Estimation of 5`-nucleotidase activity from saliva of diabetic patients with partial purification of its isoenzymes

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Abstract:

This study was performed on (60) saliva specimens, including (30) diabetic patients in addition (30) healthy saliva specimens were taken from healthy subjects as control group. The results showed a significant increase in the activity of saliva 5`-nucleotidase (5`-NT) from diabetic patients compared to healthy subjects.

This study included a partial purification of 5`-NT as well, using (35%) $(NH_4)_2SO_4$ for precipitation and gel filteration by (sephadex G-75) which gave single peak. Then 5-NT was partially also purified by using Ion exchange chromatography (DEAE-cellulos A-50), in which three isoenzymes were isolated with different degree of purity.

Introduction:

5`-nucleotidase (5`-NT) is a membrane glycoprotein enzyme that is found as a widest scope in mammalian cells[(1)], animals, plants , parasites and in human body[(2)].

5`-nucleotidase or nucleoside mono phosphate phosphohydrolases (EC 3.1.3.5) is an enzyme catalyzing the hydrolytic dephosphorylation of nucleoside monophosphate to nucleoside and orthophosphate [AMP + $H_2O \rightarrow$Nucleoside + $PO_4^{-3}$][(3,4)]. The prescence of 5`-NT in normal human erythrocytes is highly specific with pyrimidine and not purine nucleotide as substrate. The physiological role of nucleotidase is not well understood but may include the provision of nucleoside for cellular uptake and the enzyme may play a modulating role in ATP and ADP regulatory action at the cell surface[(5)] , and it participates (as catabolic enzyme) in the regulation of nucleotide level in living cells [(6,7)].

5`-NT is associated with rebuilding DNA (DNA turn-over) , multiply in bioprocess pathway of pyrimidine and purine[(8)]. There are two types of 5`-NT isoenzymes; cytoplasmic isoenzyme (soluble in cytoplasma) and associated with wall called (Ecto-5-Nucleotidase) related to outer surface of the cell. The exoenzyme associated with metabolic process of nucleotides[(4)], while cytoplasmic isoenzyme associated with adenosine tri phosphate (ATP) hydrolysis in cell to adenosine or IMP to inosine ,therefore cytoplasmic enzyme regulates hydrolysis of inner nucleotides[(9)].

5`-NT isoenzymes were purified from several sources such as rat liver, in which two isoenzymes have been isolated[(10)] ,and many isoenzymes have been isolated from kidney rat and mouse by electrophoresis[(11)] . Two 5`-NT isoenzymes separated and purified from human serum[(12)] and from liver sheep[(13)] , while Al-Salihi *et al*, (2009) purified three isoenzymes from serum of anemic patients[(14)].

5`-NT has a great clinical importance clinically and remedially, Its significance is considered for a lot of morbidity cases. So the a available information about 5`-NT is useful to diagnose or to treat the disease. The use of saliva as a diagnostic tool for a lot of diseases has been expanded , this study aimed to estimate the activity of 5`-NT in saliva of normal and pathological conditions such as diabetic patients in order to evaluate its diagnostic significance, and to partial purification of 5`-NT isoenzymes from saliva of diabetic patients.

Materials & Methods

A study was conducted taking saliva of diabetic patients, cases subjects were divided into two groups ; case group I , it included (30) samples from diabetic patients at (14-80) years age . Cases group II (control) , it included (30) samples from healthy subject at (18-70)years age.

Enzyme assay: 5`-NT activity was measured according to Fisk and Sobbarow method[(15)], using inorganic phosphate as standard. 5`-NT activity is defined as µmol inorganic phosphate that produced from substrste 5`-AMP by Fisk and Sobbarow reagent.

Estimation of protein: Protein contents were estimated by the method of lowry[(16)] with bovine serum albumin (BSA) as standard.

