

## Biochemical Study of Dipeptidylpeptidase-4 from Normal Human Serum

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### ABSTRACT

The research includes partial purification of dipeptidylpeptidase-4 (DPP-4) from serum of normal person aging 14 years in Mosul city. Gel filtration of dialysate precipitate produced by 50% ammonium sulphate saturation has given two major proteinous components. One of them (peak A) possesses a high DPP-4 activity using sephedex G-100. The apparent molecular weight of the isolated DPP-4 was 176.6 KD. High performance liquid chromatography HPLC revealed a single peak  $\tilde{A}$  at retention time 5.829 min by application the top of peak A which was isolated from gel filtration. Maximum activity of DPP-4 was obtained using 0.1 M Tris-HCl buffer at pH 8, 40°C, 4 mM of gly-pro-p-nitroanilide hydrochloride as a substrate. The concentration 0.35 mg/ml partially purified DPP-4 was used for next experiments. Maximum velocity ( $V_{max}$ ) was 50  $\mu$ M according to Line Weaver-Burk plot while Michaelis-Menten constant ( $K_m$ ) was 0.5 mM. Mercuric chloride and strontium chloride hexahydrate at 5 mM revealed maximum inhibitory effect of DPP4 activity by 30.2% and 42.9% respectively.

**Keywords:** Dipeptidylpeptidase-4, Inhibitors, Gel filtration, High performance liquid chromatography.

### دراسة كيموحيوية لإنزيم الداى ببتيدايل ببتيديز-4 من مصل دم شخص طبيعي

#### الملخص

تضمن البحث تنقية انزيم الداى ببتيدايل ببتيديز-4 من مصل دم شخص طبيعي بعمر 14 سنة من مركز مدينة الموصل. باستخدام تقنية الترشيح الهلامي تم الحصول على حزمتين بروتينيتين رئيسيتين للراسب الناتج من الترسيب بكبريتات الامونيوم المشبع ونسبة 50% والمطبق عليه عملية الديلة. اظهرت الحزمة البروتينية A فعالية عالية لإنزيم الداى ببتيدايل ببتيديز-4 وقدر الوزن الجزيئي التقريبي لها وكان بحدود 176.6 كيلو دالتون. استخدمت تقنية كروماتوغرافي السائل عالي الاداء على الحزمة  $\tilde{A}$  وكان زمن الاحتباس 5.829 دقيقة. بعد ذلك تم تحديد الظروف المثلى لعمل الإنزيم المنقى جزئياً وظهرت النتائج ان الإنزيم اعطى اعلى فعالية باستخدام المحلول المنظم Tris HCl بتركيز 0.1 مولاري وعند الاس الهيدروجيني 8 عند درجة حرارة 40 درجة مئوية وتركيز 4 ملي مولار من مادة كلايسين -برولين-بارانتروانيليد هيدروكلوريد بوصفها مادة اساس. أستخدم التركيز 0.35 ملغم بروتين/مل كمصدر للإنزيم للتجارب الاخرى. وبتطبيق علاقة لاين ويفر-برك وجد ان السرعة القصوى ( $V_{max}$ ) التي يعمل بها الانزيم تساوي 50 وحدة انزيمية/لتر وان قيمة ثابت ميكلس- منتن ( $K_m$ ) كانت 0.5 ملي مولار. كذلك اظهرت النتائج ان هناك مركبات كيميائية لها تأثير تثبيطي لفعالية الإنزيم وهي كلوريد الزئبقيك وكلوريد السترونتيوم سداسي الماء بتركيز 5 ملي مولار بنسبة (30.2%, 42.9%) على التوالي.

