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Purification and Characterization of Meprin a from Human Blood Serum

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Abstract: Meprin A is a zinc metalloendopeptidase that displays activity against a variety of biologically active peptides.Meprin α was purified from Human blood serum.
The enzyme was isolatedfrom 100mlhuman blood serum, and
further purified by fractionation with DEAE-cellulose ion exchange chromatography andSephadex G-200 gel filtration. The molecular weight of the Meprin α was estimated by gelfiltration on Sephadex G-200 to be 216271Dalton. In addition, the determining of the approximate molecular weight of Meprin α enzyme by electrical migration technology (Electrophoresis) using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (SDS-PAGE) in the presence of 2-mercaptoethanol, two majorprotein fragments with a molecular weight of (124830±3000) Dalton were observed.
Thus the enzyme is an oligomer,probably a diamer. Then, the optimum conditions of Meprin α enzyme showed the highest activity which it was at time of (10) minutes at pH = 7.2 and at (37°C), substrate concentration was about (0.625 mmol/ L) and the maximum Velocity (Vmax) was (90.17 unit / L), the value of (Km) by using Mikael's Menten and Linwifer – Burk plot was (0.067) and (0.1) mmolar respectively.

Keywords: Meprin α, purification, isolation, ion exchange, gel filtration, Electrophoresis

INTRODUCTION

Meprin α composed of alpha and/or beta subunits, is a membrane-associated neutral metalloendoprotease that belongs to the astacin family of zinc endopeptidases. It was first discovered as an azocasein(1). Previous studies indicated that meprin metalloproteases play a role in pathological conditions such as ischemic acute renal failure and urinary tract infection(2).
Meprins degrade extracellular proteins, this ability is implicated in the cell migrationof leukocytes and the invasion of tumor cells that express meprins. The zinc metallopeptidase meprin was first isolated from mouse kidney microvillar membranes (3). Subsequently,after 6 years Kenny and Ingram (1987) isolated a peptidase with similar structural and enzymatic properties from rat kidney which they named "endopeptidase-2." (4,5). In addition, both enzymes were reported to be tetramers composed of disulfide-linked subunits (6). Kenny and Ingram described meprin purified from the rat kidney yields two polypeptides fragments of 74 and 80 kDa in approximately equal amounts, whereas detergent-solubilized immunoaffinity-purified meprin results in a single band of 80 kDa as determined by SDS-PAGE electrophoresis under reducing conditions. These data support a model in which rat meprin composed of two subunits(similar but nonidentical) which gives rise to 72kDa . Both subunits have an estimated molecular weight of 82 kDa by reducing SDS-PAGE (7).

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Purification of enzyme is very important for developing a better understanding of functioning of the enzyme. Different strategies adopted for purification of enzymes are on similar lines as that of proteins. Despite the diversity in the enzymes origins they are purified using a generalized overall approach, which involves initial recovery of protein, concentration,primary purification and finally high end resolution chromatographic purification (8).For commercial purposes, crude enzyme preparations are generally employed while pure preparations are needed for pharmaceutical and other chemical analysis or medical applications. The purified enzyme is also required for property studies and better understanding of structure function relationship. Protein (enzymes as example) separation techniques have traditionally been used to isolate and to purify specific proteins in order to facilitate studies of their enzymatic, chemical, physical and structural properties(9)**.**

These types of studies are necessary in order to understand the mechanism by which the activity of specific enzymes is work and controlled. A number of Meprins from varioussources have been purified and characterized. Factors affecting properties of meprin was considered as significant in the purification and characterization of enzyme (10).

The present study describes the purification, biochemical characterization and kinetic studies of meprin α from Human blood serum.

2-Materials and methods

2.1. Collection of blood serum

The blood serum was obtained from a group of apparently healthy males, whose ages ranged between (29-38) years of non-smokers. The blood was taken directly and a centrifugation process was conducted at a speed of 3000 x g for a period of (15) minutes to obtain the blood serum that was used to perform purification steps directly on it.

2.2. Purification of meprin α

The meprin α from Human blood serum was purified by conventional purification procedures in the steps as follows.

