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The Effects of Deltamethrin and Diazinon Chemicals on Human Erythrocyte Glucose-6-Phosphate Dehydrogenase Enzyme

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Abstract
This study aims to analyze the purification of Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP+ oxidoreductase EC 1.1.1.49; G6PD) enzyme, which is important for metabolism, from human erythrocytes and to analyze the effects of some pesticides on enzymes. The purification process was performed in two steps being ammonium sulfate precipitation and 2', 5' ADP-Sepharose 4B affinity chromatography. It is identified via sodium dodecyl sulphate polyacrylamide gel (DSD-PAGE) electrophoresis that G6PD enzyme's molecular weight is about 59000 daltons. Km values for G6PD enzyme's $NADP^+$ and glucose-6-phosphate (G6P) substrates are respectively 0.14mM, 0.22mM; and, V_{max} values are respectively 2.76 U/mg and 1.94 U/mg. Enzyme activities were spectrophotometrically measured on 340 nm according to Beutler method. Pesticides analyzed in this study are deltamethrin and diazinon. After their specific concentration in IC_{50} studies, deltamethrin and diazinon substances caused turbidity in quartz vessel. Since reliable absorbance values cannot be obtained in such condition, concentration amounts of substances applied to enzyme did not reduce the activity under 50%. Because of this reason, inhibition rates could not be identified. However, it is identified that all of the pesticides show inhibition effect on enzyme activity.

Key Words: G6PD, İnhibition, purification, pesticide

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49.) enzyme functions as the first enzyme of pentose phosphate way [1]. The basic physiological work of this enzyme is the production of NADPH and ribose-5-phosphate [2]. Ribose-5-phosphate takes part in the construction of DNA and RNA, while NADPH protects several organic molecules such as nucleic acids, proteins, and membrane lipids from oxidative stress created by free radicals [3,4]. Pentose phosphate way in erythrocytes provides necessary NADPH for the reduction of oxide glutathione. The composed reduced glutathione (GSH) and GSH-dependent enzymes protect cell from endogenous and exogenous toxic compounds and reactive oxygen species (ROS) [5]. The gene, which is responsible for the synthesis of G6PD the important enzyme of pentose phosphate way, is in X-chromosome's q28 zone, and composed of 12 introns and 13 exons. G6PD gene has about 20 Kb length while mRNA on which the gene is coded has 2.269 base pair length [6].

Pesticides are the chemicals that are used to prevent or control the negative effects of harmful organisms [7]. Although pesticide has use positive sides, it also has some disadvantages such as potential toxicity for humans and animals [8]. Pesticides have been carried to clean water resources such as lakes and rivers by irrigation water or rainwater. This situation can be hazardous for all living creatures. These chemicals, which contain medicine and metal ions, affect metabolism by increasing or decreasing the enzyme activity in low concentrations [9]. Being a pesticide with organophosphore, diazinon is widely used in the control of cultivated areas against flies, pediculus, ground beetles, and nematodes [10-12]. Since diazinon prevents acetylcholinesterase (AChE) activity, it has hazardous effects on aquatic species in cultivated areas [13]. Deltamethrin is a synthetic pyrethroides that has insecticidal features, and it is a pesticide used in plant production against ectoparasites and insects [14]. It is stated that deltamethrin has some toxic effects; for example, it is observed that it is extremely toxic against fishes and aquatic insects and accordingly this effect negatively affects crustaceans in aquatic fauna by causing the increase of algae [15]. In this study, in vitro effects of diazinon and deltamethrin pesticides on G6PD enzyme, purified from human erythrocytes, were investigated.

MATERIALS AND METHODS

Chemicals

Chemicals for Glucose-6-phosphate, $NADP^+$, NADPH, protein marker, and electrophoresis used in this experimental study are supplied by Sigma Chem. 2',5'-ADP-Sepharose 4B is supplied from Pharmacia; and, deltamethrin and diazinon pesticides whose effects on G6PD enzyme activity is to be analyzed are supplied from centers where pesticides are sold.