**الكلمات الدالة:** داى ببتيدايل ببتيديز-4، المثبطات، الترشيح الهلامي، كروماتوغرافيا السائل عالي الاداء.

## INTRODUCTION

Dipeptidyl peptidase-4 (DPP-4) EC 3.4.14.5 is a protein that has a multiple functions in the body. It is known under different names depending on its location in the body. When DPP-4 is on the surface of the T- cell (lymphocyte), it is called cluster of differentiation antigen 26(CD26), which travels in the circulation and helps immune functions (Gomez *et al.*, 2013). While when it is found on the mucosal membrane lining the intestinal tract, it is known as (DPP-4). This protein is also located on the surface tissues of the pancreatic duct, bile duct, colon, and kidney (Panchapakesan *et al.*, 2013). Another name for DPP-4 is adenosine deaminase binding protein or adenosine deaminase complexing protein-2 (Prabavathy *et al.*, 2011). The protein is an intrinsic membrane glycoprotein that possessed a unique enzymatic activity which cleaved N-terminal dipeptides from many biologically active peptides, cytokines and chemokines (Detel *et al.*, 2012). The enzyme is a member of the large family of proteases (peptidases) (Edosada *et al.*, 2006). Proteases proposed as one of the key factors in the occurrence of inflammatory processes due to their ability to metabolize different biologically active molecules implicated in maintaining the integrity of mucosal barrier (Detel *et al.*, 2007). It is presented in a soluble form which circulates in body fluids of living organisms with a specific peptidase function (Panchapakesan *et al.*, 2013). It cleaves dipeptides from the N-terminus of polypeptides having proline or alanine at the penultimate position (Vanderheyden *et al.*, 2009). Since proteins containing proline in the second position of N-terminal are not easily metabolized by other protease, the action of DPP-4 is an essential step in the degradation of many polypeptides (Matteucci and Giampietro, 2009). This enzyme is unique, it specifically breaks a proline containing peptides, including casomorphin and gluteomorphin, which are generally resistant to being completely broken down by other enzymes (Iwan *et al.*, 2008).

The enzyme DPP-4 has an important role in the processing of polypeptides and protein intestinal assimilation, renal handling of proline containing peptides, adhesion and modulation of immune reactivity (West *et al.*, 2000). The enzyme appears to work as a suppressor in the development of cancers (Wesley *et al.*, 2004). Since DPP-4 plays an important role not only in tumor biology, regulation of the endocrine system, immune system and gastrointestinal system, but also in regulation of the nervous system (Gupta *et al.*, 2009). Besides being a useful marker for early diagnosis of diabetic nephropathy and tumor burden (Yu *et al.*, 2010), DPP-4 has roles in many biological processes including pancreatic cancer and rheumatoid arthritis (Ongen *et al.*, 2012). Several peptides have been identified as DPP-4 substrates. These substrates include substance-P (*in vivo* substrate) a potent inflammatory mediator, neuropeptides, chemokines and the incretin hormones such as glucose dependent insulin tropic polypeptide (GIP) and glucagon like peptide-1 (GLP-1) (Baticic *et al.*, 2011). Inhibition of plasma DPP-4 enzyme leads to enhance endogenous GLP-1 and GIP activity which ultimately results in the potentiation of insulin secretion by pancreatic  $\beta$ -cells and subsequent lowering blood glucose levels, glucagon secretion and liver glucose production (Gupta *et al.*, 2009).

**The aim of the research** is to isolate and finding the optimum conditions of dipeptidyl peptidase-4 for its important role in glucose homeostasis and breakdown of casomorphin and gluteomorphin which are considered as opioid compounds.

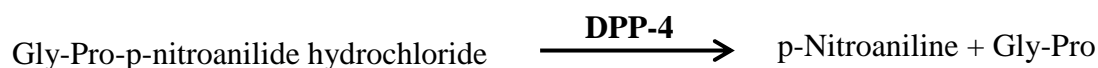
## MATERIALS AND METHODS

### -Serum sample

Fasting human serum 40 mL was obtained from normal male person aging 14 years living in Mosul city.

### -Enzymatic activity assay

The proteolytic activity was determined by p-nitro aniline as a released product (Edosada *et al.*, 2006) as shown below:



Twenty microliter of enzyme source was added to 170  $\mu$ L of Tris HCl buffer (pH 8). The mixture was incubated with 10  $\mu$ L of glycine proline-p-nitroanilide hydrochloride as a chromogenic substrate from Sigma–Aldrich Chemicals-Germany (G0513) at 2 mM for 1 hr at 37 °C. The reaction was stopped by 800  $\mu$ L of sodium acetate buffer. The absorbance was recorded at 405 nm (Kreisel *et al.*,1982).

Enzymatic unit (U) was defined as the amount of enzyme required to release 1 $\mu$ mol of product(p-Nitroaniline) in one minute.

To estimate the optimum enzymatic activity, a standard curve was constructed by plotting the absorbance mean against different concentrations of stock solutions of p-nitroaniline at 405 nm.