Step 1: Ammonium sulfate precipitation(salting out)

To the collected blood serum, solid ammonium sulfate (65% saturation) was slowly added to precipitate the enzyme (11). Precipitation was done at(4 °C).The precipitate obtained was collected by cooling centrifugation (8000*g* at 4 °C for 15 min).

Step 2: Dialysis

The dialysis process separates small molecules from large molecules by allowing diffusion of only the small molecules through selectively permeable membranes using cellulose membranes with pore sizes designed to exclude molecules above a selected molecular weight (12), which worked to increase the specific activity of the isolated enzyme in the solution obtained from dialysis.

Step 3: DEAE-Cellulose Ion exchange chromatography

The DEAE-cellulose was purchased from Sigma company and activated as per manufacturer's instructions. Then resin was packed into column (23×50 cm) care was taken to avoid trapping of air bubbles. The unbound proteins were eluted with the equilibration buffer.Fractions of(5 ml) were collected at a flow rate of (60 ml/hr) and were assayed for protein by measuring their

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absorbances at 280 nm and enzyme activity also assayed for each fraction.

Step 4: Gel filtration on Sephadex G-200

The lyophilized protein obtained after DEAE-cellulose ion exchange was dissolved in buffer (pH 7.0-7.2) and then loaded on to equilibrated Sephadex G-200 column and fractions were collected with a flow rate of 5 ml/minute by monitoring the absorbance of protein at 280 nm and activity of enzyme by standard kit assay method.

Step 5: Estimation of the approximate molecular weight of meprin α enzyme isolated from healthy human blood serum

Molecular weight of the purified meprin α was determined by gel filtration chromatography on Sephadex G-200 and SDS-PAGE .

Step 6: Kinetic studies of purifed meprin α enzyme

Characterization of the purified enzyme was carried out for several parameters like: Effect of Different Enzyme Concentrations, activity of the meprin α was measured by the presence of different concentrations (15.625-1000)ng/ml of partially purified enzyme; Effect of Buffer solution, different concentrations of the buffer solution have been used. Na_2HPO_4-NaH_2PO_4 has a range (4-19) mmol / liter at pH (7-7.2); Effect of pH on the Rate of Reaction for meprin α, activity at different pH (6.6–7.6) and temperatures (33-41 °C); Effect of reaction time, activity at different time (2–20)minutes; Effect of Different Substrate Concentrations on meprin α Activity with Determination of K_m and V_{max} , kinetic parameters for purified enzyme was calculated by using various concentration of tetra methyl benzidine range between (0.03906-1.25 mmole/L)with standard assay conditions. The kinetic rate constants, K_m and V_{max} were determined by Michaelis-Menten equation.

3. Results and Discussion

Purification of the enzyme is performed with the crude enzyme of the Human blood serum. Protein with highest specific activity was achieved at 65% ammonium sulfate saturation. The dialyzed sample was loaded on DEAE-cellulose column (Figure 1). The lyophilized sample obtained from previous step was loaded on Sephadex G-200 column and elution was carried out (Figure 2). The extent of purification at each step was shown in purification table (Table 1).

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Table (1) Steps of meprin α purification from human blood serum

Purification steps	Volume (ml)	Protein concentration (mg/ml)	Activity (ng/ml) (enzyme unit/ml)	Total activity	Specific activity (enzyme unit/mg protein)	The number of times the purification	Recovery %
Blood serum	100	71.73	55.13	5513	0.907	1.0	100
Precipitation with ammonium sulfate 65%	39	44.51	91.32	3561.48	2.051	2	64.6
Filtrate	88	4.65	0.179	15.752	0.038	0.04	0.3
Dialysis of precipitate	46	23.74	109.67	5044.82	4.632	5	91.5
The resulting peak from DEAE-cellulose ion-exchange separation column	45	11.37	114.78	5165.1	10.094	11	93.7
The resulting peak from gel-filteration sephadex G-200	40	5.66	126.12	5044.8	22.282	25	91.5

*Enzyme unit: It is the amount of meprin α enzyme that converts one micromole of tetra methyl benzidine (TMB) into product per minute / milliliter under the specified measurement conditions.

