubate at 55°C for 5 min remove the tube and all to cool to room temperature. Read the absorbance at 660 nm for each of the test tube.

**Determination of protein concentration**

Protein was determined by the method of modified lowery (Schacterle and Pollack, 1973). Bovine serum albumin was used as a standard protein.

**Isolation and purification of protenase from plasma:** We purified a protenase from the plasma of healthy man (31 year) by using these step:

**I - Protein precipitation by ammonium sulphate saturation:**

Total protein from plasma was isolated by precipitation using (75%) saturation ammonium sulphate (Dixon and weeb 1961) the mixture was left over night at 4°C then centrifuged for 20 min at 6000xg. the praciptate was dissolved in a little amount of distilled water. The protein in the precipitate determined using the modified lowry method (Schacterle and Pollack, 1973). And the protenase activity was determined in precipitate (Naidu and lakshmi, 2005).

**II - Dialysis:**

The precipitate was dissolved in distilled water and dialyzed aginst 0.1 M ammonium bicarbonate. The solution was stirred with a amagnetic stirrer over night at 4°C. The buffer was changed twice during dialysis (Robyt and White, 1987 ).The dialysat was collected and determine the concentration of protein and enzyme activity.

**III – Fractionation of total protein:**

The dialysat was applied to gel filtration column (1.6 ×100 cm)which contained Sephadex G-75. (2ml ) Elution was carried out by using distilled water and the fraction were collected at flow rate 40 ml/h. The protein compounds in each fraction collected were detected by following the absorbance at wave lenglth 280nm using UV/visible spectrophotometer and assayed for activity of protenase was determined in each fraction.

**IV – Freeze-Dryer( Lyophilization) Technique:**

The enzyme fraction peak (A) was dried using a freeze-dryer (Lyophilization) Technique to reduce the volume of fraction.

**Determination of native molecular weight by gel filtration:**

Sephadex G-75 gel filtration was used to determined the native molecular weight of protenase. A column was calibrated using the following proteins as standards: Blue dextran (2000000Da), Bovine serum albumin (BSA) (67000Da), α- amylase (58000Da), Egg albumin (45000Da), Pepsin (36000Da), Tryptophan (204Da).

**Characterization of purified protenase:**

The protenase was characterization with respect to its optimum pH, temperature, time of reaction, concentration of enzyme and concentration of substrate.

Protenase activity was assayed at different pH values (5.5 – 9.5) using phosphate buffer, different temperature values (25 – 65 °C), different time of reaction (0 – 20min), different concentration of enzyme (0 – 9.1µg) and different substrate concentration (0.14 – 0.36 mmol).

## RESULTS AND DISCUSSION

**- Isolation and purification of protenase from human plasma :**

We used gel filtration chromatography to isolate protenase from the plasma of healthy man by applied the protenase solution result from the using of the solution of saturation ammonium sulphate precipitate (75%) to a column (1.6 × 100 cm) containing Sephadex G-75. three protein peak are detected at 280 nm as shown in (Fig. 1) protenase activity was only found in the first peak (A) showed high protenase activity. The specific activity of the enzyme peak (A) show in table (1) was (14.8) fold of that in initial extract.

