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Partial Purification and Kinetic Study of the Enzyme Glucose-6-phosphate dehydrogenase from Red Blood Cells of Male Patients with Type 1 Diabetes and Determination of its Molecular Weight

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Abstract: Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease. A disorder resulting from the destruction of beta cells in the pancreas, leading to a decrease in insulin production. Insulin is the hormone responsible for regulating blood glucose levels, and its deficiency causes uncontrolled inflammation in the pancreatic islets of Langerhans because of the destruction of beta cells. Insulin is the hormone responsible for regulating blood glucose levels, and its deficiency causes uncontrolled inflammation in the pancreatic islets of Langerhans, thus causing dysfunction and destruction of beta cells. Patients with T1DM suffer serious complications in the small and large blood vessels, causing dysfunction in the heart, kidneys, retina, and nerves. In most people with T1DM, symptoms do not appear until hemolysis occurs as a result of several factors, including eating fava beans, certain medications, sepsis, kidney failure, or diabetic ketoacidosis. Purification of glucose-6-phosphate dehydrogenase (G6PD) enzyme was performed from patients' red blood cells, and several sequential steps were followed from blood sample preparation to precipitation and then dialysis. In the ion exchange technique, the purified G6PD gave two prominent and distinct peaks, (A) and (B), indicating the purity of the enzyme. G6PD activity was higher in peak (A) than in peak (B), and peak (A) was used in the gel filtration technique. A single, distinct, and prominent peak of the purified enzyme was observed. After studying the kinetics of G6PD, the values ($K_m=3.7$ mM) and ($V_{max}=1.4$ U/mL) were determined. The optimal conditions for the enzyme were: Glucose-6-phosphate substrate concentration (11 mM), (pH=8), temperature (37°C) and incubation time (7 minutes). Finally, the molecular weight of G6PD was determined by gel filtration chromatography and electrophoresis techniques and was approximately (58 KDa).

Keywords: Diabetes; Electrophoresis; G6PD; Gel filtration; Ion exchange; K_m ; Molecular weight; Optimum conditions; Purification; T1DM; V_{max} .

Introduction

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease. The body's immune cells destroy the beta cells in the pancreas, resulting in a decrease in insulin production. T1DM is widespread globally, and T cells act as attackers in the body instead of protecting it [1,2]. T1DM represents between (5-10%) of all diabetes cases and affects approximately 8.4 million people worldwide [3]. The hormone insulin works to regulate blood glucose levels, and its deficiency causes uncontrolled inflammation in the pancreatic islets of Langerhans, thus causing dysfunction and destruction of beta cells. Cytokines contribute to beta cell failure as the disease progresses. [4]. In most people with T1DM,

symptoms do not appear until hemolysis occurs due to several factors, including fava bean consumption, certain medications, sepsis, kidney failure, or diabetic ketoacidosis.

Elderly patients with T1DM over the age of 60 suffer from depression, weakness, and cognitive decline as a result of severe hypoglycemia [2]. Patients with T1DM suffer from serious complications in micro- and macro-vascular diseases, impaired cardiac function, and kidney, retinal, and neurological disorders [3,4]. In most people with T1DM, symptoms do not appear until hemolysis occurs due to several factors, including fava bean consumption, certain medications, sepsis, kidney failure, or diabetic ketoacidosis [5]. Important technologies have been used to reduce disease severity, including insulin pumps and continuous glucose monitoring devices to control blood sugar levels. These advancements have collectively contributed to better management of risk factors in T1DM, reduced complications, and improved quality of life [2].

The enzyme Glucose-6-phosphate dehydrogenase (G6PD) [Oxidoreductases, EC 1.1.1.49] is found in the cell cytoplasm and in all tissues. G6PD is considered to be responsible for determining the reaction rate in the pentose phosphate pathway. Glucose 6-phosphate is converted to 6-phosphogluconolactone to form nicotinamide adenine dinucleotide phosphate (NADPH), which is one of the most important intracellular coenzymes. [6,7]. G6PD protects cells from oxidative stress generated by reactive oxygen species (ROS). Red blood cells are exposed to ROS because they are responsible for transporting oxygen, and once they mature, they cannot replace damaged proteins [8]. NADPH is used for the synthesis of fatty acids, and to maintain the oxidation-reduction state in cells. NADPH is a reducing agent for thiol-dependent enzymes and is involved in glutathione (GSH) recycling. GSH acts as an antioxidant to combat oxidative damage caused by metabolic processes or inflammation [6]. ROS is based on the oxidation of hemoglobin and membrane structure, resulting in hemoglobin denaturation and the formation of Heinz bodies and lipid peroxides. These changes cause a decrease in red blood cell flexibility along with membrane stiffening [8].

In humans, approximately (500 million) patients suffer from G6PD enzyme deficiency, and affecting males more than females. This deficiency is considered a genetic condition resulting from a structural defect caused by genetic mutations in the G6PD genes associated with the X chromosome, which are responsible for encoding the G6PD enzyme [9]. Most disease-causing mutations produce deficient proteins as a result of a single nucleotide substitution. Severe mutations that destabilize cells manifest as chronic non-spherocytic hemolytic anemia instead of embryonic death [8]. G6PD deficiency is closely associated with clinical hemolytic anemia following fava bean ingestion, certain medications, or infection. Abnormalities in the complete blood count (CBC) are also an important indicator for the early detection of hemolysis [8,9].