#### **- Protein estimation**

Protein concentration was estimated by the method of modified Lowry (Schacterle and Pollack, 1973) where bovine serum albumin was used as a standard protein.

#### **- Partial purification of serum DPP-4**

Dipeptidylpeptidase-4 was partially purified from a serum of fasting normal human by the following steps:-

##### **1- Precipitation**

The protein from 40 mL serum was precipitated using 50% ammonium sulphate (Robyt and White, 1987). The mixture was left over night at 4 °C. The proteinous precipitate was isolated using cooling centrifuge for 45min at 10000 rpm.

##### **2- Dialysis**

The precipitate was dialyzed against 0.1M ammonium bicarbonate. The solution was stirred slowly with a magnetic stirrer over night at 4 °C. The buffer was changed three times during dialysis (Robyt and White, 1987). The protein concentration and enzymatic activity were performed.

##### **3- Gel filtration chromatography**

The dialysate 2 mL was applied to gel filtration column (2.2 x 100 cm) which contained sephadex G-100 to a height (90 cm). Elution was carried out using phosphate buffer 0.1 M, pH 7.4 and the fractions were collected at a flow rate (38.4 mL/hr) with a definite time 5 min by automatic fraction collector L C100, Haake Buchler Instruments, USA.

The proteinous compounds were detected by following their absorbance at 280nm using UV/Visible spectrophotometer PD-303UV. The DPP-4 activity was estimated in each fraction.

##### **4- Molecular weight determination**

Sephadex G-100was used to determine the approximate molecular weight of DPP-4 using the following proteins as standard materials: phosphatase (140000 Da), hexokinase (100000 Da), bovine serum albumin (67000 Da), egg albumin (45000 Da), papain (23000 Da).

#### **- High Performance Liquid Chromatography**

The top fraction of peak **A** which was separated by gel filtration was dissolved in 1mL of 0.1% trifloroacetic acid and 90  $\mu$ L of the mixture then injected into C<sub>18</sub>, 4.6X250 mm, stainless steel column.

The analysis was performed using Shimadzu corporation DGU-20A5, prominenceLC-20AD-LiquidChromatographyin AL-Kindy State Company/Chemical Analysis Lab in Mosul city.

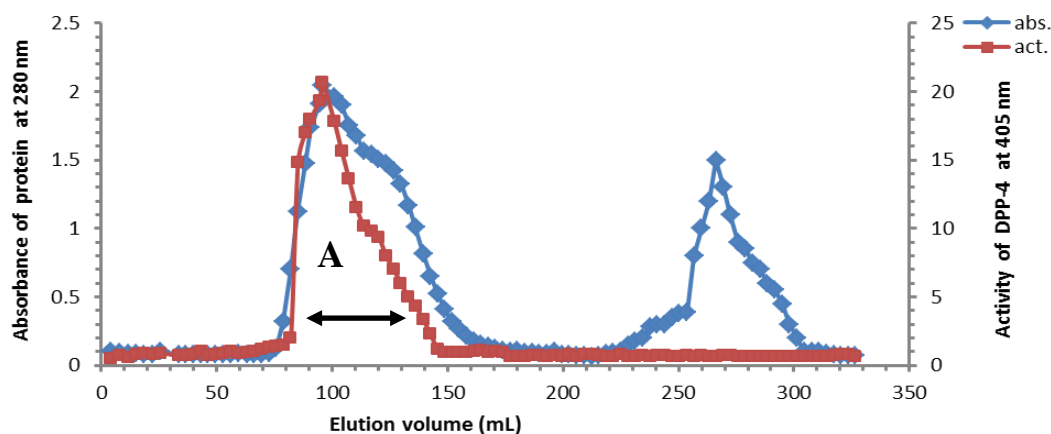
#### **-The optimal conditions of partial purified DPP-4**

Dipeptidylpeptidase-4 was characterized with respect to its optimum temperature, buffer concentration, pH of Tris HCl buffer, substrate concentration, effect of different concentrations of some DPP-4 inhibitors.

## RESULTS AND DISCUSSION

### I. Isolation and partial purification of DPP-4 from human serum

As mentioned in experimental part, many techniques were used to purify normal human serum DPP-4. Gel filtration chromatography was used to isolate and to find the molecular weight of normal human serum DPP-4. The results showed that there were two proteinous peaks detected at 280 nm. The dipeptidyl peptidase-4 activity was found in the first peak **A** as the major peak at elution volume 95.8 mL as indicated in Figure 1.