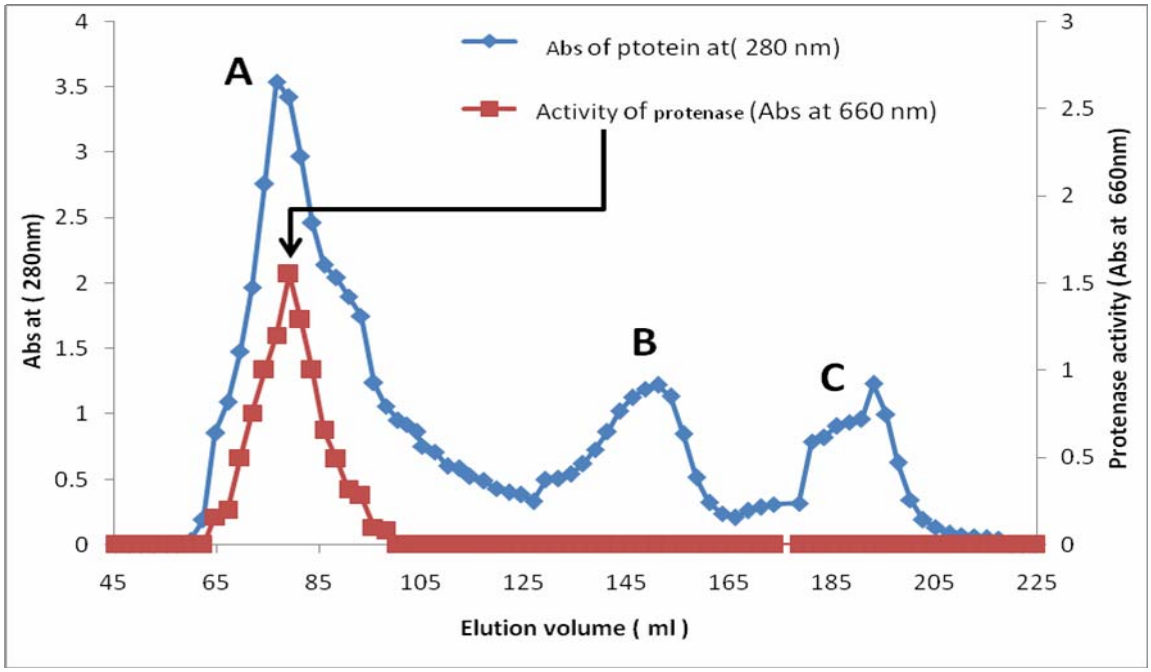


Fig. 1: Elution profile of protenase purified by saturation mmonium sulphate precipitation (75%) on gel filtration column (1.6 × 100 cm) A, B and C protein peaks

Table 1: Purification steps of protenase from human plasma.

| **Purification stages** | Volume ml | Protein mg/ml | Enzyme activity •**U/ml** | Specific activity unit/ mg protein | Fold of purification | Recovery % |
|---|---|---|---|---|---|---|
| **Plasma** | 25 | 78 | 175 | 2.24 | 1.0 | 100 |
| **Precipitate ammonium sulphate** | 20 | 62 | 170 | 2.74 | 1.24 | 97.1 |
| **Dialysis** | 18 | 40.5 | 158 | 3.9 | 1.74 | 90.2 |
| **Sephadex G-75 fraction peak( A) after Lyophilization** | 97 | 0.078 | 2.6 | 33.3 | 14.8 | 1.49 |

•U/ml: One unit of protenase activity was defined as the amount of enzyme that catalyzing the formation one micromole of product per min under of optimum conditions.

**II - The Molecular weight determination of protenase by gel filtration:**

The apparent molecular weight of protenase (peak A) which was separated by gel filtration chromatography could be determined from the standard curve which was represented by (Fig. 2). It has been found that the apparent molecular weight of protenase (peak A) was (75000 ± 1000) Da. This result is in agreement with other studies like (Bang and Fielder, 1997).

**III – Characteristics of purified protenase**

The optimal condition for enzymatic reaction were studied systemically.

**1- Effect of enzyme concentration on protenase activity:**

The activity of enzyme was measured in the presence of different concentration of partially purified enzyme (peak A) shown in ( Fig. 3). maximum activity was obtained using (8.5 µg/ml) of a protein as a source of the enzyme and these concentration was used for the next experiment.

