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Biochemical Study of Dipeptidyl Peptidase-4 in Autistic's Patients

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Abstract: The research includes estimation of autistics' serum dipeptidyl peptidase-4 (DPP-4) activity. The results indicated a significant ($p \leq 0.05$) decrease in (DPP-4) activity of autism spectrum disorder groups, 57.85, 42.86, 36.47 µmol/L respectively compared to control group. By statistical analysis, the study revealed a significant ($p \leq 0.05$) relationship between DPP-4 activity with gastrointestinal disorder on one hand and with various inflammation incidence on the other hand. Partial purification of DPP-4 from serum of normal person age 14 years in Mosul city was done. 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. Then SDS-PAGE was performed. HPLC revealed a single peak 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. Maximum velocity (Vmax) was 50 µM according to Line Weaver-Burk plot while Michaelis–Menten 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: Dipeptidyl peptidase 4, Autism spectrum disorder, Gel filtration, Electrophoresis, High performance liquid chromatography

Introduction

Autism spectrum disorder (ASD) can be classified into five types of neuro developmental disorders which have the same main symptoms and different severity. Autism spectrum disorder (ASD) was first described in (1943) by Leo Kanner (Evans et al., 2008) and officially recognized in (1980) (Volkmar and Pauls, 2003).

ASD is beginning before the age of three years. It is classified as pervasive disorders of childhood, characterized by behavioral abnormalities in social interactions, communication and restricted repetitive and stereotyped patterns of behaviors, interest and activities which are thought to be due to abnormal brain function or structure and are also thought to have genetic bases (Rose et al., 2012). Children with ASD often present an abnormal immune and digestive system such as inflammation of the bowel, changes in digestive enzymes and suffer from bowel problems such as constipation. The cause of these changes is not entirely known (Randolph-Gips and Srinivasan, 2012).

Under conditions of high levels of oxidative stress, opioids from casein and gluten may bind too tightly to receptors in the brain. In few words, the opioid-excess theory suggests that excessive levels of incompletely metabolized peptides from foods that contain proteins, gluten and casein, pass through the intestinal and blood brain barrier (BBB) into the brain (Shattock and Whitely, 2002).

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Dipeptidylpeptidase-IV (DPP-4) is the only known enzyme to break down small protein fractions from foods called peptides. The inability to break down certain peptides from gluten and casein leads to elevation of certain potentially neurotoxic peptides that formed in the gut like casomorphin, gliadorphin, dermorphin, deltorphin. These in turn are absorbed and may adversely affect neurological and immune functions. The enzyme (DPP-4), which is CD26 on T-lymphocytes, also appears on the surface of certain immune cells and serves to signal the cell into activity. When this enzyme is dysfunctional, the immune system is compromised (Shaw, 2008).

Dipeptidylpeptidase-IV (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 CD26, which travels in the circulation and helps support immune functions (Lad, 2007). While when this enzyme 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). Adenosine deaminase binding protein another name for DPP-4 or adenosine deaminase complexing protein-2 (Prabavathy et al., 2011).

In human, DPP-4 is encoded by the DPP-4 gene and that gene is associated with immune regulation, signal transduction and apoptosis. DPP-4 gene is located on chromosome 2q23 and spanning 70 kb. It is composed of 26 exon that encode a protein of 766 amino acids (King 2012). It is intrinsic membrane glycoprotein which possesses a unique enzymatic activity that can cleave N-terminal dipeptides from many biologically active peptides, cytokines and chemokine (Detel et al., 2012).

The enzyme DPP-4 is a member of the large family of proteases (peptidases)(Edosada et al., 2006). 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).

Methods

This study included (67) subjects (controls and autism spectrum disorder patient. patients group included (37) cases of both sexes (6 females, 31 males) aging 2-12 years with autism spectrum disorder recruited from psychiatric research unit in Medicine College - Mosul University from January to April 2012. While control group included (30) healthy subjects of both sexes (10 females, 20 males) with the same age of patients.To estimate dipeptidyl peptidase-4, fasting blood samples were collected at (8:00-8:30 am) from the same previous groups (patients & control) from September 2012 to January 2013.