Enzyme purification: The purification of 5`-NT included several steps described as below:

- **Ammonium sulphate option**: (35)% Ammonium sulphate was added to the saliva (salting out) and the solution centrifuged at (3500rpm). The pellet was suspended in (3.5)ml of (50)mM Tris-HCl buffer (pH 7.2) and dialysed against the same buffer for overnight with continues changing of the buffer.
- **Gel filtration on sephadex G-75** : (1.5)ml from isolated 5`-NT above was loaded on sephadex G-75 (30 × 2cm) , which was swollen in (50)mM Tris-HCl buffer (pH 7.2 ), and the 5`-NT was eluted with (50) mM Tris-Hcl buffer pH 7.2, and collected into different fractions (5)ml.
- **Ion Exchange Chromatography**: (2.5)ml eluant from gel filtration column above was loaded on DEAE Cellulose A-50 column (ion exchange chromatography) (30 × 1.5)cm , which was swollen in (50)mM Tris-HCl pH 7.2 with using NaCl gradient (0.02-0.4)M , Eech fraction collected (10) ml.

Statistical analysis : Values were calculated as mean±SD and the statistical analysis was done using SPSS 17.0 software. Student unpaired t-test was used for comparison between two groups. The p-value of

less than 0.05 was considered as statistically significant.

Results and Discussion

In routine clinical analysis , 5`-NT activity has been measured in serum ,which was increased in hepatobiliary diseases and malignancy. Here in this study, saliva was used as a diagnostic tool to estimate 5`-NT activity level in diabetic patients. The results showed a significant increase in the activity of 5`-NT in saliva of diabetic patients compared to control , as shown in Table (1). Results of this study were consistent with Nagendra *et al.*, 2014, in which enzyme activity was elevated with increased blood sugar level[17] .

Table (1) :Salivary 5`-NT activity levels & glucose concentration in blood of diabetic patients

State	No.	Glucose conc. (mg/dl) Mean ± SD	5`-NT (IU/L) Mean ± SD	P value
Control	30	90.77 ± 8.2	122.05 ± 47.911	0.05
Diabetic patient	30	288.57 ± 46.933	199.981 ± 49.1	

Table (2) shows a statically significant increase ($p \leq 0.05$) in 5`-NT activity of male or female diabetic patients compared to healthy control , and the results revealed a marked increase in 5`-NT activity in male compared to female diabetic patients as well,(Fig.1) .

Table (2) : Salivary 5`-NT activity in females and males diabetic patients

State	Male	Female
	5`-NT (IU/L) Mean ± SD	5`-NT (IU/L) Mean ± SD
Control	120.8 ± 45.11 (NO.13)	118.134 ± 51.574 (NO.17)
Diabetic patient	208.78 ± 40.64 (NO.13)	185.152 ± 54.11 (NO.17)
P value	0.05	0.05

5`-NT catalyzes the hydrolytic dephosphorylation of nucleoside monophosphates. As catabolic enzyme which contributes significantly to the regulation of cellular nucleotide levels; so misregulation of nucleotide metabolism and nucleotidase defect were associated with a number of disease[3]. Increased 5`-NT activity in diabetic patients may be caused also by oxidative stress due to increased glucose level and decreased antioxidant levels [18].