Symptoms of G6PD deficiency do not appear in patients until they are exposed to an oxidizing stimulus that results in hemolysis. G6PD deficiency in T1DM patients can be predicted by warning signs and serves as an indicator for disease detection and minimizing the risk of clinical symptoms such as dark urine, jaundice, or sudden fatigue [8,9]. G6PD expression has also been considered a biomarker for detecting various types of cancer, including breast, cervical, ovarian, renal cell, and prostate cancer [10].

G6PD enzyme and its association with Type 1 Diabetes

The close relationship can be summarized in the following points:

1- The role of the G6PD enzyme with beta cells and oxidative stress: Under normal conditions, high levels of glucose-6-phosphate and adenosine triphosphate (ATP) act as positive stimulators to activate the G6PD enzyme, triggering cell proliferation or nitrogenous compound synthesis [11]. The compound NADPH plays a key role in protecting cellular structures from damage caused by ROS. NADPH supports the production of reduced glutathione, which converts hydrogen peroxide into water, thus

protecting cell components, especially the red blood cell membrane, from oxidative damage [12]. Hyperglycemia leads to inhibition of G6PD enzyme activity [13].

2- G6PD deficiency, risk of acute hemolytic crises, and risk of developing proliferative diabetic retinopathy: Common complications in T1DM patients include a life-threatening medical emergency resulting from ketoacidosis due to severe insulin deficiency [13]. The body is unable to produce energy, and resorts to burning fat quickly, causing the accumulation of ketones and raising blood acidity to toxic levels. A sudden, acute attack of hemolysis occurs as a result of damage to small blood vessels several days after treatment [5,12].

3- -Genetic deficiency of the G6PD enzyme and low HbA1c (glycosuria): Red blood cells break down and regenerate at a faster rate than normal in cases of genetic G6PD deficiency (favism). This gives a false low HbA1c level, while in reality the patients suffer from a severe and hidden high blood sugar [11].

Methods and Materials

Sample Collection

Blood samples were collected from (60) male patients with type 1 diabetes. They were aged 50 and above. Their illness was confirmed through medical reports, doctors' diagnoses, and insulin treatment. As an ethical requirement, permission was obtained from the patients before blood was drawn from them. It was also confirmed that they did not suffer from high blood pressure or heart disease, and they do not consume alcohol or cigarettes.

Systematic steps for purifying the G6PD enzyme

1- Step for preparing the blood sample

(5 ml) of blood was drawn from male patients with type 1 diabetes intravenously. It is placed directly into tubes containing anticoagulants such as EDTA or heparin to preserve cell integrity. All laboratory steps are carried out under complete cooling at (4°C), to prevent enzyme damage, to ensure separation of components, and to obtain a pure protein precipitate. Red blood cells are isolated from the blood by washing them with (0.9%) NaCl saline solution at a ratio of (1:3). Place the ingredients in a centrifuge at (10000 RPM) for (10 minutes) to remove the plasma, the top liquid, and the white layer (Buffy coat). The process is repeated (3 times) to obtain red blood cells, then (3 volumes) of pure, cold distilled water are added to dissolve the washed cells. The ingredients are left in the refrigerator for (60 minutes), then placed in a centrifuge at (10000 RPM) for (20 minutes) to remove cell membrane debris at (4°C). The upper liquid (clear) containing the dissolved G6PD enzyme is collected and the precipitate (cell membrane remnants) is discarded [10,14,15].

2- Step to get rid of hemoglobin

Hemoglobin must be removed immediately because it represents (90%) of the proteins of red blood cells, as it interferes with the purification components. The (DEAE-Cellulose) type ion exchange resin, which was previously prepared with the buffer solution (Phosphate buffer solution, pH 6.5), is added directly to the clear liquid containing G6PD. Then the G6PD enzyme binds directly to the resin, leaving hemoglobin which does not bind or has very weak binding. The resin is then washed several times with the same buffer solution to completely remove the hemoglobin until the washed liquid becomes colorless. Finally, the G6PD enzyme can be recovered by separating it from the resin using a buffer solution (phosphate buffer, pH 7.5) containing a higher salt concentration than the first. (10 ml) of G6PD enzyme was obtained, G6PD activity was measured at (≈ 340 nm λ) and total protein at (≈ 280 nm λ) [15].

3- Sedimentation step: The sedimentation process takes place in two stages at (4°C)

A- Primary sedimentation: Unwanted proteins are precipitated first, with a saturation level of (40%). Ammonium sulfate salt is added in powder form slowly, gently

and gradually with continuous stirring at (4C°) with a magnetic motor. The mixture is left in the refrigerator for (30 minutes) and then placed in a centrifuge at (10000 rpm) for (20 minutes). Unwanted proteins settle as a precipitate, which we then discard. The upper (clear) fluid containing the soluble G6PD enzyme is taken [14].

B- Secondary sedimentation: Ammonium sulfate powder was added to (6 ml) of the clear liquid containing G6PD to be precipitated at a saturation level of (70%) [14,16].

4- Dialysis step

The dialysis process was carried out at (4C°) to remove ammonium sulfate and other unwanted salts from the precipitation step. (5 ml) of the protein sample was placed in the dialysis bag and immersed in a flask with a capacity of (1000 ml). The flask contains the Tris-HCl buffer solution with a concentration of (1M) and a pH of (7.8). The solution was moved by magnetic motor for (24 hours) with the buffer solution being replaced every (6 hours). G6PD activity and total protein were measured [14].

