**In Vitro Effects of Clindamycin Antibiotic on Glucose-6-Phosphate Dehydrogenase Enzyme Purified from Sheep Spleen Tissue**

**Abstract**

In our study, the in vitro effects of clindamycin antibiotic on glucose-6-phosphate dehydrogenase enzyme (G6PD; E.C. 1.1.1.49) purified from sheep spleen tissue were investigated. Firsty, G6PD enzyme was partially purified from sheep spleen tissue by ammonium sulfate precipitation. The enzyme sample obtained as a result of ammonium sulfate precipitation was purified by 2', 5' ADP-Sepharose 4B gel affinity chromatography and the purity of the enzyme was controlled by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, the effects of clindamycin antibiotic on enzyme activity were investigated. The IC50 value was calculated as 28.75 mM by plotting the % Activity-[I] graph for the clindamycin antibiotic, which showed an inhibitory effect on the enzyme activity. In addition, in order to determine the Ki constant and inhibition type, preliminary experiments were carried out to determine the most suitable five different substrate concentrations and Lineweaver-Burk graphs were drawn by making measurements for each substrate concentration with the three most appropriate inhibitor concentrations. With the help of graphic, the Ki constant of the clindamycin antibiotic was determined as 41.64±11.89 mM and the inhibition type was determined competitively.

**Keywords:** Glucose 6-phosphate dehydrogenase, clindamycin, inhibition

**1. INTRODUCTION**

Glucose-6-phosphate dehydrogenase enzyme (G6PD; E.C. 1.1.1.49) in the presence of nicotinamide adenine dinucleotide (NAD+) catalyzes the first reaction of the pentose phosphate pathway, producing nicotinamide adenine dinucleotide phosphate (NADPH), as well as synthesizing ribose phosphates (one of the enzymes at the key point of metabolism). Adem et al., 2012). Ribose phosphates are components of vital biomolecules such as NAD+ and deoxyribonucleic acid (DNA) adenosine triphosphate (ATP), flavin adenine dinucleotide (FAD), ribonucleic acid (RNA), acetyl coenzyme A (CoA) (Çiftci et al., 2000; Sevki and Ciftci, 2012). NADPH released during the reaction catalyzed by G6PD functions as the substrate of the Glutathione reductase (GR) enzyme, which is responsible for the synthesis of the glutathione (GSH) molecule, which acts primarily as an antioxidant in cell metabolism. In addition to serving as an antioxidant, the GSH molecule is also involved in DNA and protein synthesis, amino acid transport, detoxification of some antineoplastic drugs and some metabolic end products. As can be understood from here, the G6PD enzyme, which is involved in the production of the GSH molecule, which is the intracellular defense system, acts indirectly in protecting cells against the harmful effects of oxidized molecules (Lu et al., 2014; Akkemik et al., 2011). In addition, NADPH acts as a coenzyme in the reduction of another antioxidant, thioredoxin (Trx) (Kose et al., 2015). The thioredoxin system has an important role in DNA synthesis, prevention of oxidative stress and peroxide damage, apoptosis, cellular growth and stimulation of transcription factor activity (Carlberg and Mannervik., 1981; Çiftci et al., 2000; Altikat et al., 2002; Keha et al., 2002). Küfrevioğlu.,2010). Due to this mentioned importance, in this study, it was aimed to investigate the effects of clindamycin antibiotic on G6PD enzyme activity purified from sheep spleen tissue.

Clindamycin, a narrow-spectrum antibiotic, is effective against most gram-positive species and gram-negative anaerobic species of some pathogenic bacteria and inhibits these pathogens by binding to the 50S ribosomal subunits. It is an alternative drug to penicillin used in the treatment of bacterial infections caused by a number of gram-positive odors, including bone or joint infections, strep throat, pneumonia, middle ear infections, pelvic inflammatory disease, and endocarditis (Kayaalp 2002).