Preparation of Hemolysate

Fresh human blood taken into tubes that include ethylenediaminetetraacetic acid (EDTA) was daily used by bringing into the laboratory at +4 ^oC. With the aim of erythrocytes separation, blood sample was centrifuged for 10 minutes on 5000 rpm. Serums and erythrocytes were separated from each other. Plasma and leukocyte layers on tubes upper parts were taken via a suction bulb. Acquired

erythrocytes were hemolyzed with iced pure water that is five times more than their volume. With the aim of removing erythrocytes' cell membranes, samples were centrifuged for 30 minutes at +4 ^{o}C and 15000 rpm. Supernatant was taken via a suction bulb [16]. All processes were performed at +4 ^{o}C.

Ammonium Sulfate Precipitation and Dialysis

For the hemolysate prepared from the blood sample, ammonium sulfate precipitation was practiced first between the ranges of 30-0% then between the ranges of 30-70%. Samples were centrifuged for 30 minutes on 15000 rpm. Then, the precipitation processes were completed by adding necessary potassium phosphate buffer (50 mM, pH=7,0) to the precipitation; and, it is dialyzed at +4 ^{o}C against 50Mm potassium acetate/50Mm potassium sulfate buffer [17].

2', 5' ADP-Sepharose 4B Affinity Chromatography

To prepare affinity gel, 2 gr dry 2', 5' ADP-Sepharose 4B colon material was weighed to form 10 ml deposit volume, and washed for a few times in 400 ml pure water to remove the additives. After the gel, which inflated throughout the washing process, was deflated, it was balanced being 25% balancing buffer (0.1 M Potassium acetate/ 0.1 M Potassium phosphate pH=6.0) and 75% gel. Balanced gel was packed in a 1x10 cm closed jacketed colon. The gel packed in the colon was washed with balancing buffer by using peristaltic pump. Balancing and washing was practiced being 50 ml/hour flow speed [18]. By considering the equalization of the absorbance of eluate, with which the colon was balanced, and the balancing buffer applied to the colon from the top on 280 nm, it was determined whether the colon was balanced or not. Concentrated sample acquired from ammonium sulfate precipitation was applied to the affinity colon, which was balanced with 0.1 M Potassium acetate/0.1 M Potassium phosphate (pH=6,0) buffer solution. The colon was washed respectively with 25 mL 0.1 M Potassium acetate/0.1 M potassium phosphate (pH=6.0), 25 mL 0.1 M potassium acetate /0.1 M potassium phosphate (pH=7.85) and 0.1 M KCl/ (how much ml) 0.1 M potassium phosphate (pH=7.85) solutions. Flow speed was kept under control via peristaltic pump. In this way, a large part of the enzyme held on to the gel and it was removed from other contaminations. Later, enzyme held on to the colon was eluated by using 80 mM potassium phosphate + 80 mM KCl + 0.5 mM NADP + 10 mM EDTA (pH=7.85) elution solution [17].

Glucose-6-Phosphate Dehydrogenase Enzyme's Activity Determination

Enzyme activities were practiced in UV-Vis spectrophotometer at 25 ˚C according to Beutler method [16]. This method is based on the principal that NADPH, formed through the reduction of nicotinamide adenine dinucleotide phosphate (NADP+), gives absorbance on 340 nm. Lowry method was used for the protein quantitative determination [19]. With this method, bovine serum albumin protein was used as standard.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were divided into pieces on a 12% SDS-PAGE gel by using a Minigel system (Bio-Ras Laboratories, USA) [20]. 0.1 Coomassie Brillant Blue R-250 (Sigma) was used as the coloration solution and protein bands were acquired.

In Vitro Inhibitor Studies

In this study, diazinon and deltamethrin insecticides were chosen with the aim of identifying their effects on G6PD enzyme. To identify IC_{50} value (IC_{50}; inhibitor concentrations causing 50% inhibition), enzyme activity values were used through applying pesticides in different concentrations. Enzyme activities were measured in 0.60mM, 3.04mM, 5.60mM, 5.77mM, 6.08mM and 6.38mM vessel concentrations for diazinon; and in 0.76mM, 0.91mM, 1.14mM and 1.52mM vessel concentrations for deltamethrin. As control group, enzyme activity without pesticide was measured and the activity was taken as 100% in the graphic. Through drawing an inhibition graphic against pesticides concentrations, IC_{50} values were calculated.