The diagnosis of autism spectrum disorder was applied by specialist psychiatric doctors, and the criteria of ASD are defined in the Diagnostic and Statistical Manuals of Mental Disorder, Fourth Edition (DSM-IV, 1994).
The patients group is divided into three subgroups: mild, moderate and severe according to symptoms severity grade and PDD scale. If the scores > 50 it is considered normal, between 50-100 it mild, between 100-150 it moderate and < 150 is considered as severe ASD (AL-Hayaly, 2010).

Venous blood samples (3-5 mL) were collected from (control and ASD) groups in clean dry plain tubes without anticoagulant and incubated in water bath at 37○C for 10 minutes to allow the blood to clot. Centrifugation was then done at (3000 rpm) for (15 mins). Serum samples were transferred immediately by micropipette and stored at -20○C to be analyzed later.

Also, serum sample (40 mL) was obtained from fasting normal human volunteer aging 14 years for dipeptidyl peptidase-4 activity study. The study included separation, partial purification and optimum conditions.

Assay of serum DPP-4 activity

Serum DPP-4 activity was estimated by measuring the release of light yellow product of 4-nitro aniline from an assay mixture (Kreisel *et al*., 1982). Glycine-proline-p-nitroanilide hydrochloride was used as a substrate *in vitro*.

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.

Enzyme Purification

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

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.

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.

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.

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).

Sodium Dodecyl Sulphate Gel Electrophoresis

Protein sample, Top peak A, which was separated by gel filtration technique of normal human serum and prepared previousely, applied on SDS-Electrophoresis.

High Performance Liquid Chromatography

The top fraction of peak A which was separated by gel filtration injected into C18, 4.6X250 mm, stainless steel column. The analysis was performed using Shimadzu corporation DGU-20A5, prominenceLC 20AD LiquidChromatography in 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

Figure 1. demonstrated the reduction percentage of enzymatic activity of DPP-4 in mild, moderate and severe autism spectrum disorder (57.85±2.35, 42.86±0.7, 35.47±0.94) µmol/L compared to control group (68.23 ± 3.6) µmol/L. The percentage of serum DPP-4 reduction was (48%) in severe ASD group compared to controls.

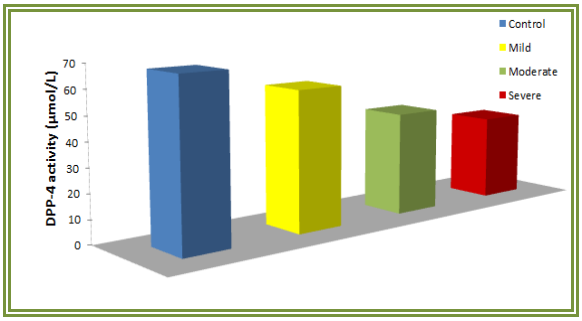

Figure 1. Serum dipeptidyl peptidase-4 activity in mild, moderate and severe ASD patients groups compared to control group.

The present study revealed a significant ($p<0.05$) relationship between DPP-4 activity and gastrointestinal disorders which occurred in ASD patients. Data revealed that the GI disorders incidence among ASD patients was (69.7%) as indicated in Table (1). Also, the results showed the percentage of various inflammations (87.9 %) occurred in ASD patients compared to controls.

Table 1:- Relationship between DPP-4 activity and some pathological disorders which occurred in ASD patients and % of disorder incidence.

Relationship between DPP-4 and pathological disorders	P value	% incidence
DPP-4 & inflammation	0.0001*	87.9
DPP-4 & gastrointestindisorders	0.035*	69.7

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 2.