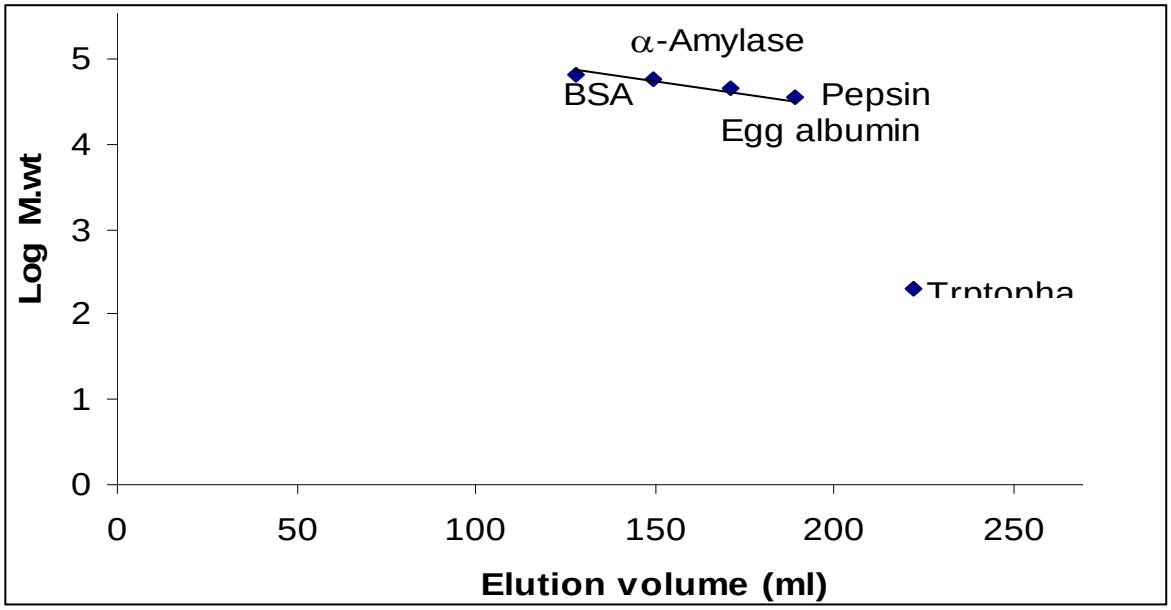


Fig. 2: The standard curve for the M. wt by using gel filtration chromatography.

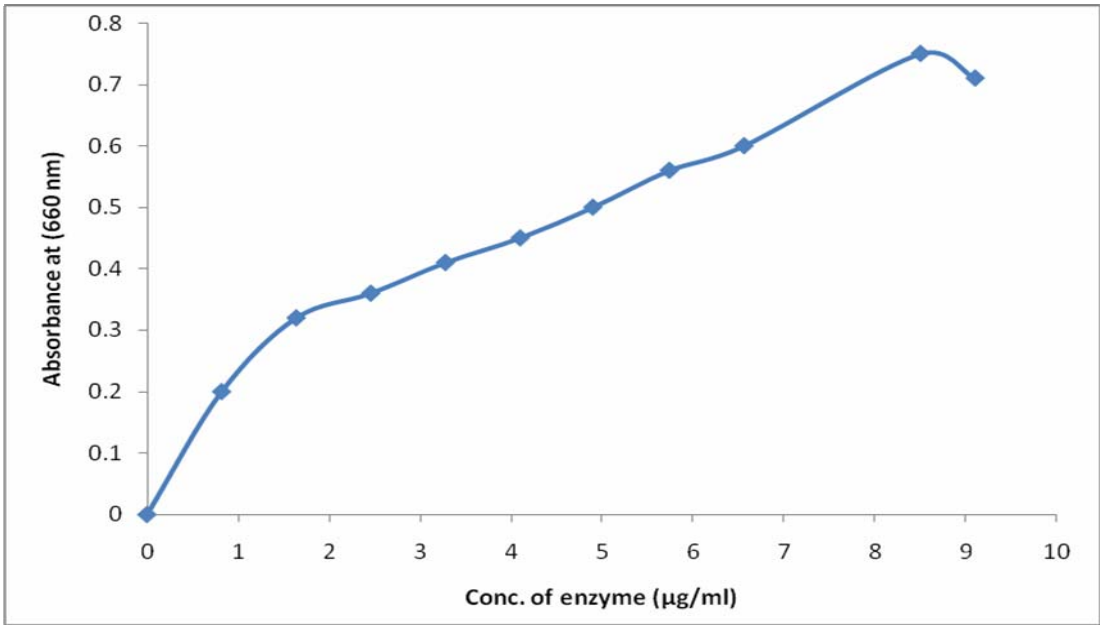


Fig. 3 : Effect of different protein concentration on protenase activity (peak A).