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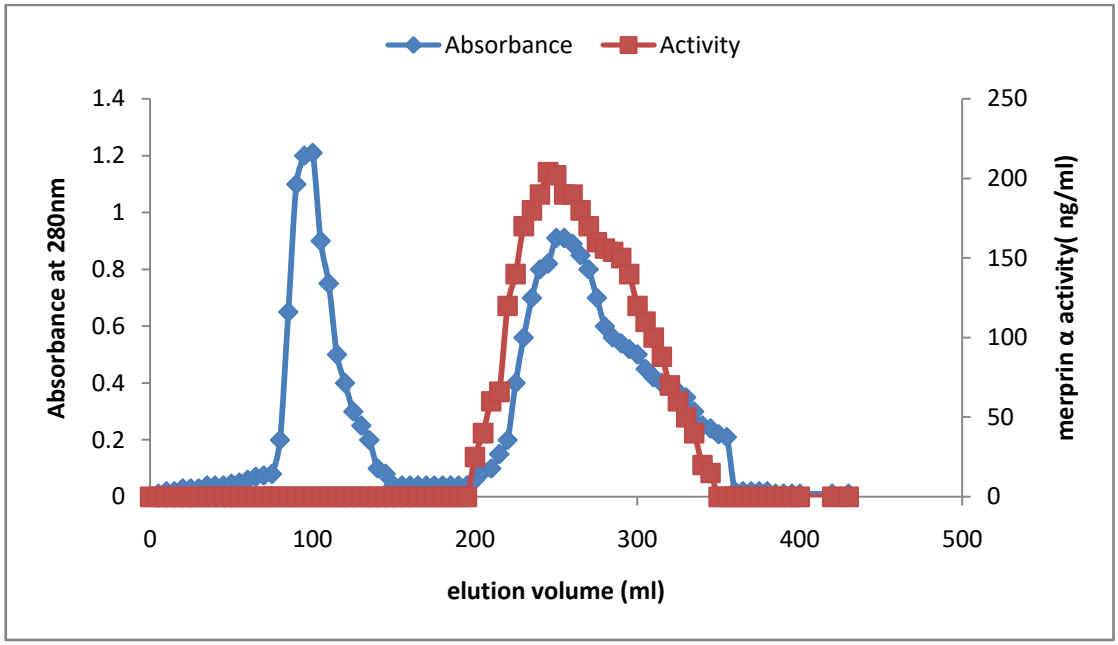
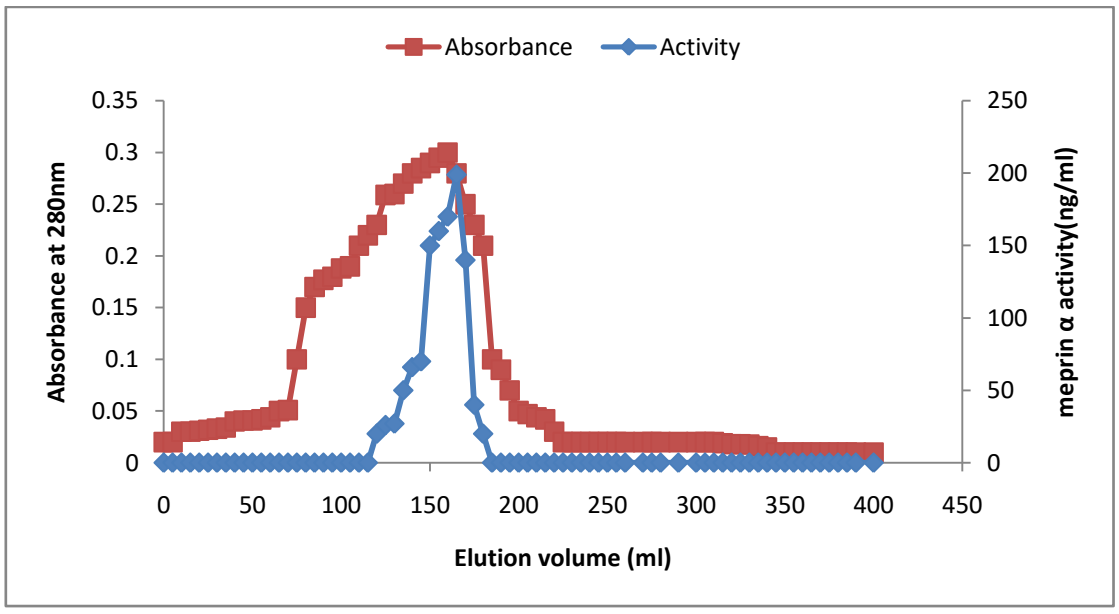


Figure(1) The elution of the meprin α proteinous peak from the ion exchange DEAE-Cellulose column of human blood serum.