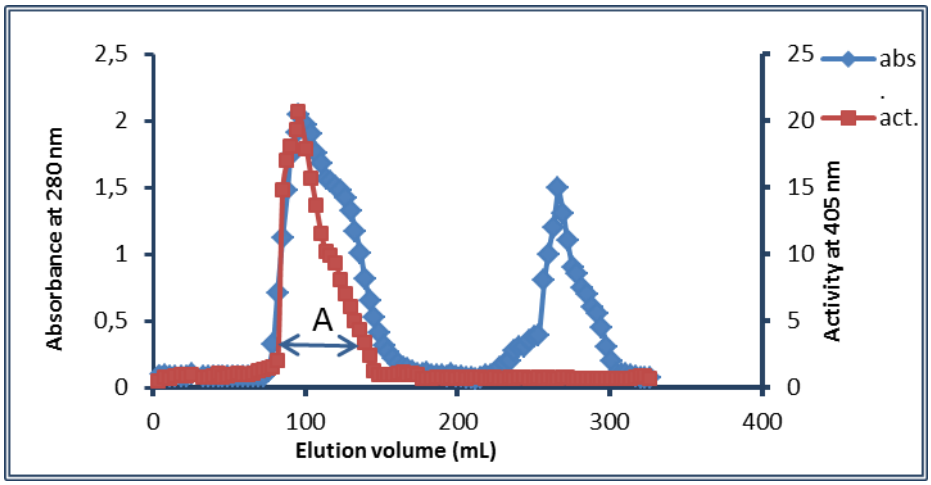

Figure 2. Elution profile of DPP-4 purified by ammonium sulphate precipitation (50%) on gel filtration column (2.2 × 90 cm). A represents the enzyme source collections

While Table 2 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 2. Purification steps of DPP-4 from human serum

Purification steps	Volume (mL)	Total protein (mg)	Total activity (µmol/L)	Specific activity	Fold of purification	% Recovery
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

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.

The top of peak **A** was applied to HPLC technique, a single peak (**Ã**) 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 as indicated in Figure 3.

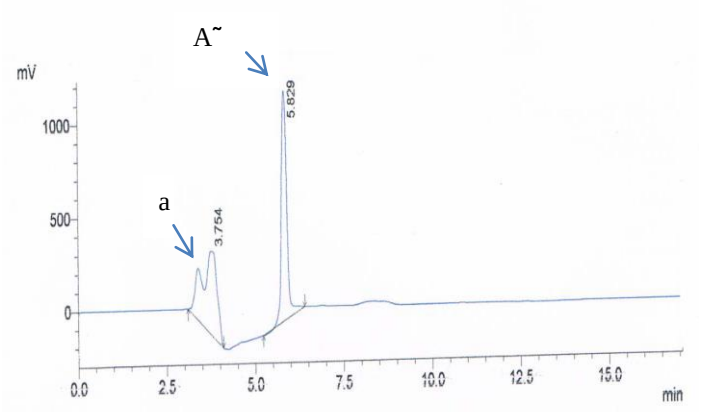

Figure 3. Chromatogram of top of the peak which was isolated from gel filtration, peak a represents the solvent triflouroacetic acid while peak Ã represents DPP-4

In this study, the top of peak A from sephadex G-100 gel filtration chromatography of normal human serum dipeptidyl peptidase-4 as indicated in Figure 2 was used in SDS-PAGE. Two bands were detected after staining and destaining with coomassie blue as shown in Figure 4 This result might indicate that DPP-4 has two

subunits. Similar results were published in the literature where DPP-4 has two subunits in SDS-PAGE (Shibuya-Saruta et al., 1996 ; Davy et al., 2000 ; Sanz and Toldra, 2001).

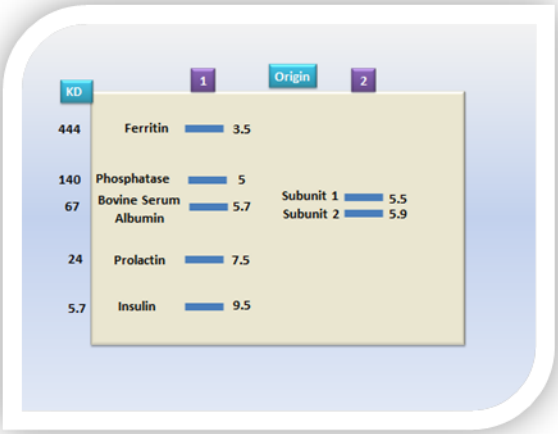

Figure 4: Representative profile of SDS-PAGE of the top peak A from sephadex G-100 of normal human serum (lane 2) compared to known materials (Lane 1). The numbers on the right hand represented relative mobility in(cm) of each material from the origin.