**Fig. 1: Elution profile of DPP-4 purified by gel filtration. The dimensions of the column are (2.2 × 90 cm).**

Table 1 indicates the purification steps of dipeptidyl peptidase-4. The results show that the specific activity of normal human serum DPP-4 and the increment was about four folds. These results were in agreement with previous studies (Duke-Cohan *et al.*, 2001) which showed a significant increase of DPP-4 activity.

**Table 1: Purification steps of DPP-4 from human serum**

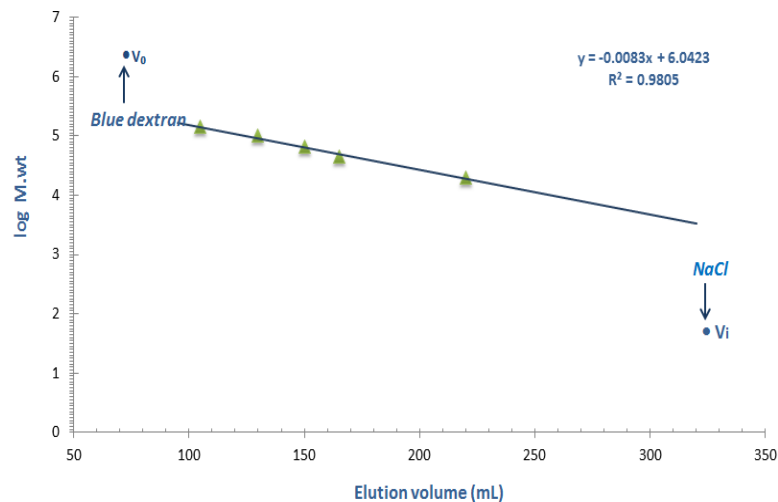
Purification steps	Volume (mL)	Total protein (mg)	Total activity U*	Specific activity U/mg protein	Fold of purification	% Recovery
Crude Serum	40	3920	4466	1.14	1	100
Precipitate (50% ) amm.sulphate	12	948	2256	2.38	2.1	50.5
Gel filtration (peak A)	26	351	1745	5.0	4.38	39.1

U\*: Enzymatic unit which was defined as the amount of enzyme required to release 1  $\mu$ mol of product(p-Nitroaniline) in one minute.

### II. Molecular weight determination of DPP-4 by gel filtration

The apparent molecular weight of DPP-4 (peak **A**) which was separated by gel filtration was determined from the calibration curve (Fig. 2). It has been found that the apparent molecular weight of DPP-4 was 176.6 KD. This result is in agreement with other reported studies (Iwaki-Egawa *et*

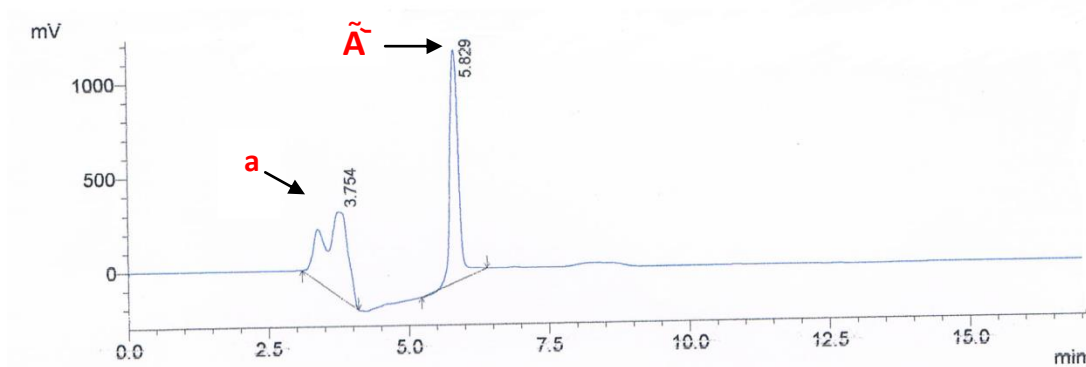
*al.*, 1998; Duke-Cohan *et al.*, 2001) where they found that molecular weight of normal human serum DPP-4 was 175 KD.