Figure(2) The elution of the meprin α proteinous peak from the gel filtration G-200 column of human blood serum.

3.1. Determination of the molecular weight

By reducing SDS-PAGE of the purified protein sample resulted in two protein bands of molecular weights 127.78 kDa and 121.83 kDa respectively (Figure 3). It indicates the existence of the enzyme as a dimer (linked by disulphide bridges) in its native state and further studies were carried out with purified protein . Gel filtration on Sephadex G-200 was carried out (Figure 2) and the molecular weight of the enzyme, as calculated from the plot (elution volume versus log molecular weight) was 216.271 kDa (Figure 4).

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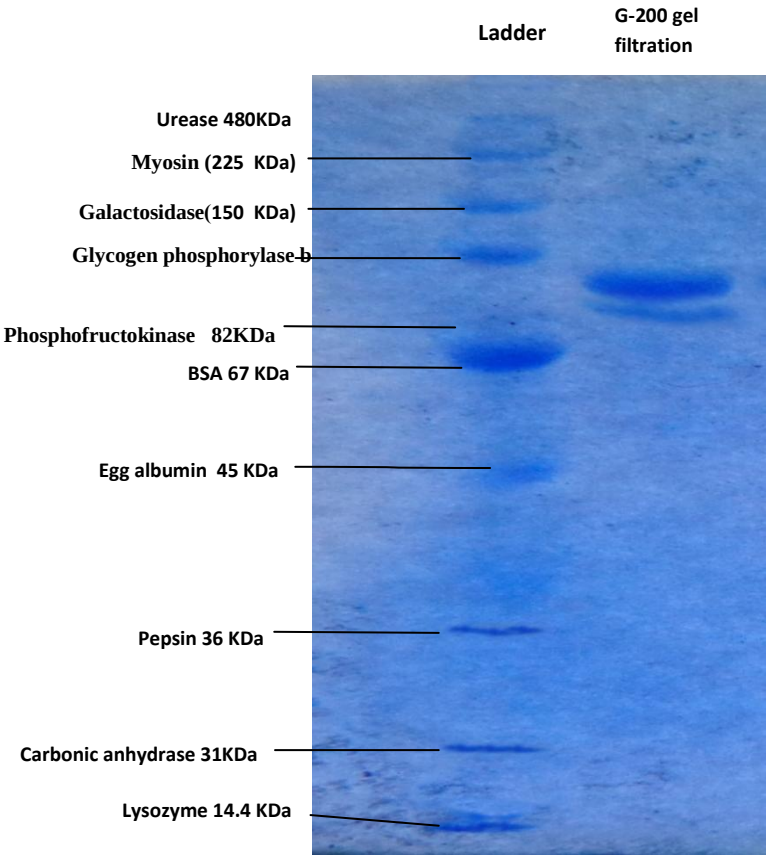
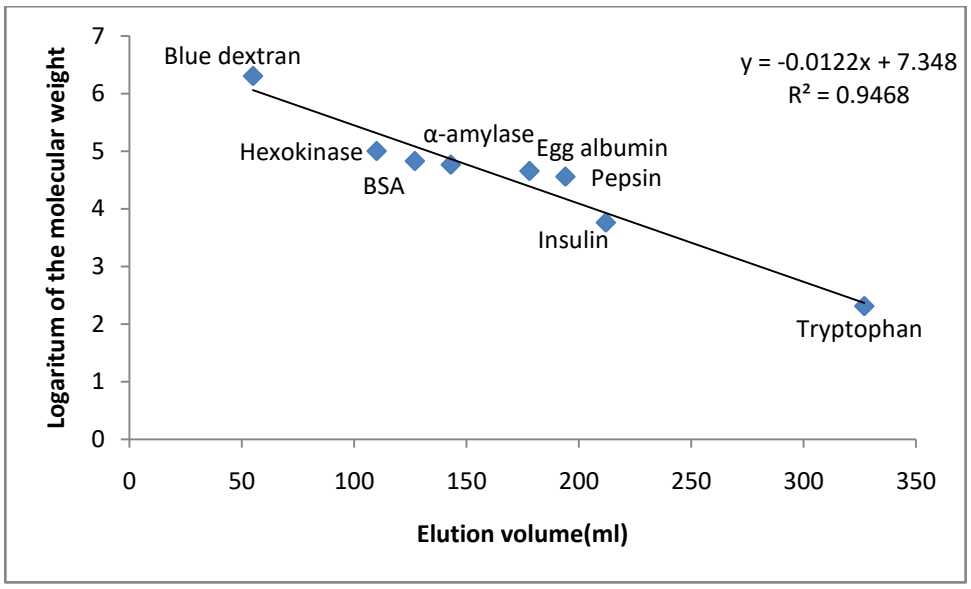


