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Partial purification of topoisomerase IB from serum of diabetic patients and study it's kinetic properties and molecular weight

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Abstract

This study was done to partially purification of topoisomerase IB from serum of diabetic patients using Gel filtration technique, by using Sephadex G 100 gel. A single peak in fraction four has been obtained, and the degree of purification (17.1) fold, enzyme yield (108.2%) and specific activity (0.189ng/mg). Kinetics studies for the partial purified enzyme were carried out which showed optimal concentration of substrate which was (0.1ng/ml), Michael's - Menten constant (K_m=0.033ng) and maximum velocity (V_{max}=0.90 ng/ml), while optimum Temperature was (37C°) and optimum pH was (7.5). The molecular weight of the partial purified enzyme has been determined by gel electrophoresis method, in presence of polyacrylamide gel and sodium dodecyl sulphate (SDS-PAGE) which showed that the approximated molecular weight was (66KD).

1-Introduction

Diabetes mellitus (diabetes) has been defined as a metabolic disorder with heterogeneous etiologies, which is characterized by chronic hyperglycemia and disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both. The long-term relatively specific effects of diabetes include development of retinopathy, nephropathy and neuropathy. People with diabetes are also at a risk of other diseases, including cardiac, peripheral arterial and cerebrovascular disease [**1,2**]. Diabetes can be classified into four clinical categories[**3**]:

1.Type 1 diabetes (due to β-cell destruction, usually leading to absolute insulin deficiency).

2.Type 2 diabetes (due to a progressive insulin secretory defect on the background of insulin resistance).

3.Other specific types of diabetes due to other causes, e.g., genetic defects in β-cell function, genetic defects in insulin action, diseases of the exocrine pancreas (such as cystic fibrosis), and drug- or chemical-induced (such as in the treatment of HIV/AIDS or after organ transplantation).

4 .Gestational diabetes mellitus (GDM) (diabetes diagnosed during pregnancy that is not clearly overt diabetes).

At 2015 the International Diabetes Federation (IDF) suggest that the number of adults with diabetes in the world will increase to 55%, and at 2040 may be increased from 415 to 642million [**4**].

Human DNA topoisomerases are essential cellular enzymes in all living cells [**5**], The enzymes regulate the topological stress of DNA during replication and recombination, gene transcription and other cellular processes by transiently breaking one or two strands of DNA, passing single- or double-stranded DNA through the break and finally resealing the DNA strand breaks [**6**]. DNA topoisomerase I accomplish this feat by either passing one strand of the DNA through a break in the opposing strand, other type as a (type II topoisomerase) by passing a region of duplex from the same or a different molecule through a double-stranded gap generated in a DNA. Topoisomerases are known that relax only negative supercoils, that relax supercoils of both signs, or that introduce either negative or positive supercoils into the DNA. Besides altering the supercoiling of a closed DNA domain, the strand passing activities of topoisomerases can be promote the catenation and decatenation of circular DNAs or the disentanglement of intertwined linear chromosomes[**7**]. Strand cleavage is achieved by the nucleophilic attack of the active site tyrosine on a DNA phosphodiester bond. The resulting formation of a phosphodiester bond between the tyrosine and the 3_ end of the cleaved

strand enables the enzyme to reseal the DNA by simple reversal of the cleavage
Reaction[**8**]. Human DNA Topoisomerase IB Structure Based on conservation of sequence, sensitivity to limited proteolysis, hydrodynamic properties, and fragment reconstitution experiments, the 91-kDa human topoisomerase IB protein has been subdivided into four distinct domains[**9**], The N-terminal 214 amino acids of the human enzyme are dispensable for relaxation activity in vitro and constitute a hydrophilic, unstructured, and highly protease-sensitive region of the protein [**10**].There are many of clinical significant of topoisomerase IB where DNA Topoisomerases are the targets of important anticancer and antibacterial drugs. These enzymes are the molecular targets of a class of compound with anticancer effects so they play an important role in clinical terms. The research in this field has allowed to better understand the mechanism of action of Topoisomerase and carried out the possibility to produce more effective and specific therapeutic drugs[**11**].