It is known that many antibiotics and drugs are used in human and animal treatment in studies (Dilek et al., 2013). The target of many of these drugs is specifically regulatory enzymes in metabolism. Drug inhibitors are used in the treatment of diseases by binding to enzymes reversibly or irreversibly. Therefore, enzymes constitute an active research area in pharmacology (Kose et al., 2015). It is known that the G6PD enzyme is purified and characterized from many living tissues in the literature searches, and inhibition studies of many drug molecules on enzyme activity are carried out (Çoban and Ciftci., 2022; Çiftci et al., 2000; Sevki and Ciftci., 2012). However, the effects of clindamycin antibiotic on G6PD enzyme purified from sheep spleen tissue have not been investigated.

**2. MATERIAL AND METHOD**

**2.1. Material**

The clindamycin antibiotic used in our study was commercially available, TEMED, EDTA protein standards, ammonium sulfate, Tris, 2', 5' ADP-Sepharose 4B affinity gel G6P, NADP+, electrophoresis chemicals, and other chemicals from Sigma-Aldrich Com. (St. Louis, MO) and Merck (Darmstadt, Germany).

**2.2. Preparation of Homogeneous**

The sheep spleen tissue used in this study was obtained from the Combined Meat and Milk Institution of Bingöl according to the cold chain rules. To prepare G6PD enzyme homogenate, spleen tissue was brought to room temperature and 10 grams of tissue were cut into small pieces and suspended in 30 mL of 1 M Tris-HCl (pH= 8.0) buffer. The tissue, which was thoroughly shredded in the homogenator, was centrifuged for 1 hour at 10,000xg, and the precipitate was discarded and homogenate was formed (Çoban and Ciftci, 2022; Smith, 1987; Keha and Küfrevioğlu, 2010).

**2.3. Ammonium Sulphate Precipitation and Dialysis**

For the prepared homogenate, ammonium sulfate precipitation was performed at 40%-60% saturation concentrations according to the salting-out method. The precipitate in this range was dissolved in 1 M Tris-HCl buffer (pH= 8.0) and the enzyme solution was transferred to dialysis bags and dialyzed against 50 mM K-acetate/ 50 mM K-phosphate (pH= 7.0) buffer for 2 hours (Çoban et al. Ciftci, 2022; Smith, 1987; Keha and Küfrevioğlu, 2010).

**2.4. of 2',5'-ADP Sepharose-4B Affinity Column and Purification of Sheep spleen G6PD Enzyme**

Partially purified enzyme sample after ammonium sulfate precipitation was purified by 2', 5' ADP Sepharose-4B affinity chromatography. First of all, an affinity column was prepared. For a bed volume of 10 mL, 2 g of dry 2', 5'-ADP sepharose 4B gel was weighed and washed several times in 400 mL of distilled water to remove solids. At the same time, the gel was swollen with the washed water. After deaerating the gel with vacuum using a water trumpet, it was suspended with equilibration buffer (0.1M KH2PO4 + 0.1M K-acetate pH= 6.0). The suspended gel was packed into a cooled column consisting of a 1x10 cm closed system. After the gel precipitated, it was washed with wash and equilibration buffer with the help of a peristaltic pump. The equilibration of the column was understood from the approximate equalization of the absorbance and pH of the eluate and buffer at 280 nm. Thus, the affinity column was prepared. 11 mL of G6PD enzyme solution was loaded onto the prepared column. Affinity column 25 mL 0.1M KH2PO4 +0.1M K-acetate (pH= 6.0 ), 25 mL,0.1M KH2PO4 +0.1M K-acetate (pH= 7.85) and 25 mL, respectively It was washed with solutions of 0.1M KH2PO4 + 0.1M KCl (pH= 7.85). The washing process was followed in the spectrophotometer and the absorbance values ​​were determined to be approximately equal to the blank. After washing the column, the G6PD enzyme was eluted from the affinity column with elution buffer (80 mM KH2PO4 + 80 mM KCl + 0.5 mM NADP+ + 10 mM EDTA pH= 7.85) (Çoban and Ciftci, 2022; Bradford, 1976; Smith, 1987). ; Demir et al., 2006; Keha and Küfrevioğlu, 2010, Temel and Taysı, 2019).

**2.5. Measurement of Glucose 6-Phosphate Dehydrogenase Enzyme Activity**

G6PD enzyme activity was measured spectrophotometrically according to the Beutler method. This method is based on the principle that NADPH, which is formed as a result of the reduction of coenzyme NADP+ with GSH in the reaction catalyzed by the G6PD enzyme, gives maximum absorbance at 340 nm (Beutler, 1971).