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. 5 and the concentration 0.35 mg/mL of enzyme was used for next experiments (Medium range value).

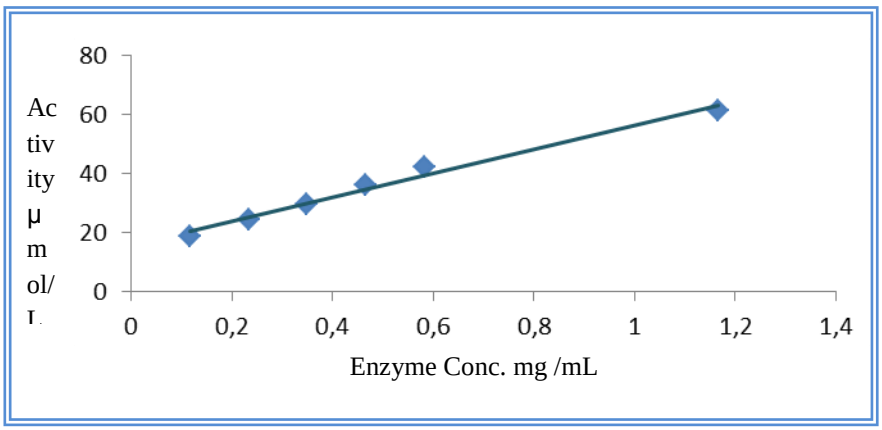

Figure 5. Effect of different concentrations of enzyme on DPP-4 activity

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. (6) and (Table 3). 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).

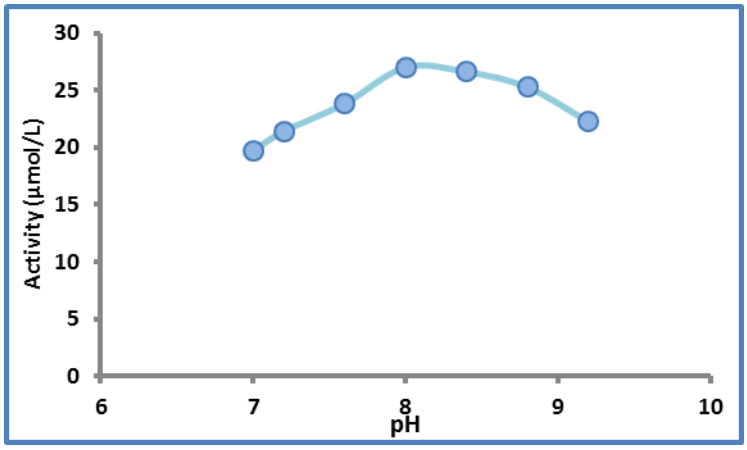

Figure 6. Effect of different pH on DPP-4 activity

Table 3: DPP-4 activity at different concentrations of Tris HCl at pH 8

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

Effect of temperature on DPP-4 activity

The effect of temperature on DPP-4 activity was assayed at a temp. ranging between 25-60 ºC. 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. (7). 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 ºC.

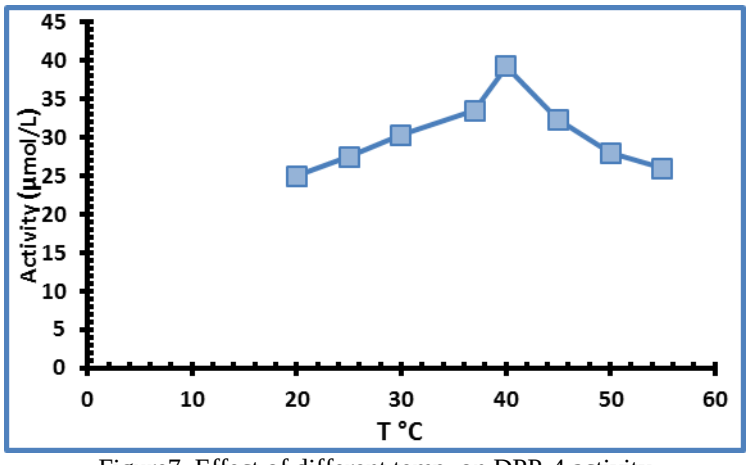

Figure7. Effect of different temp. on DPP-4 activity

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 as indicated in Figure 8.