Figure(3) :SDS-PAGE Analysis Bands of the Purified meprin α and the Standard Proteins.



Figure(4) Standard curve for determining the approximate molecular weight of meprin α by G-200 gel filtration technique.

3.2. Characterization of purified meprin α

studies on the purified enzyme from Human blood serum showed that meprin α exhibits highest

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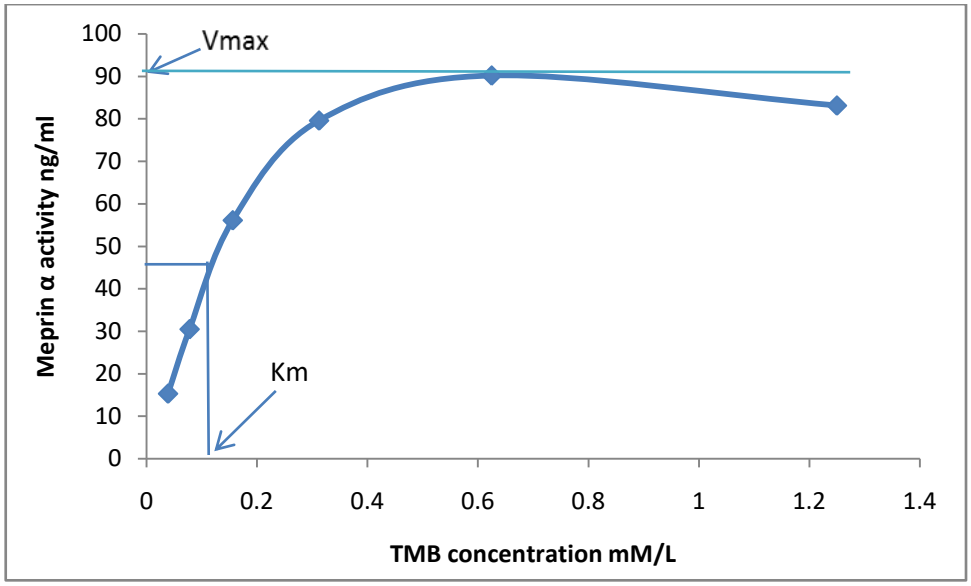
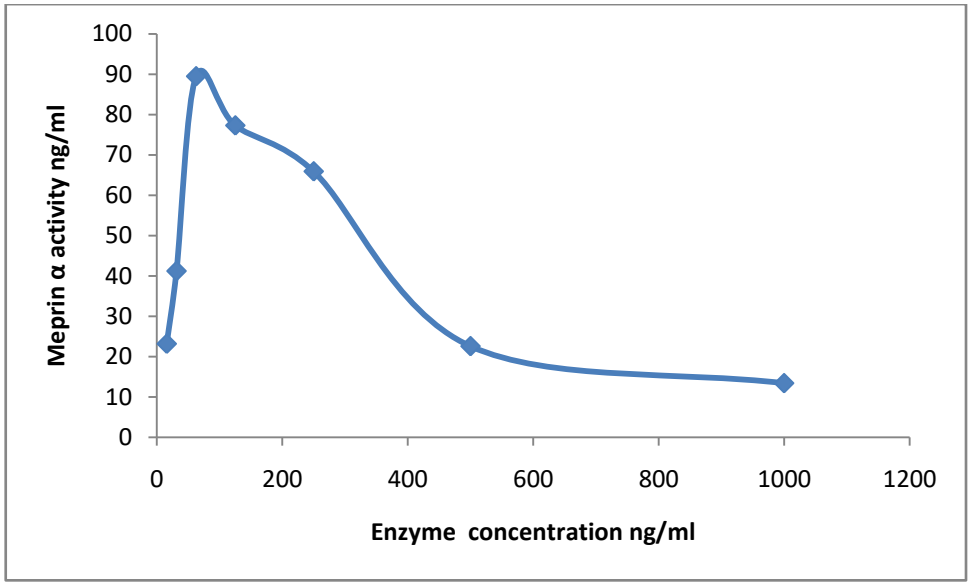
activity with TMB (0.625mmole/L)(Figure 5) with optimum activity at 62.5ng/ml of enzyme concentration (Figure 6), pH 7.2 and good activity in the pH range(6.6–7.6)(Figure 7) of Na_2HPO_4-NaH_2PO_4 (10mmole/L)(Figure 8) . It is active at all tested temperatures (33–41 °C) with maximum activity at 37 °C (Figure 9), qualifying it to be designated as a significant thermoactive enzyme.