2- Experimental

2.1.Collection of sample: The total number of these samples was (70) samples, serum samples were collected from diabetic patients for both sexes .Type I was(30) sample while type II was (40)sample. Blood was drawn from the vein using a 5 ml plastic syringe with one use. The blood was placed in clean and free anticoagulant tubes, and left to coagulate at room temperature. The blood serum was then separated by centrifuge at a velocity of 5000 G for 15 minutes to ensure adequate serum red blood cell extraction .The effectiveness of the enzyme was measured directly and the study was done outside the body (in vitro).

2.2.Diagnosis tests : Glucose concentration in serum was measured by using (kit AMS Italia) depending on enzyme method that stated on Trinder reaction[**12**]. Total protein level in serum was measured by using (kit spectrum Egypt) depending on enzyme method(Biuret method) [**13**]. Topoisomerase I activity in serum was measured by using Elisa kit supplied from Cloud clone company -USA [**14**].

2.3.Separation and purification of Topoisomerase IB from serum of diabetes patients

Topoisomerase I was purified from the serum of diabetic patients using the following steps**:** 1-Addition ammonium sulphate (80%) 2-Dialysis 3-Gel Filtration Chromatography(using Sephadex G100)

2.4.Kinetics of topoisomerase IB

The kinetics of topoisomerase I were studied after its separation and partially purified from serum of diabetic patients by gel filtration. These included:

1- Effect of substrate concentration (supercoiled DNA): by using different concentrations of substrate (0.1 ,0.05 ,0.01,0.001,0.004,0.00008,0.000016 ng)

2- Effect of temperature: using to measure the effectiveness of topoisomerase I. The reaction was conducted at different temperatures (7,17,27,37,47 and 57 C°)

3- Effect of pH: The pH effect of the topoisomerase I reaction. Different pH solutions (4.5, 5.5, 6.5, 7.5, 8.5, 9.5) were used with topoisomerase I at 0.1ng and 37 C° .

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis to Measurement molecular weight of Purified Enzyme (SDS-PAGE)

Followed the way to the researcher Laemmli [**15**] to prepare polyacrylamide gel with some modifications.

3- Results and Discussion

3.1.Partially purification of topoisomerase I from serum of diabetic patients

The basic principle is to equalize the charges on the surface of the protein(enzyme) and the degradation of the water layer surrounding the protein and reduce the degree of watering, solubility of the protein and sedimentation [**16**]. Therefore separation and purification process of topoisomerase I was made from serum of diabetic patient by steps where in the first stage of purification the enzyme was precipitated using ammonium sulphate salts $(NH_4)_2SO_4$ to concentrated enzyme and the access of the resulting salts was removed by using dialysis technique using buffer solution 1Mm Tris-HCl pH 7.4,wherase the degree of purification of enzyme was (4.40)folds and yield of enzyme 95.1% , specific activity0.0485ng/ml showing the result in table (1), the stages of purification were complete by using gel filtration using Sephadex G100 which showed a single peak in fraction four with the degree of purification was (17.1) fold and enzyme yield was(108.2%) while specific activity was(0.189ng/mg).

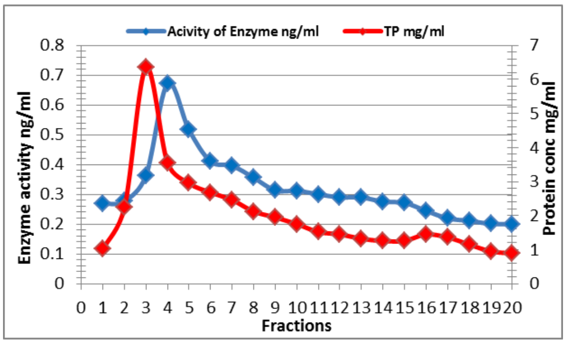


Figure (1) purification of topoisomerase I with Gel filtration on Sephadex G-100 (Elution curve)