5- Ion exchange chromatography step

(5 ml) of the dialyzed sample was added to the ion exchange column with dimensions (2 cm × 45 cm). The positively charged ion exchange resin DEAE-Cellulose-A50 was used and added to the column up to a height of 40 cm. The flow rate of the leached portions of the column was set to 1 ml/min. The column was then washed with a graduated buffer solution of NaCl salt (0-0.5 M). Fractions are collected and G6PD activity is measured at (≈340 nmλ) and total protein at (≈280 nmλ) [17].

6- Gel filtration chromatography step

(4 ml) of the concentrated sample was placed into the gel filter column with dimensions (2 cm × 100 cm) resulting from the ion exchange step. The column was filled with (Sephadex G-100) gel. The flow rate of the column components was set to (1 ml/min) and each exudate fraction was collected from the column after measuring G6PD activity and total protein [18].

Kinetic study of the G6PD enzyme and optimal conditions

The enzyme is kept at (-20°C) in Deep Freeze until the kinetics of the G6PD enzyme are studied in order to finding the value of the Michaelis-Menten constant (Km) and the maximum speed (Vmax). Then determine the optimal conditions for G6PD action in terms of pH, temperature, substrate concentration and incubation period.

Estimation of the Molecular Weight (MW) of Pure G6PD Enzyme

There are two methods for determining the molecular weight (MW) of G6PD.

A- Gel filtration technology: In this method, standard proteins of known MW were used [19]. The gel filtration column was washed several times with the buffer solution (Tris-HCl), then the standard proteins were gently added to the column. The flow rate was set (1 ml/min) and G6PD activity and total proteins were measured at each of the fractions collected at the bottom of the column. The calibration curve was plotted between the logarithm of the In this method, standard proteins of known MW were used (y-axis) and the elution volume (x-axis).

B- Electrophoresis technology: This technique was used to precisely separate the purified G6PD enzyme via SDS-PAGE gel based on its molecular weight [20].

Estimation of G6PD Enzyme Activity and Total Protein

G6PD enzyme activity was estimated using the UV-Kinetic Method at a wavelength of (340 nm) with a diagnostic kit supplied by the French company Biolabo [21]. Total protein activity was measured using the Lowry method [22].

Results and Discussion

Table (1) shows the steps for purifying the G6PD enzyme from the red blood cells of male patients with type 1 diabetes. In the precipitation step with (70%) ammonium sulfate salt, the specific activity was (0.80 U/mg), the folds was (1.09), and the yield was (85.33%). In the dialysis step, G6PD activity increased to (0.85 U/mL) compared to crude erythrocyte which was (0.45 U/mL). The specific activity also doubled to (1.76 U/mg), and the Fold value reached (2.39) and the Yield value reached (94.44%). Total protein concentrations decreased from (6.11mg) to (2.41mg), indicating the removal of unwanted proteins, salts, and impurities from dialysis.

Table 1. G6PD enzyme purification from red blood cells in male patients with T1DM.

No.	Steps	Volume (ml)	Total protein (mg)	Activity of G6PD (U* / mL)	Total activity (U)	Specific activity (U / mg)	Fold	Yield %
1	Red Blood Cells	10	6.11	0.45	4.5	0.74	1	100
2	ammonium sulphate (70%)	6	4.8	0.64	3.84	0.80	1.09	85.33
3	Dialysis	5	2.41	0.85	4.25	1.76	2.39	94.44
4	Ion Exchange DEAE-Cellulose-A50							
	Peak A	5	1.01	0.88	4.4	4.36	5.92	97.78
	Peak B	5	1.21	0.75	3.75	3.10	4.21	83.33
5	Gel Filtration Sephadex G-100	4	0.3	1.12	4.48	14.93	20.28	99.56

In the ion exchange technique step, the positively charged DEAE-Cellulose-A50 ion exchanger was used, which gave excellent results in purification. Two prominent and distinct peaks of the G6PD enzyme appeared, peak (A) and peak (B) as shown in Figure (1).