Figure(5) : The effect of the substrate (3,3′,5,5′-Tetramethylbenzidine (TMB))concentration on the activity of the purified meprin α



Figure(6): The Effect of the Enzyme Concentration on the Velocity of the Purified meprin α

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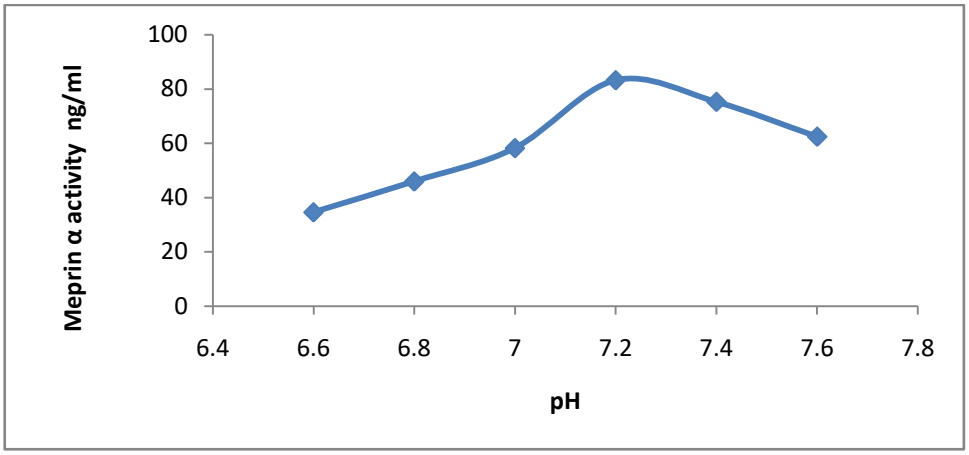
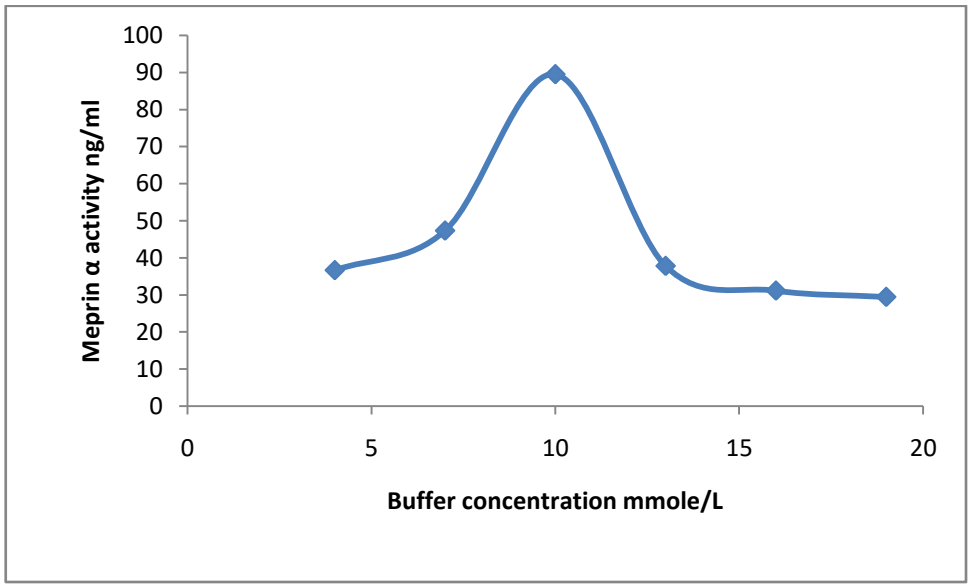


