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 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 (Vmax) was 50 μM according to Line Weaver-Burk plot while Michaelis–Menton constant (Km) 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 الداي ببتيدايل ببتيديز-4 من مصل دم شخص طبيعي

تتضمن البحث تنقيه إنزيم الداي 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 للراس الناتج من الترسيب بكميات الأمونيوم المشبع بنسبة 50% والمطبق عليه عملية الديلزة. ظهرت الحزمة البروتينية A فعالية عالية لإنزيم الداي ببتيدايل ببتيديز-4 وكان الوزن الجزيئي التقريبي لها كان حوالي 176.6 كيلو دلما. استخدمت تقنية كروماتوغرافية السائل عالي الاداء على الحزمة A و كان زمن الاحتباس 5.829 دقيقة. بعد ذلك تم تحديد الظروف المثلى لعمل الإنزيم المنقى جزئيا وأظهرت النتائج ان الإنزيم أعطي أعلى فعالية باستخدام المحلول المنظم Tris HCl بتركيز 0.1 مولاري عند الادس الهايدروجيني 8 عند درجة حرارة 40 درجة مئوية وتركيز 4 ملي مولار من مادة كلاريسين –برولين–باراتروينيل هيدروكلوريد بوصفها مادة أساس. استخدم التركيز 0.35 ملم بروتين/مل كمصدر للإنزيم للتجارب الأخرى. وتطبيق علاقة لاي وير-برك جد ان السرعة القصوى (Vmax) التي يعمل بها الإنزيم تساوي 50 وحدة إنزيمية/لتر/وحدة إنزيمية/لتر. كان نتيجة تأثير ميكسيك -منتن (Km) كانت 0.5 ملي مولار. كذلك أظهرت النتائج أن 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: داي ببتيدايل ببتيد-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 β-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:

\[ \text{Gly-Pro-p-nitroanilide hydrochloride} \xrightarrow{\text{DPP-4}} \text{p-Nitroaniline} + \text{Gly-Pro} \]
Twenty microliter of enzyme source was added to 170 µL of Tris HCl buffer (pH 8). The mixture was incubated with 10 µL of glycine proline-p-nitroanilide hydrochloride as a chromogenic substrate from Sigma–Aldrich Chemicals-Germany (G0513) at 2 mM for 1 hr at 37 ºC. The reaction was stopped by 800 µL 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 1µmol 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 ºC. 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 ºC. 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 µL of the mixture then injected into C18, 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 x 90 cm).](image)

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.

| 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% )       | 12          | 948                | 2256              | 2.38                          | 2.1                 | 50.5       |
| amm.sulphate             |             |                    |                   |                               |                     |            |
| 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 μmol 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
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al., 1998; Duke-Cohan et al., 2001) where they found that molecular weight of normal human serum DPP-4 was 175 KD.

III. High performance liquid chromatography

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

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).

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

Fig. 3: Chromatogram of top of the peak with HPLC, a the solvent triflouroacetic acid, Å the DPP-4.
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).

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 1μmol 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](image_url)

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](image_url)

A limiting value of substrate concentration of 0.5 mmol/L as shown in Figure 7 as Km value. Line Waver-Burk plot (Fig. 8) revealed that the values of Km and Vmax 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 Km 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 Km value of barley DPP-4 was 0.59 mM.
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 cysteinyil domain (Shibuya-Saruta et al., 1996).

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

| Conc. of $\text{HgCl}_2$(mM) | Activity ($\text{U/L}$) | 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                  |

$\text{U}^*$: Enzymatic unit was defined as the amount of enzyme required to release 1 μmol of product (p-Nitroaniline) in one minute.
Table 4: Inhibition effect of strontium chloride hexahydrate on DPP-4 activity

| Conc. of SrCl₂.6H₂O (mM) | Activity (U/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                  |

U*: Enzymatic unit was defined as the amount of enzyme required to release 1μmol of product(p-Nitraniiline) in one minute.

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