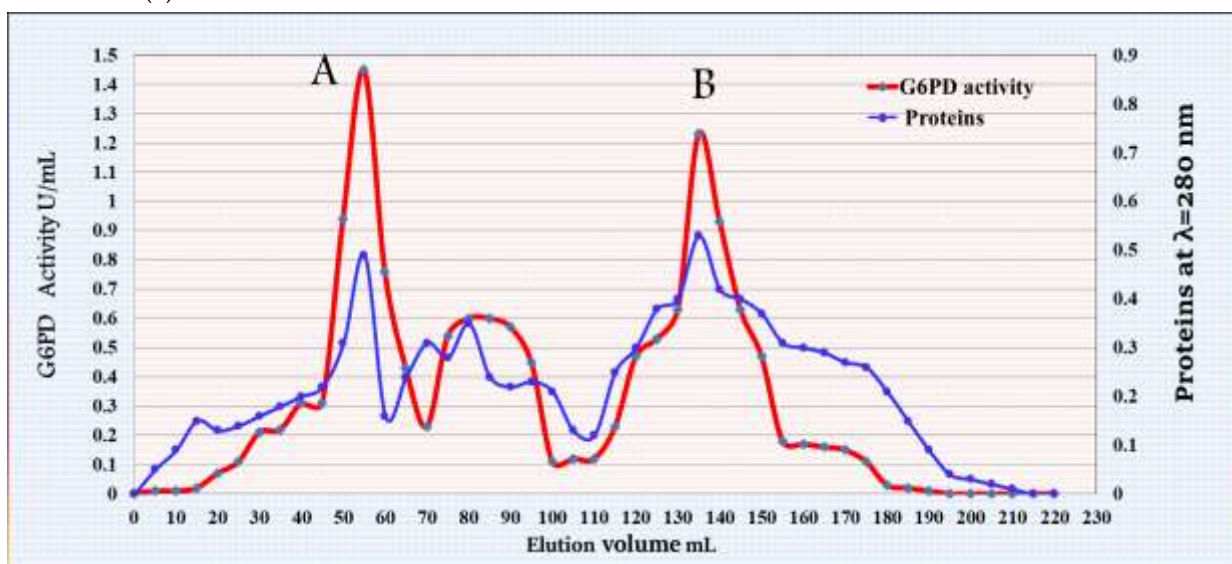


Figure 1. Ion exchange technique for G6PD enzyme purified from red blood cells of male patients with T1DM.

G6PD activity was at peak A (0.88 U/mL) and at peak B (0.75 U/mL), while the qualitative activity was at peak A (4.36 U/mg) and at peak B (3.10 U/mg). According to the results, G6PD activity at peak A was higher than at peak B, and for this reason, peak A was

relied upon in the subsequent purification steps. To obtain high purity of the enzyme, an additional technique was performed, namely the gel filtration technique.

In the gel filtration technique step, (Sephadex G-100) gel was used. Excellent results were also obtained, as a single prominent and distinctive peak of G6PD activity appeared, as shown in Figure (2).

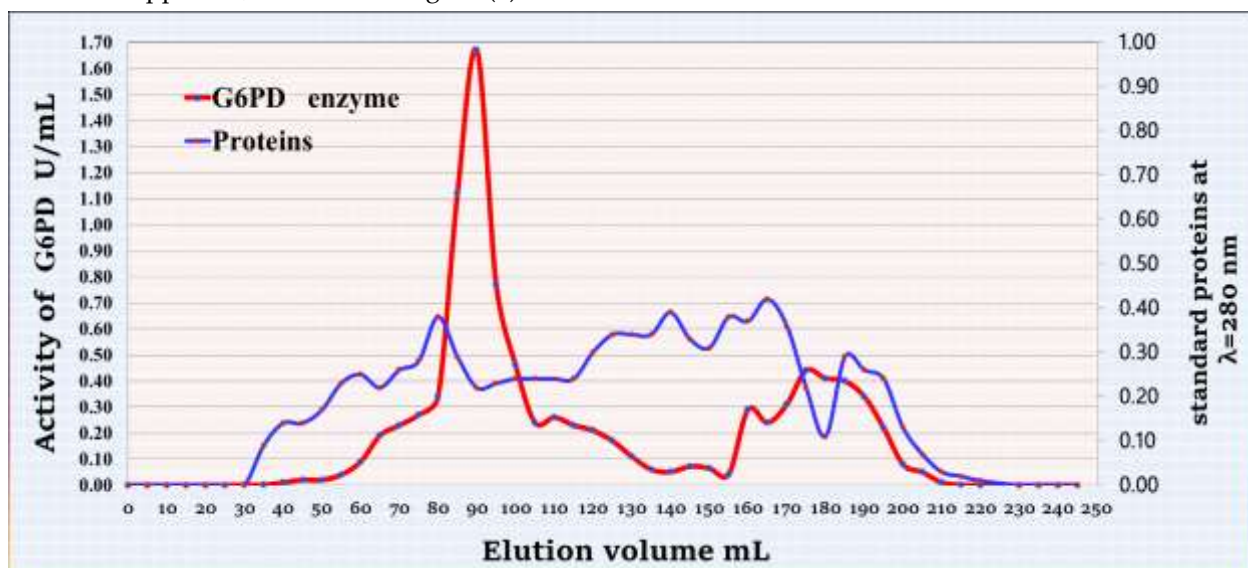


Figure 2. Gel filtration technique for G6PD enzyme from red blood cells of male patients with T1DM.

G6PD activity increased from (0.45 U/mL) in crude red blood cells to (1.12 U/mL) in gel filtration. The specific activity also increased from (0.74U/mg) to (14.93U/mg), which is about (14 times) compared to crude erythrocyte. The number of times the Fold was purified reached (20.28) and became (20 times) compared to Fold at the beginning of the purification. The results show that a very high purity G6PD enzyme was obtained due to the appearance of a single high and distinctive peak.

Estimation of the molecular weight (MW) of the G6PD enzyme

There are two methods for determining the MW of G6PD.

A- Gel filtration chromatography technique: Proteins with known MW were used as standard proteins, as shown in Table (2) [19].

Table 2. Standard proteins used in gel filtration to find the molecular weight of the G6PD enzyme.

standard proteins	Molecular Weight of Proteins	Elution Volume	Log M.wt
Blue dextrin	2000000	25	6.30
Bovine serum albumin	67000	55	4.83
Egg albumin	45000	80	4.65
Pepsin	36000	95	4.56
Papain	23400	110	4.36
Insulin	5800	135	3.76
Oxytocin Hormone	1000	180	3.00
Tryptophan	204	215	2.31
G6PD Unknown	58.117	160	4.74

The separation column in gel filtration chromatography is filled with these proteins, The flow rate of the separation column is set to (1 ml/min). Then the purified G6PD enzyme is gently placed onto the column. G6PD activity and total protein are measured in all the mutated fractions at the bottom of the column as shown in Figure (3).