**2 – Effect of pH on the protenase activity:**

The activity of enzyme was measured in the presence of different pH of (50 mmol) potassium phosphate buffer. maximum activity of protenase was ablatined at PH (7.5) as shown in (Fig. 4). These results were similar to those how found by other investigators (Fujimura and Nakamura, 1987; Jram *et al.*, 2007).

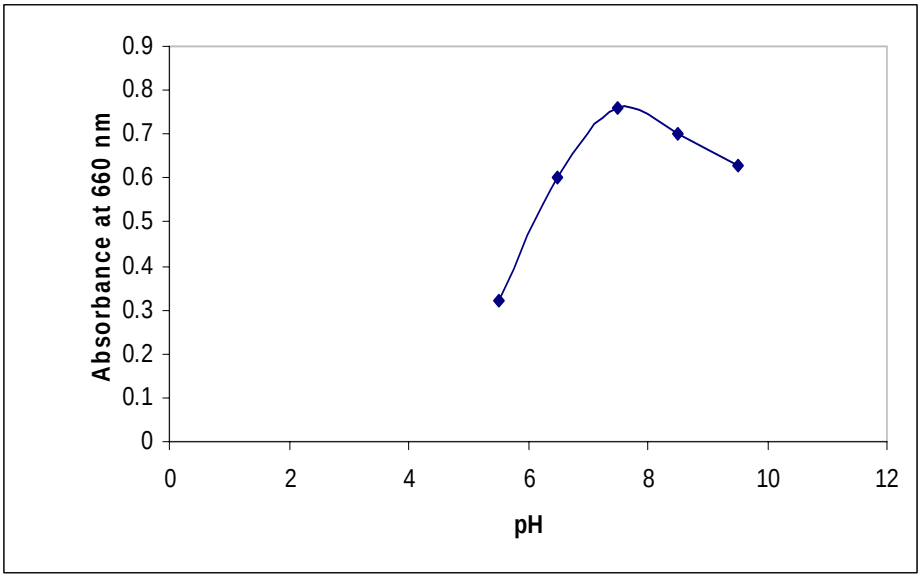


Fig. 4: Effect of pH on protenase activity(peak A) using (50 mmol) potassium phosphate buffer.

**3 – Effect of incubation time on the protenase activity:**

The activity of enzyme was measured in the different incubation time. The result indicate that maximum enzyme activity (peak A)was obtained after (10) min in 37 °C as shown in (Fig.5).

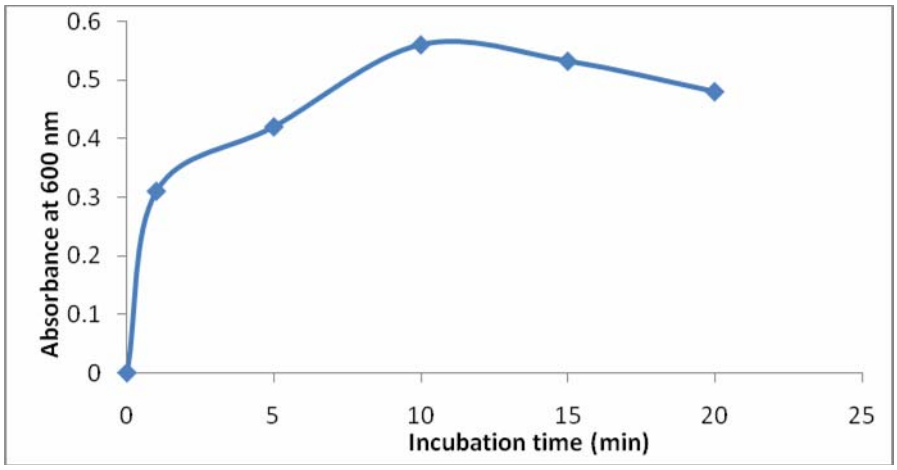


Fig.5: Effect of incubation time on protenase activity (peak A).