Figure (7) : The effect of the pH on the activity of the purified meprin α



Figure(8) : The Effect of the Buffer solution Concentration on the activity of the Purified meprin α

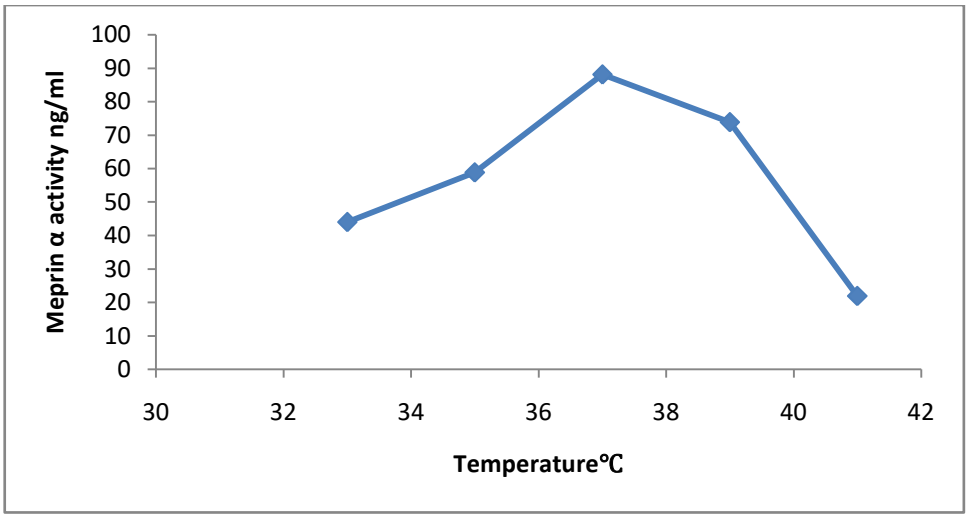


Figure (9) : The effect of the temperature on the activity of the purified meprin α

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3.4. Kinetic studies of meprin α

Effect of TMB concentration on meprin α activity was determined by evaluating the meprin α activity with 0.03906 to 1.25mmole/L of TMB at temperature 37 °C and pH 7.2. It was observed that the meprin α activity was increased with increase in substrate concentration and maximum protease activity was observed at 0.03906mmole/L of TMB, beyond this concentration a slight decrease in activity was recorded (Figure 5).Kinetic studies of meprin α from Human blood serum revealed that lower K_m value of meprin α reflects a stronger binding affinity of this meprin α to substrate (Figure 10).