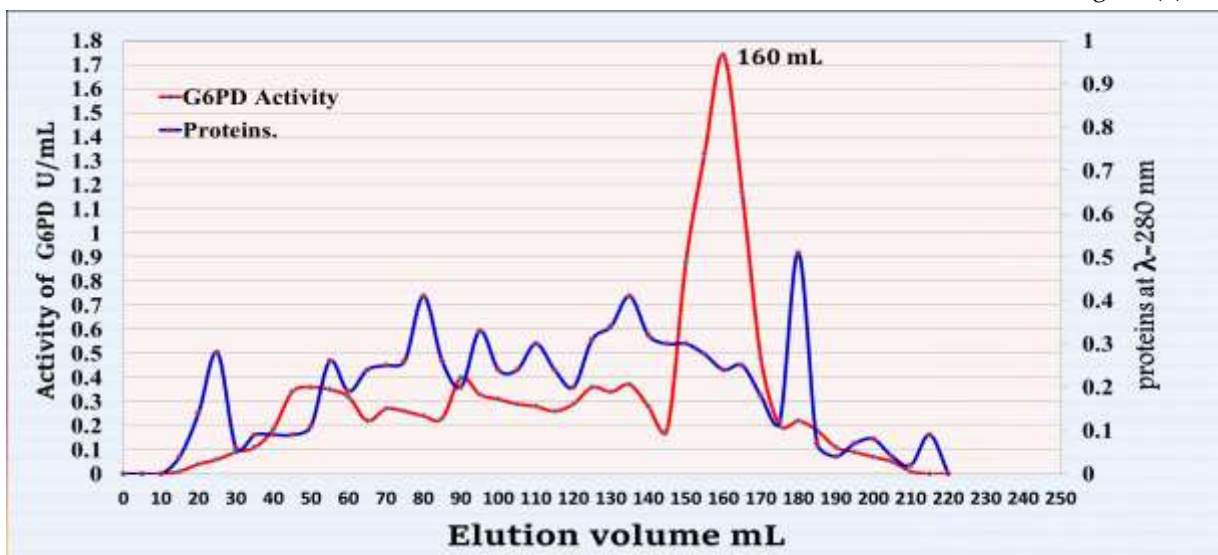


Figure 3. Determination of Elution Volume by Gel Filtration Technique for G6PD Activity to Find Molecular Weight.

The calibration curve is drawn between the Elution Volume on the (x-axis) and the logarithm of the MW of proteins on the (y-axis) to obtain the straight line equation ($y = -0.0186x + 4.7403$) as in Figure (4). The molecular weight of G6PD is determined by substituting the elution volume value (160 mL) from Figure (3) into the straight-line equation in Figure (4). Finally, The molecular weight of G6PD was estimated after compensation and was (58.117 kDa).

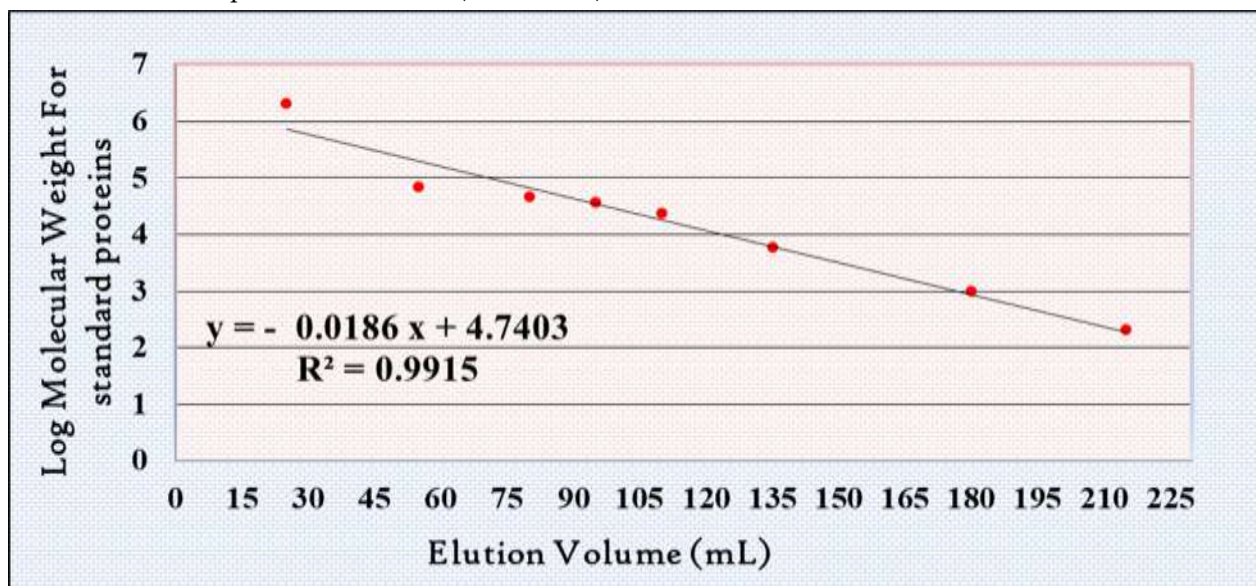


Figure 4. is a calibration curve for finding the straight-line equation of standard proteins for MW estimation.