Table(1) steps of purification

Steps of purifications	Volume ml	Activity ng/ml	Total Activity	Protein Conc Mg/ml	Specific activity ng/mg	Yield %	Folds	Total protein mg
Crude serum	5	0.6201	3.1006	56.3	0.0110	100	1	281.5
Ammonium sulphate	3.5	0.7418	2.5963	42.1	0.0176	83.7	1.59	147.3
Dialysis	4	0.7372	2.9488	15.2	0.0485	95.1	4.40	60.8
Gel filtration	5	0.6715	3.3579	3.55	0.1891	108.2	17.17	17.75

3.2.Detemination of molecular weight of topoisomerase I using polyacrylamide gel and SDS (SDS-PAGE)

Molecular weight of the enzyme determined by Polyacrylamide gel electrophoresis(PAGE), describes a technique widely used in biochemistry, forensics, genetics, molecular biology. (SDS-PAGE) is a technique for separating proteins based on their ability to move within an electrical current, which is a function of the length of their polypeptide chains or of their molecular weight. This is achieved by adding sodium dodecyl sulphate (SDS) detergent to remove secondary and tertiary protein structures and to maintain the proteins as polypeptide chains. The SDS coats the proteins, mostly proportional to their molecular weight, and confers the same negative electrical charge across all proteins in the sample [**17**]. figure (2) showed the band of topoisomerase I compared with standard solutions bands which known it's molecular weight to found molecular weight of enzyme which was(66KD).

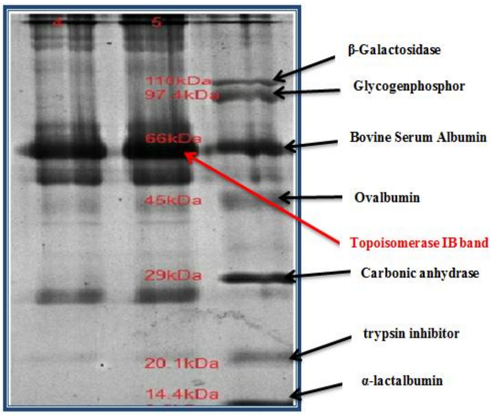
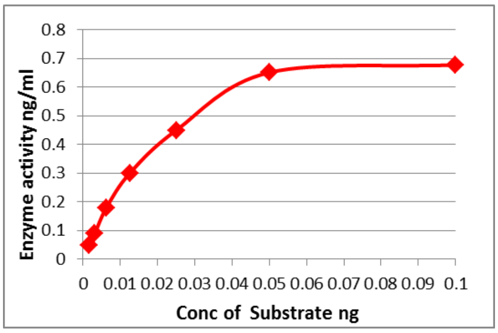


Figure (2) electrophoresis of topoisomerase IB to determined molecular weight compared with standard protein

3.3.Study of kinetic properties of topoisomerase I partially purified of diabetic patients

3.3.1.Concentration of substrate

The effect of substrate was study on the activity of enzyme, whereas the optimal concentration of substrate (supercoiled DNA) 0.1ng and figure (3) showed that. Also determined Michael's-minten constant(K_m) that mean as the affinity between the enzyme and substrate [**18**] . The constant value of the Michael's-Minten of substrate was (0.033ng) and the maximum velocity value V_{max} (0.90ng/ml). The differences between all these studies were clear and almost natural as a result of the different sources of enzyme which were cleared and the different methods In one study to purify the enzyme from E-Coli K_m was7.52nM and V_{max} was 7.98nM/min [**19**] .