**Fig. 2: A standard curve for molecular weight determination of DPP-4 using G-100 sephadex.**

### III. High performance liquid chromatography

The top of peak **A** was applied to HPLC technique, a single peak ( $\tilde{A}$ ) which appeared at retention time of (5.829 min) was obtained with flow rate 0.7 mL/min. The minor peak (a) represents the elution of the solvent trifluoroacetic acid.

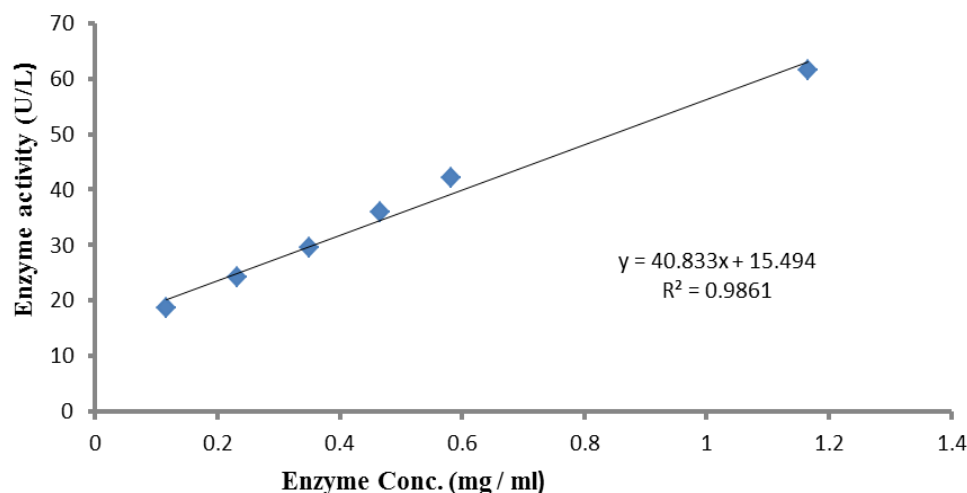


**Fig. 3: Chromatogram of top of the peak with HPLC, **a** the solvent trifluoroacetic acid,  $\tilde{A}$  the DPP-4.**

### IV. Kinetic study

#### -Effect of enzyme concentration on DPP-4 activity

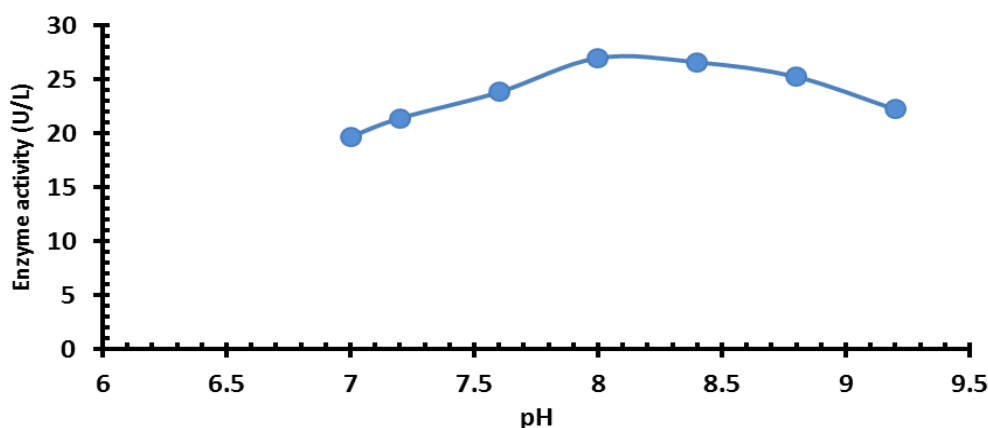
The activity of enzyme was measured in the presence of different concentrations of partially purified enzyme peak **A**, ranged 0.1-1.2 mg/mL as shown in Fig. 4 and the concentration 0.35 mg/mL of enzyme was used for next experiments (Medium range value).