B- Electrophoresis technology: The purified G6PD enzyme was separated using SDS-PAGE gel [20]. The separation is based solely on molecular mass, as the negatively charged G6PD moves towards the positive electrode after the passage of electric current to give a molecular weight of (58 KDa), as shown in Figure (5).



Figure 4. Electrophoresis for the separation of the G6PD enzyme via SDS-PAGE. gel from red blood cells in male patients with T1DM

The molecular weight of the G6PD enzyme varies in living organisms according to the difference in the number of amino acids, as well as according to the number of units that make up the peptide chains (monomer, dimer and tetramer) [23,24]. The molecular weight of the G6PD enzyme in humans, animals, plants, fungi and bacteria ranged between (43-79 KDa). In humans and animals, the molecular weight was approximately (55-68-KDa). Therefore, this study is consistent with several studies conducted on the same enzyme [14,20,24,25,26].

Studies have shown that the weight of a single monomer in humans is approximately (59 KDa) [27,28]. While the molecular weight of a single monomer of the G6PD enzyme in red blood cells using SDS-PAGE gel ranged (56-64 KDa) [15]. One study showed that the molecular weight of G6PD in children with favism was (56KDa) when separated by electrophoresis. When G6PD was separated by gel filtration, it was (63KDa) [20].

The molecular weight of a single monomer in pig liver is approximately (67.50 KDa), and in the pituitary gland of cows and the liver of mice (34 KDa) [29]. And in the liver of sheep it was (55KDa) [25]. And in the camel's liver it was (64KDa) [14]. The liver of the yellow catfish contained (68.89 KDa) [30].

Plants possess different forms of G6PD within a molecular weight range of (60-79 KDa) for a single unit, some of which function within the chloroplasts and others within the cytoplasm. In the pea plant, the single unit was (40KDa) and it combines to give an active enzyme with a molecular weight of (79KDa) [31,32].

In fungi and yeasts such as baker's yeast, the weight of a single unit is (55-60 KDa) and it is active in the form of a homologous dimer. In bacteria and prokaryotes, the weight of a single unit is only (50 KDa), and in *Leuconostoc mesenteroides* bacteria it is (52 KDa) per unit [24].

Kinetic study of purified G6PD enzyme and optimal conditions for its action

1- Studying the effect of the substrate (Glucose-6-phosphate) on the activity of purified G6PD enzyme

Several different concentrations ranging from (1-14 mM) were prepared from the glucose-6-phosphate substrate. An increase in G6PD activity was observed with increasing substrate concentration, as shown in Figure (5). G6PD activity continues to increase until the enzyme is saturated with the substrate, at which point the rate remains constant regardless of the substrate concentration; this is called the maximum velocity (V_{max}). The optimal substrate concentration (11 mM) was observed at the highest G6PD activity.

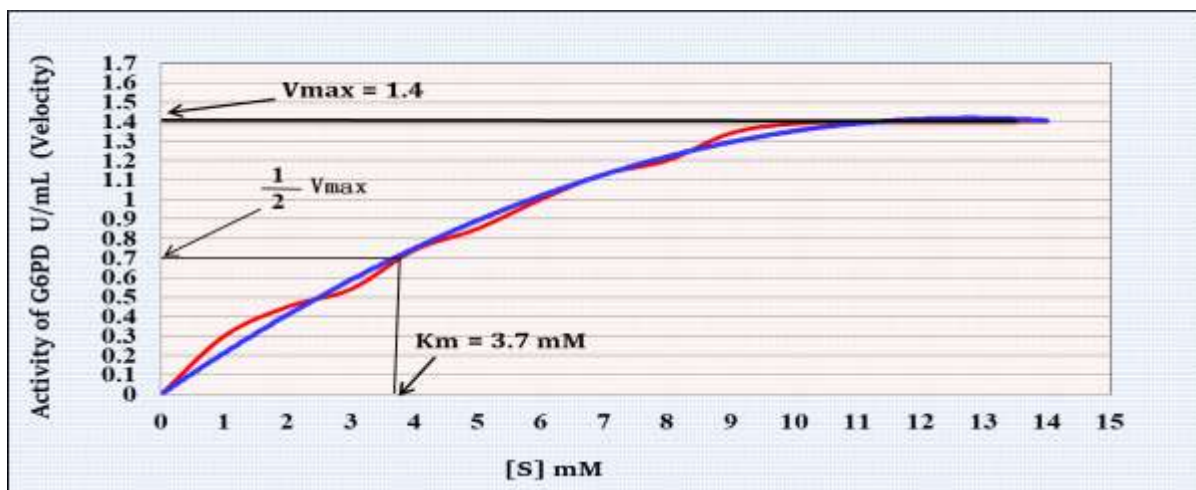


Figure 5. Effect of Glucose-6-phosphate substrate on purified G6PD activity.

The Linover-Berke K diagram was drawn as shown in Figure (6), to find the value of Km (Michaelis-Menten constant), which was ($K_m=3.7$ mM), and the value of the maximum speed (V_{max}), which was ($V_{max}=1.4$ U/mL).