**4- Effect of temperature on protenase activity:**

The effect of temperature on protenase activity (peak A) were assayed at a temperature range between (25 to 65 )°C (Fig. 6). The result shown maximum activity of enzyme at 45

°C and these is a sharp decrease in the enzyme activity at 65 °C. these result were in agreement with the other results (Fujimura and Nakamura, 1987; Jram *et al.*, 2007) .

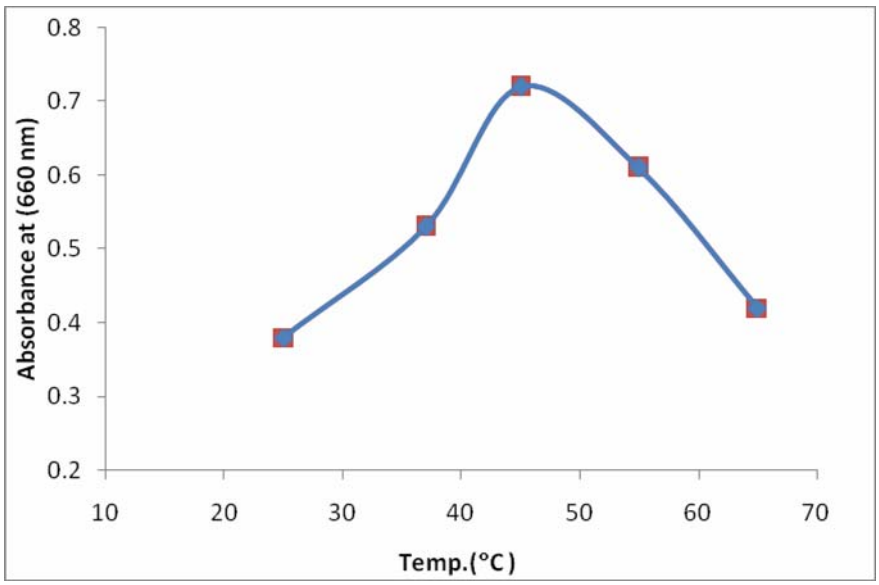


Fig. 6 : Effect of temperature on the protenase activity(peak A)

**5- Effect of substrate concentration:**

To determine the effect of substrate concentration on the enzyme activity, series of experiments were performed at different concentration of casein as a substrate (Fig. 7 ).

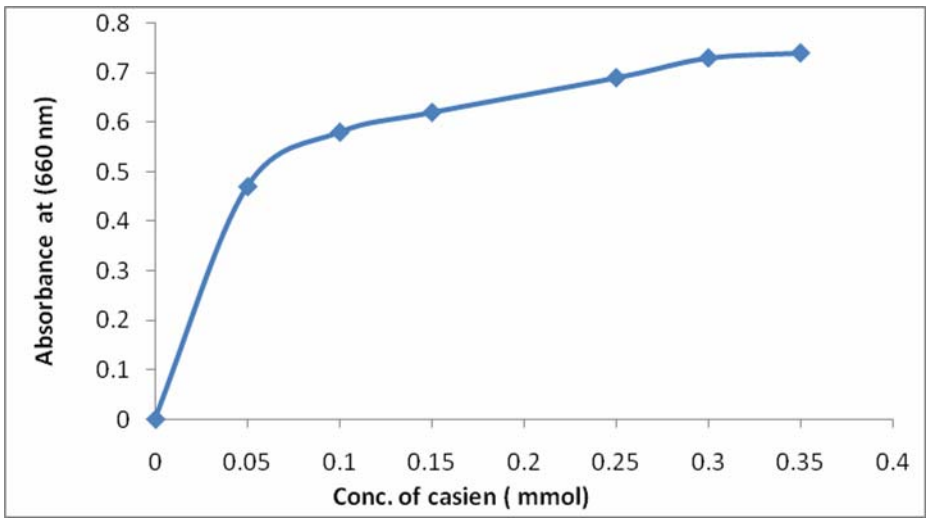


Fig . 7: Effect of casein Conc . on the a protenase activity (peak A).