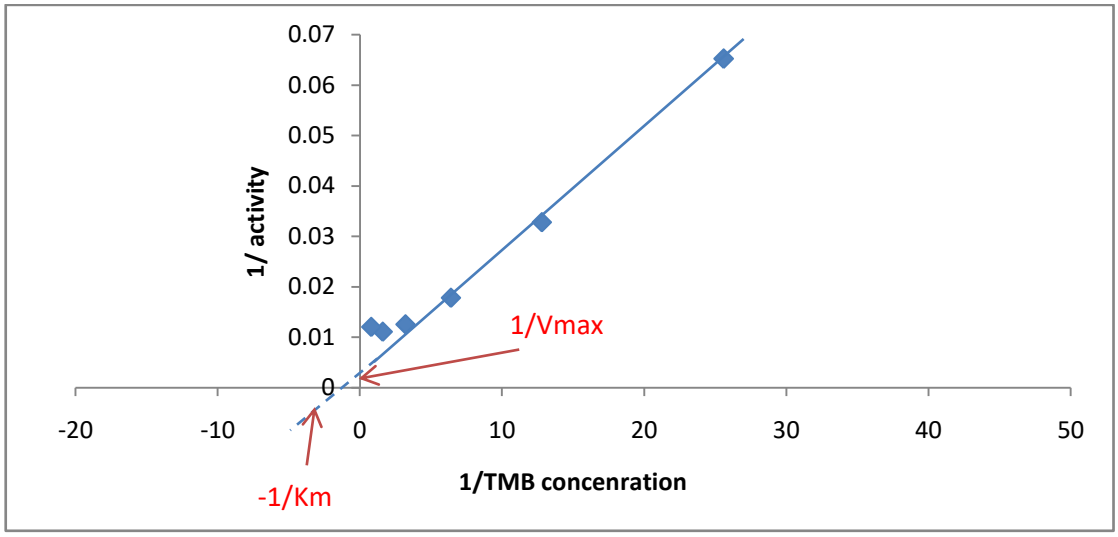

Figure (10): Lineweaver –Burk Plot to calculate Michaelis -Menten constant (K_m) of meprin α

The optimum conditions for measuring the activity of Meprin α enzyme purified from healthy human serum are shown in the following table (2).

Table 2: Optimum conditions for the partially purified enzyme

Substrate concentration (mmole/L)	Temperature (°C)	Buffer concentration (mmole/L)	pH	Reaction Time (minute)	Enzyme (ng/ml)concentration
0.625	37	10	7.2	25	200

Purification of meprin α is very important for developing a better understanding of the enzyme functioning. It also required for property studies and better understanding of structure function relationship. meprin α can be purified by a combination of chromatographic procedures like Ion exchange chromatography and gel filtration chromatography.

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In our studymeprin αfrom Human blood serum was purified to 11 and 25 fold of purification by employing DEAE- cellulose ion exchange and gel filtration chromatographic techniques respectively. Meprin α with unique properties like thermostability, pH stability, reported that purified Meprin α was active over wide range of pH between(7.5– 9.5) depending on substrate(13). Other study reported that an enzyme from rat kidney, with highest activity at 45 °C (14).

The Kinetic parameters (K_m and V_{max}) were determined for meprin α from mouse kidney by (15) at 40 °C and pH 9.0 for concentrations ranging between (0.5 - 7.5) mg/ml of casein. The K_m and V_{max} of purified meprin α was found to be 3.3 mg/ml and 15 U/mg protein respectively(15). Meprin A cleaved IL-6 with micromolar affinities (Km of 4.7 and 12.0 μM, respectively) and with high efficiencies (kcat/Km of 0.2 and 2.5 (M^{-1} /s^{-1}) ×10 ^{6}, respectively). These efficiency constants are among the highest for known meprin substrates The results for meprin α (16) showed that the enzyme followed a typical Michaelis-Menten kinetics from a double reciprocal plot and the apparent K_m of the enzyme for substrate was found to be 3.2 mg/ml. Iqbal et al. (14) reported purified meprin α with K_m and V_{max} values were 0.03564 μM and 69.76 U/mL using casein as substrate.

The result of the present study was fruitful and it reveals that there is a pronounced increase in specific activity of meprins was obtained after the ammonium sulfate precipitation, ion exchange and gel filtration chromatography. The purified meprin α was further characterized by electrophoresis and found that molecular weight of the meprin α was (124.83±3) kDa.

Characterization studies of the purified meprin α reveals that important features exhibited by this meprin α like, high activity at neutral pH and temperature, as well as there are no reports available until now with these prominent features exhibited in a single enzyme and it proves the novelty of the work. Low K_m particularly exhibited by this meprin α (compared with other meprin α from literature) can be very useful to design a future study on competitive inhibitory effects for different chemical compounds or ions to reduce or inhibt meprin α activity which is very desired in medical field as an attempt to protect from some cancer kinds which related to significant high activity of meprin α.Meprin may has function to degrade large proteins in vivo, which require multiple copies of the active protomer in close proximity(17). The presence of multi subunits may serve in increaseing the efficiency of proteolysis. Linkage of these subunits together in a specified geometry form could serve to digest proteins effectively (18). Moreover, Meprin shares similarities to other proteases such as aminopeptidase I,in which is large multimeric complexe composed of homo-oligomers that function is digesting polypeptides and involved in protein turnover within the cell (19–22). However, unlike these proteases, the meprin α homo-oligomer is secreted from the cell and degrades extracellular proteins and peptides.

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