**2.6.** **Protein Determination**

Quantitative protein amount, enzyme homogenate, ammonium sulfate precipitate and pure enzyme sample were determined spectrophotometrically at 595 nm according to the Bradford method. The standard chart was created using bovine serum albumin protein (Bradford, 1976).

**2.7. Enzyme Purity Control with SDS-PAGE**

Enzyme purity control 3-8% batch sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS-PAGE) was carried out according to the method of Laemmli (Laemmali, 1970).

**2.8. Kinetic Studies**

Inhibition effects of clindamycin antibiotic on G6PD enzyme activity were investigated in kinetic studies. The % Inhibition-[I] graph was drawn to calculate the IC50 value by measuring the antibiotic, which showed an inhibitory effect as a result of the activity measurements, at different inhibitor concentrations. In addition, preliminary experiments were carried out to determine the Ki constant and inhibition type, and the most suitable five different substrate concentrations were determined. Ki constant and inhibition type were determined from the graph obtained (Smith, 1987; Keha and Küfrevioğlu, 2010).

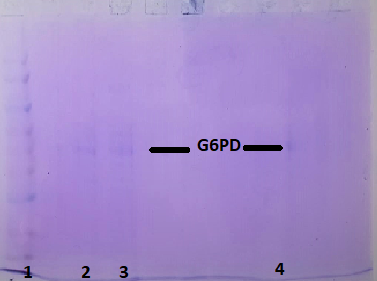
**3. RESULTS**

Using ammonium sulfate precipitation and 2', 5', ADP-Sepharose-4B affinity chromatography, G6PD enzyme, which has 15.24 EU/mg protein specific activity, was purified 1120.5 times with 40% yield and the results are shown in Table 1.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Purification step | Total  Volume  (mL) | Activity (EU/mL) | Protein  (mg/mL) | Total  Protein  (mg) | Total  Activity | Specific  Activity  (EU/mg protein) | % Yield | Purification  Number of floors |
| Homogenate | 25 | 0,932 | 97,47 | 2437 | 23,300 | 0,0096 | 100 | 1 |
| Ammonium sulfate precipitation (40-70%) | 11 | 1,484 | 108,81 | 1197 | 16,324 | 0,0136 | 70,06 | 1,416 |
| Affinity  Chromatography | 7,5 | 1,265 | 0,083 | 0,63 | 9,4875 | 15,24 | 40,71 | 1120,5 |

**Table 1.** Sheep spleen tissue G6PD enzyme purification steps

SDS-PAGE was performed to determine the enzyme purity. The single band obtained on the gel is shown in figure 1.



**Figure 1.** Well 1: standard proteins (14kDa - 175 kDa), wells 2, 3 and 4: Pure G6PD enzyme eluted from the affinity column.

In kinetic studies, clindamycin antibiotic was used at 1.75, 3.5, 7.04, 17.5, 35, 52.5 and 70 mM concentrations on G6PD enzyme activity purified from sheep spleen tissue. The IC50 value was calculated as 28.75 mM by plotting the % Activity-[I] graphs of this antibiotic with an inhibitory effect (Figure 2). In addition, Linevawer-Burk plot was drawn for G6PD at 3 different inhibitory clindamycin and 5 different substrate concentrations, and the Ki constant for clindamycin was 41.64±11.89 and the inhibition type was determined competitively (Figure 3).

**Figure 2.** [Clindamycin]-% Activity graph on sheep spleen G6PD enzyme.

**Figure 3.** Lineweaver-Burk plot plotted for sheep spleen G6PD enzyme at 5 different substrates and 3 different clindamycin concentrations.

**4. ARGUMENT**

G6PD, the first enzyme of the pentose phosphate pathway, is responsible for the production of NADPH, which acts as a coenzyme in the antioxidant system, together with the 6-phosphogluconate dehydrogenase enzyme, as seen in the following reaction.