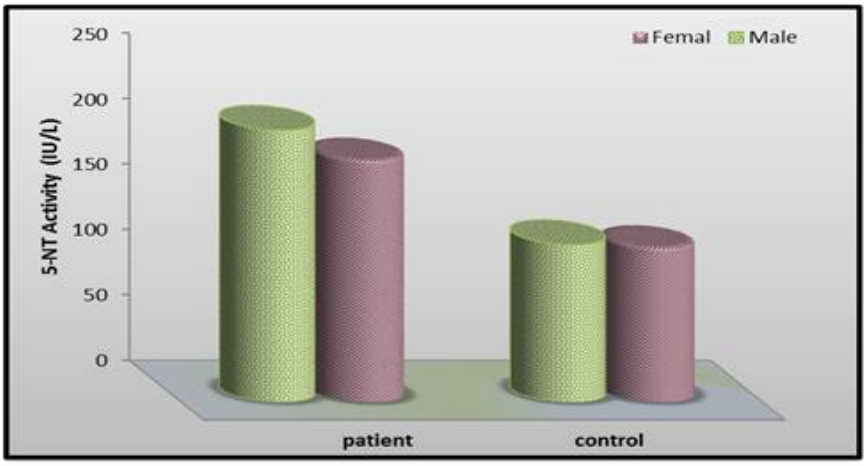

Figure (1) : Salivary 5`-NT activity in diabetic patients

5`-NT is implicated in inflammatory condition as well as micro and macro vascular complications of diabetes mellitus. Adenosine mimics the action of insulin on glucose and lipid metabolism , while it inhibits the insulin effect on total hepatic glucose output suggesting that adenosine causes local insulin resistance in liver tissue[17].

Diabetes mellitus leads to many changes and complication on liver concerning its function and shape. Liver has great role on diabetes mellitus for its important role in metabolism carbohydrates, and It is known that 5`-NT is considered as a diagnostic enzyme for liver diseases and that is in concomitant with the results of this study[19,20,21].

Partial purification of 5`-NT isoenzymes from diabetic's saliva:

In an attempt to more completely understanding the nature of salivary 5`-NT, this enzyme was partially purified from saliva of diabetic patients by different steps ,as shown in table (3), which includes option

with ammonium sulphate , dialysis and gel filteration chromatography with sephadex G-75 column and single peak was obtained with purification degree of (2.98) folds , as shown in figure (2). Then 5`-NT was further fractionated into its isoenzymes by using ion-exchange chromatography (DEAE-Cellulose A-50 column) with NaCl-gradient, in which three isoenzymes with various degrees of purity were obtained as shown in figure(3).

Table (3) : Steps of 5`-NT purification from diabetic patient's saliva

State	Elute (ml)	5`-NT activity (IU/L)	Protein conc. (mg/l)	Total Recovery		Specific activity (IU/mg)	Purification (fold)	Yield (%)
				5`-NT activity (IU)	Protein conc (mg)			
Crud	5	389.67	801.215	1.45	4.01	0.49	-	100
$(NH_4)_2SO_4$	4.5	240.713	326.136	1.1	1.47	0.74	1.5	75.9
Dialysis	3.5	277	273.669	0.97	0.96	1.01	2.1	66.9
Gel filtration	5	183.08	125.669	0.92	0.63	1.46	2.98	63.45
Ion Exchange Isoenzyme –I	10	85.3	54.654	0.853	0.55	1.561	3.19	58.83
Isoenzyme –II	10	78.1533	43.44	0.782	0.434	1.799	3.7	53.9
Isoenzyme –III	10	70.33	32.354	0.7033	0.324	2.174	4.44	48.5