Figure(3) effect of substrate on topoisomerase IB activity

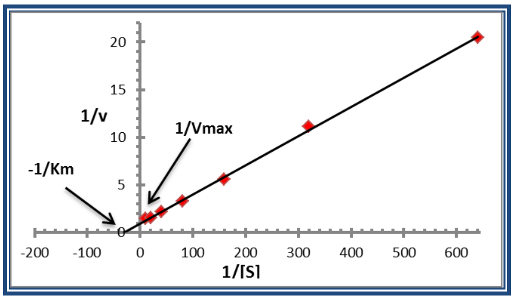


Figure (4) Line weaver-Burk to calculate (K_m) and (V_{max}) of topoisomerase IB

3.3.2.Effect of temperature

The optimum temperature was study on the activity, the highest temperature, where the rate of enzymatic reaction rate is maximal, while the enzyme is highly effective, and is affected by pH and other factors[**20**]. The optimum temperature of the enzyme activity when the pH was confirmed and the concentration of substrate was (37C°). The results varied with the other studies carried out, including a study on the enzymatic extracted from Calf thymus mitochondria (30C) [**21**]. and another study indicated that the optimum degree of the enzyme purified from Tobacco cell (30C°) [**22**]. The figure (5) showed that.

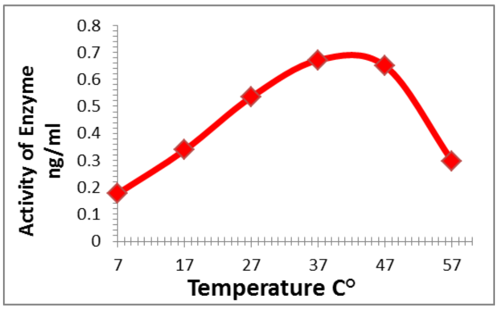


Figure (5) effect of temperature on topoisomerase IB activity

3.3.3.Effect of pH

Effect of pH was studied on the enzyme activity whereas pH has a significant effect on the enzyme's effectiveness for controlling ionization Ionic aggregates at the active site of the enzyme The optimal pH of enzyme stability is an important characteristic of enzymes [**23**]. The results of the kinetic study of enzyme showed that the optimal pH of topoisomerase I was (7.5) as showing in figure (6). The results of pH varied with the other studies carried out, including a study on the enzymatic extracted from Tobacco cell was (7.8) **[22]** and from E-Coli was (8.2) **[19]**.

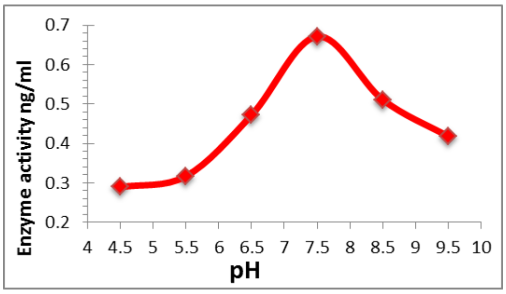


Figure (6) effect of pH on topoisomerase IB activity

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تنقية انزيم التوبوايزومريز IB جزئياً من مصل مرضى داء السكري ودراسة خواصه الحركية ووزنه الجزيئي

سيف محمود حسن ، فراس طاهر ماهر ، نغم قاسم كاظم
قسم الكيمياء ، كلية العلوم ، جامعة تكريت ، تكريت ، العراق

الملخص

اجريت الدراسة الحالية لتنقية انزيم التوبوايزومريز IB جزئياً من مصل المرضى المصابين بداء السكري باستخدام تقنية الترشيح الهلامي وباستخدام هلام السيفادكس G100. تم الحصول على قمة واحدة في الجزء الرابع حيث بلغت درجة التنقية 17.1 وبحصيلة انزيمية 108.2% وفعالية نوعية (0.189ng/mg) واجريت الدراسات الحركية للأنزيم المنقى جزئياً حيث كان التركيز الامثل للمادة الاساس (0.1ng/ml) وبلغت قيمة ثابت ميكالس– منتن K_m (0.033ng) والسرعة القصوى V_{max} (0.90ng/ml), بينما درجة الحرارة المثلى لعمل الانزيم(37C°) والاس الهيدروجيني الامثل pH (7.5). تم تقدير الوزن للأنزيم المنقى جزئياً من المرضى بطريقة الترحيل الكهربائي على هلام متعدد الاكريل امايد بوجود كبريتات دوديكايل الصوديوم (SDS-PAGE)حيث بلغ الوزن الجزيئي التقريبي للأنزيم (66KD).