G6PD

Glukoz 6-fosfat + NADP+  6- fosfoglukonat + NADPH + H

In the researches, it has been determined that antioxidant systems degenerate due to reasons such as drugs, nutrition and oxidative stress. The increase in malondialdehyde (MDA), an indicator of lipid peroxidation, showed that these cells were senescent (Stocks et al., 1972). This situation is related to the decrease of GSH-related enzymes such as G6PD, GR (Mieyal et al., 1991). In addition, abnormal activation of G6PD is associated with cancer. Fast-growing cancer cells increase the production of NADPH they need by constantly stimulating G6PD (Patra et al., 2014). The liver tissue, which is the most important organ of the antioxidant system, and its enzymes have been extensively studied for GSH molecule studies in the literature. In experiments on animals, it has been determined that GSH biosynthesis is reduced by various xenobiotics such as butionine, sulfoximine, acetaminophen and bromobenzene. In hepatocytes exposed to these, GSH is depleted and liver damage occurs. It has been stated that glutathione sources should be increased in order to correct this situation (Atamer et al., 2000).

Since the G6PD enzyme is directly or indirectly associated with many diseases, the research and development of G6PD inhibitors is important (Luo et al., 2021). In our study, G6PD enzyme was purified from sheep spleen tissue by ammonium sulfate precipitation and 2',5' ADP-Sepharose-4B affinity chromatography. Affinity chromatography is a chromatography method based on specific interactions, which is common in enzyme purifications, used in the purification of protein, DNA, RNA and other macromolecules in a short time and with high purity (Ciftci et al., 2001). In the second stage of the study, the effects of the antibiotic clindamycin, which is widely used in the treatment of humans and animals, on the enzyme were investigated. As a result of kinetic studies, IC50 values ​​for clindamycin were calculated as 28.75 mM, Ki constant was calculated as 41.64 ± 11.89 mM, and inhibition type was also determined competitively.

In the literature review on G6PD, it was determined that the G6PD enzyme was purified from sheep erythrocyte tissue and the effects of penicillin G potassium, gentamicin sulfate and amikacin antibiotics on enzyme activity were investigated. In this study, it was observed that gentamicin sulfate inhibited the G6PD enzyme with IC50 values ​​of 10.01 mM, penicillin G potassium 12.83 mM and amicasin 41.88 mM (Beydemir et al., 2002). In addition, in the study investigating the inhibitory effects of lincomycin, amoxicillin, ampicillin, iveral, gentamicin, streptomycin sulfate, novamizole ketogenic, cefuroxime sodium, cefazolin sodium and tylosin on the G6PD enzyme activity purified from sheep spleen tissue, iveral was the most effective on the enzyme with an IC50 value of 0.62 mM. It was determined that lincomycin had the lowest inhibitory effect on the enzyme with an IC50 value of 231 mM (Çoban and Ciftci., 2022). In another study, the effect of the hormone melatonin on the G6PD enzyme activity purified from human erythrocytes was investigated. As a result, it has been observed that this hormone increases enzyme activity (Bayindir et al., 2018). In a study in which the G6PD enzyme was purified from rat erythrocytes, clindamycin antibiotic was used on enzyme activity and it was observed that clindamycin inhibited the G6PD enzyme competitively with an IC50 value of 34.65 mM and a Ki constant of 39.8mM (Temel et al., 2020). Our study is in agreement with this study. In addition, clindamycin antibiotic was studied on the GR enzyme activity purified from sheep spleen tissue and it was observed that it did not show any activation or inhibition on the enzyme (Çoban, 2022). In another study, G6PD enzyme was purified from rat heart and lung tissues and the effects of some drugs on its activities were investigated. These drugs; gentamicin, ceftazidime, digoxin, cefuroxime, methylprednisol, teicoplanin furosemide, dopamine, furosemidine, Adrenaline, lidocaine, metoprolol tartrate, verapamil HCl, levofloxacin, cefazolin and cotin. Of these drugs, cefazolin, ceftazidime, cefuroxime furosemide, gentamicin, levofloxacin, methylprednisol, and teicoplanin inhibited the G6PD enzyme in rat lung tissue (Adem, 2011).

As a result, it would be beneficial to adjust the dose considering the values ​​obtained in the therapeutic use of clindamycin antibiotic, which has a competitive inhibition effect for the sheep spleen G6PD enzyme.

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