**Fig. 4: Effect of enzyme concentration on DPP-4 activity**

#### **-Effect of pH and conc. of buffer solution on DPP-4 activity**

The effect of pH upon DPP-4 activity was investigated using different pH of Tris-HCl buffer between 7.2-8.8. Maximum activity was obtained at pH 8.0 which is used for the following experiments. The different concentrations 0.05-0.2M of Tris-HCl pH 8 were examined. Maximum activity was detected at concentration 0.1M of Tris-HCl pH 8 as illustrated in Fig. (5) and (Table 2). The current results are in agreement with others (Durinx *et al.*, 2000; Davy *et al.*, 2000). However, other investigators found that DPP-4 exhibited its highest activity over the pH range 6.7-8.9 (Scharpe *et al.*, 1998) and it had no activity below pH 5 (Durinx *et al.*, 2000).



**Fig 5. Effect of pH on DPP-4 activity**

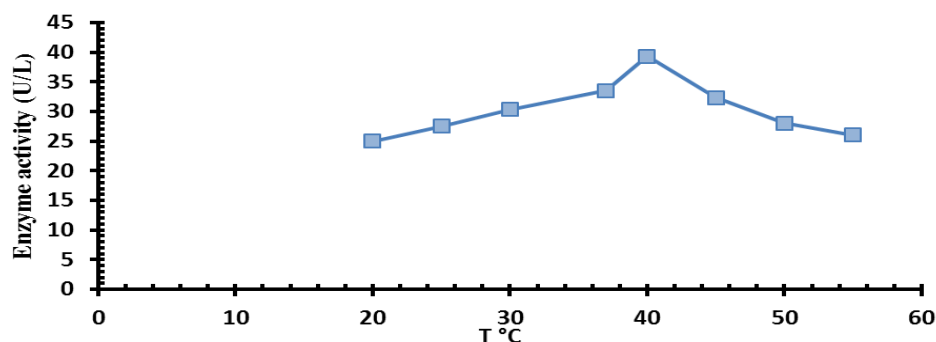
**Table 2: DPP-4 activity at different concentrations of Tris HCl at pH 8**

Concentration of Tris HCl (M), pH 8	DPP-4 activity (U*/L)
0.05	22.9
0.1	28.3
0.2	27.7

U\*: Enzymatic unit was defined as the amount of enzyme required to release 1 $\mu$ mol of product(p-Nitroaniline) in one minute.

#### **-Effect of temperature on DPP-4 activity**

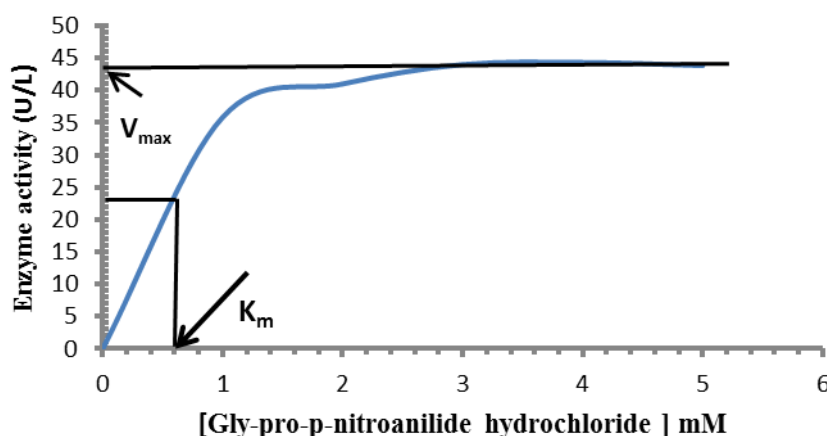
The effect of temperature on DPP-4 activity was assayed at a temp. ranging between 25-60 °C. The results showed a maximum activity of enzyme at 40 °C then dropped gradually until most of it was lost as indicated in the Fig. (6). The current results was similar to what was obtained by other investigators (Durinx *et al.*, 2000) where they found that optimum temperature for DPP-4 activity from normal human serum is 40 °C.