The activity was increased with increase of substrate concentration there reached a limiting value at substrate concentration of (0.36) mmol. The follow Michaels Minton kinetics using LineWeaver – bark plot (Fig 8) in maximum velocity (Vmax) and Michaels Minton constant (Km) were found to be (0.796) U /ml and ( 0.035 ) mmol respectively.

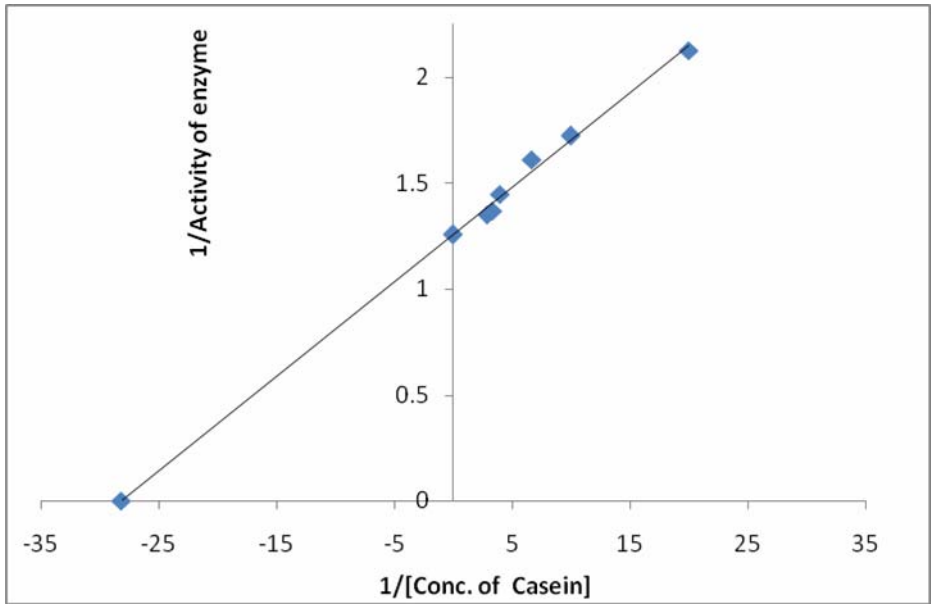


Fig. 8: LineWeaver – burk polt of protenase activity ( peak A)

In condition of protenase was isolated, partially purified from human plasma and its optimum condition for activity were determined and summarized in the following Table 2.

Table 2: The optimum condition of activity protenase (peak A).

| **Substrate Conc. mg/ml** | **Vmax** U/ml | **Km** mmol | **Temp. °C** | **pH** | **Time of reaction min** | **Enzyme Conc. µg/ml** | **protenase** |
|---|---|---|---|---|---|---|---|
| 0.36 | 0.796 | 0.035 | 45 | 7.5 | 10 | 8.5 | peak A |

**Effect of some inhibitors and inactivators on protenase activity**

The effect of EDTA, thiourea, magnesium sulfate, calcium chloride (3 mmol) concentration as inhibtors or activator were studied on the activity of protenase. The results in Table (3) were shown increase protenase activity using magnesium sulfate, calcium chloride, similar results were published by others (Iswanson and Nichols, 1971; Sunga *et al.,* 1993) and also decrease, protenase activity using EDTA and thiourea (Iswanson and Nichols, 1971; Aoyama and Chen, 1990).

Table 3: Effect of some Substances on activity protenase (peak A).

| **Substances** | ***% Increase and Decrease Activity** |
|---|---|
| EDTA | 8.8- |
| Thiourea | 6.4- |
| Magnesium sulfate | 25.3+ |
| Calcium chloride | 12+ |

***%**: Calculated according to the activity without of these substance equal to 100 % .

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