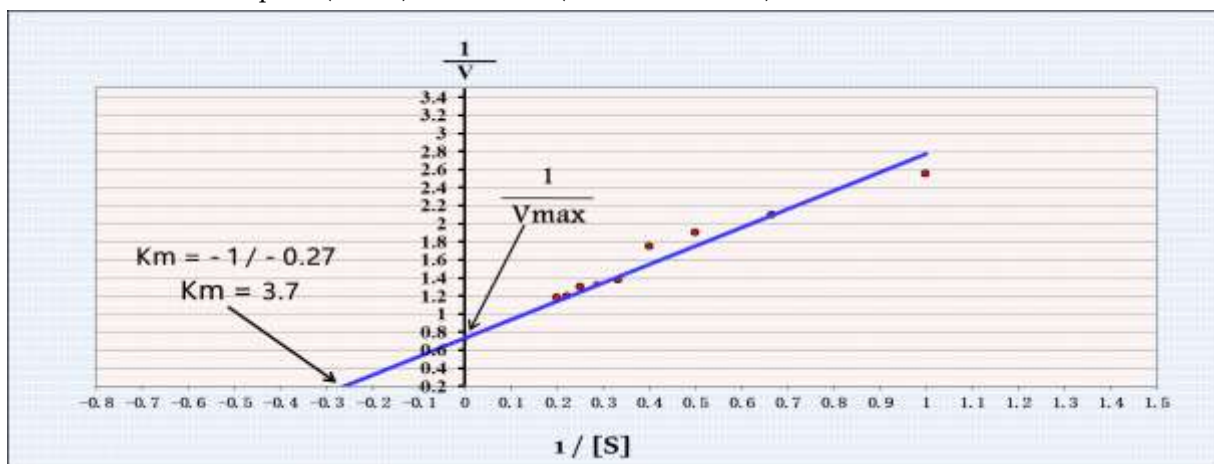


Figure 6. shows a Lineover-Berke diagram for finding the values of V_{max} and K_m .

2- Studying the influence of pH value on the enzymatic reaction rate of purified G6PD activity

Several solutions with different pH values ranging from (pH=3-10) were prepared. It was observed that the activity of G6PD increased at the optimum pH value (pH = 8) as shown in Figure (7).

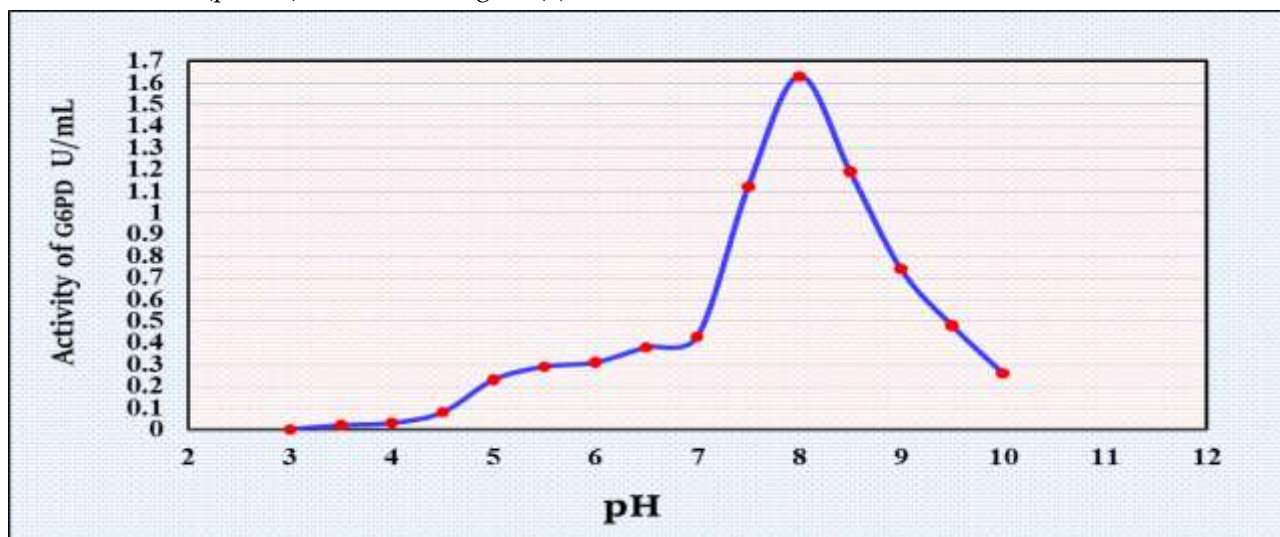


Figure 7. Effect of optimum pH on purified G6PD activity.

3- Studying the effect of incubation period on the activity of purified G6PD enzyme

G6PD activity was measured during incubation for different time periods ranging from (one minute to 12 minutes), and the optimal time period for the highest G6PD activity was (7 minutes). As shown in Figure (8).