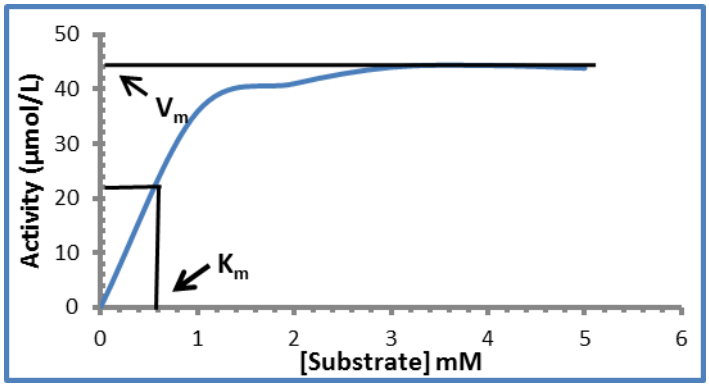

Figure 8. Effect of different concentrations of substrate on DPP-4 activity

A limiting value of substrate concentration of 0.5 mmol/L as shown in Figure 8 as Km value. Line Waver-Burk plot (Fig. 9) revealed that the values of Km and Vmax were 0.5 mM and 50 U/Lrespectively. 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.

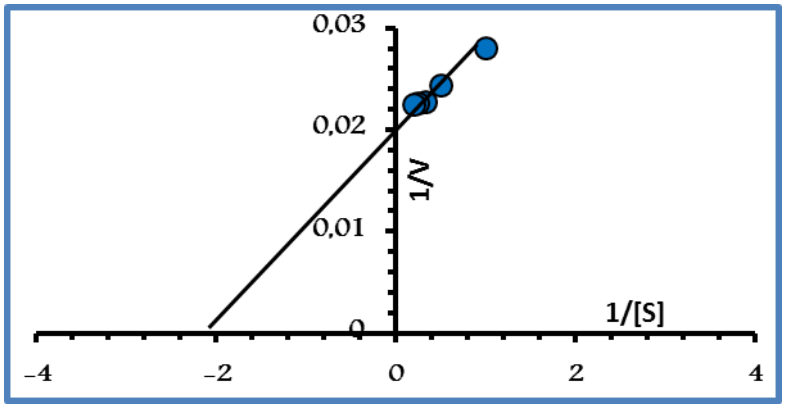

Figure 9. Lineweaver-Burk plot for DPP-4 activity

Table 4. The optimum conditions of DPP-4 activity

M DPP-4	enzyme conc. (mg/mL)	Tris HCl buffer conc. (M)	pH of Tris HCl buffer	Temp. (°C)	Km (mM)	Vmax (µM)
Peak A	0.35	0.1	8	40	0.5	50

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 5 and 6 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 Zn+2 and Ca+2 inhibited the enzyme. While (Shibuya-Saruta et al., 1996) explained activity inhibition of normal human serum DPP-4 by Ba+2, Mn+2, Cd+2, Hg+2, Co+2 and 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 5. Effect of mercuric chloride on DPP-4 activity

Concentration mM	Activity µmol/L	%Inhibition
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

Table 6. Effect of strontium chloride hexahydrate on DPP-4 activity

Concentration mM	Activity µmol/L	%Inhibition
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

Conclusion

Dipeptidyl peptidase4 activity decreases in autistics' patients. Also, DPP-4 has a significant relationship with gastrointestinal disorders and different inflammations. 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.

Recommendations

Genetic mutations of *DPP-4*, in autistic children must be studied since mutation in this enzymes might be involved in ASD.

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