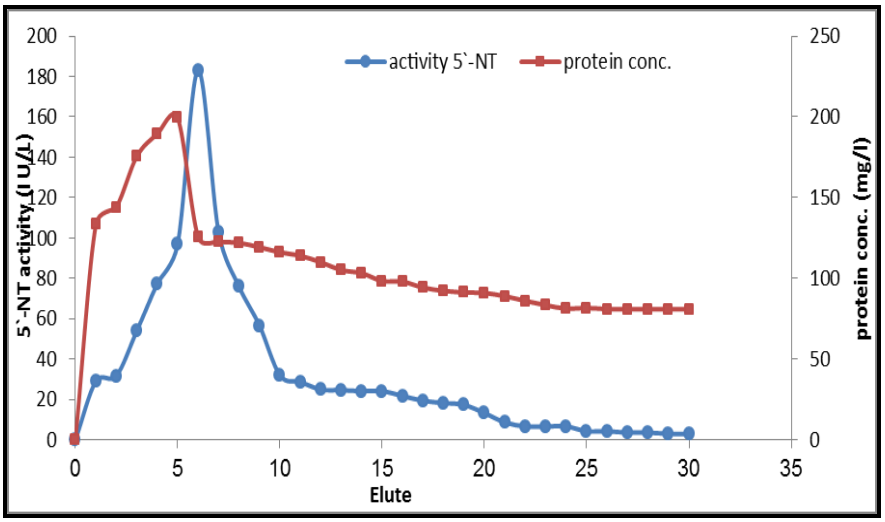

Figure (2) : purification of 5`-NT from diabetic patient's saliva by gel filtration using (sephadex G-75)

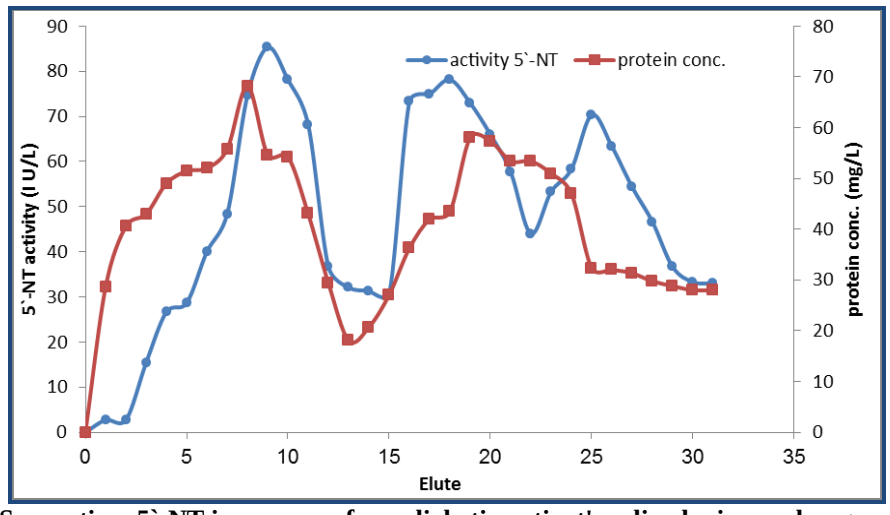

Figure (3) : Separation 5`-NT isoenzymes from diabetic patient's saliva by ion exchange using (DEAE-Cellulose A-50)

The most important problems facing the researchers to study and purify 5`-NT from saliva are associated with its presence in low concentration in saliva , in addition to its contamination with some impurities . This study has an attempt to overcome these problems through a suitable resin (DEAE-Cellulose), which assists in isolating three isoenzymes for 5`-NT from saliva of diabetic patients.

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تقدير فعالية انزيم `5 – نيوكليوتايديز في لعاب مرضى السكري وتنقية متناظراته جزئيا

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الملخص

تضمنت الدراسة الحالية قياس مستوى انزيم `5– نيوكليوتايديز (5`-NT) في لعاب مرضى السكري ، اذ شملت الدراسة (60) عينة لعاب ،تضمنت (30) عينة من لعاب مرضى السكري اضافة الى (30) عينة لعاب من الاشخاص الاصحاء كمجموعة سيطرة ومن كلا الجنسين. اظهرت نتائج الدراسة الارتفاع المعنوي في فعالية 5`-NT من لعاب مرضى السكري مقارنة مع الاصحاء.

و تضمنت الدراسة ايضا تنقية جزئية لانزيم 5`-NT من لعاب مرضى السكري ، حيث شملت ترسيب الانزيم بتركيز 30 % كبريتات الامونيوم وكذلك امرار البروتين المترسب والمذاب بالمحلول المنظم Tris-HCl pH 7.2 على هلام sephadex G-75 حيث تم الحصول على قمة منفـردة . وباستخدام تقنية التبادل الايوني (DEAE-Cellulose A-50) تم فصل ثلاث متناظرات وبدرجات نقاوة متفاوتة.