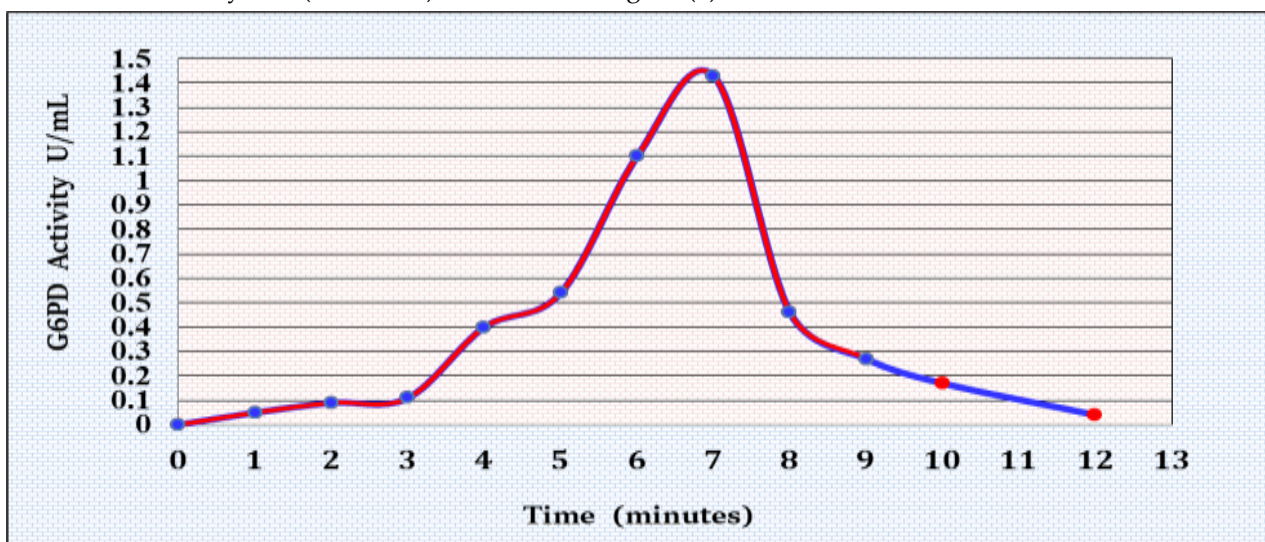


Figure 8. Impact of incubation duration on pure G6PD activity.

4- Studying the influence of different temperatures on G6PD activity

G6PD activity was measured at various temperatures ranging from (30-43 °C) and G6PD activity was high at the optimum temperature (37 °C). See Figure (9).

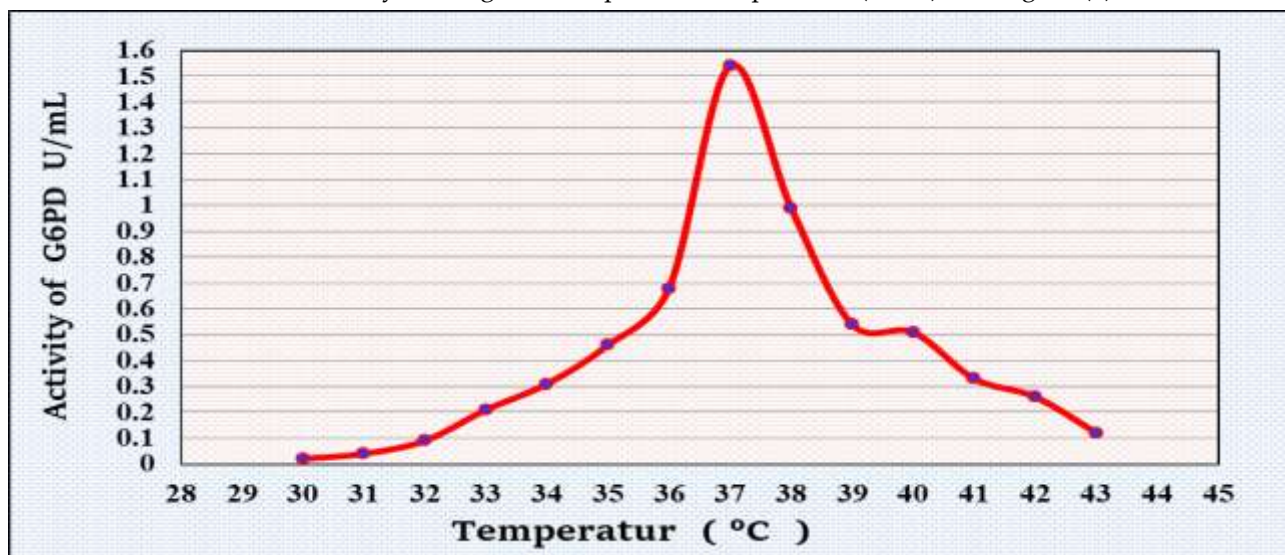


Figure 9. Impact of different temperatures on the G6PD activity.

The optimal conditions for the G6PD enzyme purified from red blood cells of male patients with type 1 diabetes can be summarized in Table No. (3).

Table 3. Optimal conditions for G6PD enzyme purification from red blood cells in male patients with T1DM.

Km (mM)	Vmax U/mL	[S] mM	pH	Time (minutes)	Tempertur (Celsius)
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3.7	1.4	11	8	7	37°C
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Conclusion

We conclude that the low level of the G6PD enzyme in type 1 diabetic patients is due to the development of diabetic complications and increased oxidative stress, leading to decreased production of NADPH, which is responsible for protecting red blood cells. G6PD is considered a biomarker for detecting the disease. Severe hemolytic anemia occurs in type 1 diabetic patients as a result of the breakdown of red blood cells, especially after the initiation of insulin therapy and the stabilization of blood glucose levels.

We conclude that when purifying G6PD, and through the appearance of two prominent peaks (A,B) during ion exchange, the purified enzyme has high purity. The activity of G6PD in (peak A) was higher than in (peak B). The (A peak) was adopted in the gel filtration step, as it gave a single, a distinctive peak indicates enzyme purity. The enzyme's kinetic constants were ($K_m=3.7$ mM) and ($V_{max}=1.4$ U/mL). The optimal conditions for the G6PD enzyme were pH (8), temperature (37°C), an incubation time of (7 minutes), and a substrate concentration of (11 mM). The molecular weight after purification of G6PD was estimated to be (58 kDa).

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