**Fig. 6: Effect of temp. on DPP-4 activity**

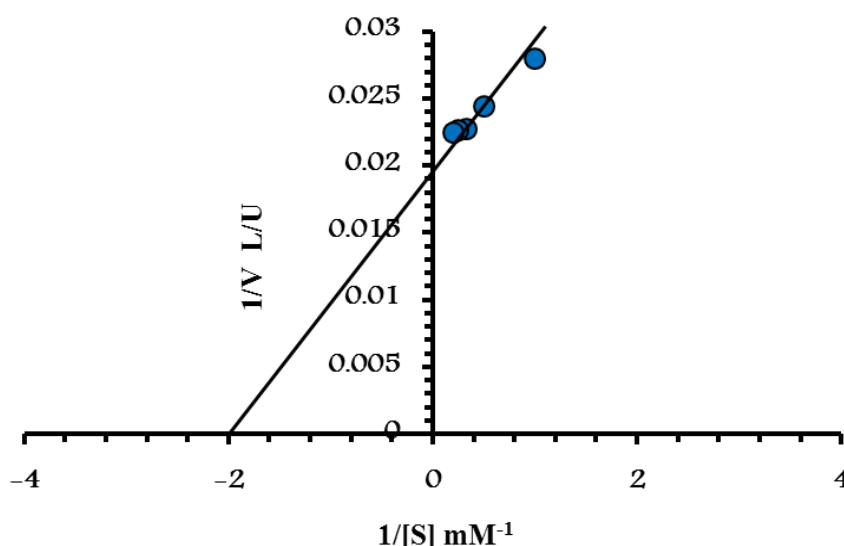
#### -Effect of substrate concentration

Different concentrations of gly-pro-p-nitroanilide as a substrate for DPP-4 were used ranging between (0-5 mM) and 4 mM gave a maximum activity of DPP-4.



**Fig. 7: Effect of gly-pro-p-nitroanilide conc. on DPP-4 activity**

A limiting value of substrate concentration of 0.5 mmol/L as shown in Figure 7 as  $K_m$  value. Line Waver-Burk plot (Fig. 8) revealed that the values of  $K_m$  and  $V_{max}$  were 0.5 mM and 50 U/L respectively. The current results are in agreement with reported studies (Pereira *et al.*, 2003) where they indicated that the values of  $K_m$  of DPP-4 are ranging between 0.43-0.98 mM in human connective tissues and with (Davy *et al.*, 2000) who revealed that the  $K_m$  value of barley DPP-4 was 0.59 mM.



**Fig 8. Lineweaver-Burk plot for DPP-4 activity**

#### **-Effect of certain compounds on DPP-4 activity**

Different conc. of mercuric chloride and strontium chloride hexahydrate on DPP-4 activity have been studied. The results in Table 3 and 4 showed that the activity was decreased with increasing their concentrations. The current results are in accord with others (Gomez *et al.*, 2013) who they studied the effect of divalent ions on DPP-4 of rat kidney and found that  $\text{Zn}^{+2}$  and  $\text{Ca}^{+2}$  inhibited the enzyme. While (Shibuya-Saruta *et al.*, 1996) explained activity inhibition of normal human serum DPP-4 by  $\text{Ba}^{+2}$ ,  $\text{Mn}^{+2}$ ,  $\text{Cd}^{+2}$ ,  $\text{Hg}^{+2}$ ,  $\text{Co}^{+2}$  and  $\text{Sr}^{+2}$ . So, environmental toxicants such as mercury and strontium have been found to strongly inhibit the activity of dipeptidyl peptidase (DPP-4) which are required in the digestion of the milk protein (casein) or wheat protein (gluten). The dipeptidyl peptidase-4 and cluster differentiation antigen 26 (CD26) are the same. It helps in T lymphocyte - activation. CD26 or DPP-4 is a cell surface glycoprotein that is very susceptible to inactivation by mercury binding to its cysteinyl domain (Shibuya-Saruta *et al.*, 1996).

**Table 3: Inhibition effect of mercuric chloride on DPP-4 activity**

Conc. of $\text{HgCl}_2$ (mM)	Activity (U*/L)	Inhibitory effect (%)
0	42.5	---
1	36.66	13.7
2	35.50	16.47
3	32	24.7
4	31.5	25.9
5	29.66	30.2

U\*: Enzymatic unit was defined as the amount of enzyme required to release 1 $\mu$ mol of product(p-Nitroaniline) in one minute.



**Table 4: Inhibition effect of strontium chloride hexahydrate on DPP-4 activity**

Conc. of $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ (mM)	Activity ( $\text{U}^*/\text{L}$ )	Inhibitory effect (%)
0	42.5	---
1	37.16	11.5
2	31.66	24.5
3	31.33	25.4
4	29.5	29.8
5	24	42.9

$\text{U}^*$ : Enzymatic unit was defined as the amount of enzyme required to release 1 $\mu\text{mol}$  of product(p-Nitraniline) in one minute.

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