RESULTS AND DISCUSSION

G6PD enzyme in human erythrocytes was purified by using ammonium sulfate and 2', 5' ADP-Sepharose 4B affinity chromatography. Through practicing ammonium sulfate precipitation between the ranges of 30-70%, impurity in the total protein was reduced. Precipitation was thawed for G6PD activity and it was applied to 2', 5' ADP-Sepharose 4B affinity chromatography. In Figure 1, G6PD enzyme's elution graphic is seen from 2', 5' ADP-Sepharose 4B affinity colon. G6PD enzyme was purified 7068 times with 33.65% efficiency and its activity was found as 70.7 U/mg (Table 1).

Purified with SDS PAGE analysis, G6PD enzyme's molecular weight is 59 kDa and weight standard is demonstrated in Figure 2. For the reaction kinetics of the purified G6PD, K_m and V_{max} values were found through using G6P and $NADP^+$ substrates and through drawing Linewear-Bork graphic. G6P and $NADP^+$ substrates' K_m values are respectively 0.221 and 0.141; and, V_{max} values are respectively 1.94 and 2.76 EU/ml. As it can be seen in Table 2, enzyme's relevance to $NADP^+$, whose K_m value is lower, is higher than G6P. It was identified that known as catalytic activity, also the rate of V_{max}/K_m is higher in NADPH compared to G6P.

It is known that G6PD plays the basic role in metabolism activities [21]. Existing in all cells and being produced in enzyme pentose phosphate way, NADPH's take part in cholesterol, steroid, fatty acid, nucleic acid, amino acid and nucleotide-structured coenzyme synthesis, and in the reduction of methaemoglobin erythrocytes [22]. Pesticides don't damage humans, domestic animals, and wildlife if they are used against pests in an adequate dose [23]. However, the increase in pesticide use, and the unconscious use of pesticides are threatening for the environment. These sprayed insecticides and herbicides affect the non-targeted creatures rather than targeted creatures [24]. On the basis of this situation's importance, diazinon and deltamethrin pesticides, whose inhibition effects on G6PD enzyme were analyzed, were solved with DMSO. In Table 3, enzyme activities % activity change in pesticides different concentrations were demonstrated. After their specific concentration in IC_{50} studies, deltamethrin and diazinon substances caused turbidity in quartz vessel. Since reliable absorbance values cannot be obtained in such condition, concentration amounts of substances applied to enzyme didn't reduce the activity under 50% (Figure 3 and Figure 4). Inhibition rates for these pesticides could not be identified.

Table 1. Purification of human erythrocyte glucose-6-phosphate dehydrogenase

	Total protein (mg/ml)	Specific activity (U/mg)	Yield (%)	Purification factor
Hemolysate	732,270	0,01	100	1
Ammonium sulfate	391,125	0,012	68,18	1,2
2',5'ADP-Sepharose 4B affinity chromatography	0,035	70,681	33,65	7068,1

Table 2. Kinetic parameters of human erythrocyte glucose-6- phosphate dehydrogenase

Substrat	K_m (mM)	V_{max} (EU/ml)	V_{max} (EU/ml)/ K_m (mM)
$NADP^+$	0,141	2,76	19,57
G6P	0,221	1,94	8,77

Table 3. Enzyme activities' % activity change in pesticides different concentrations

Chemicals	C (mM)	% Act.	C (mM)	% Act.	C (mM)	% Act.	C (mM)	% Act.	C (mM)	% Act.	C (mM)	% Act.
Deltamethrin	0,76	90	0,91	76	1,14	64	1,52	56	-	-	-	-
Diazinon	0,60	96	3,04	93	5,60	84	5,77	78	6,08	69	6,38	66

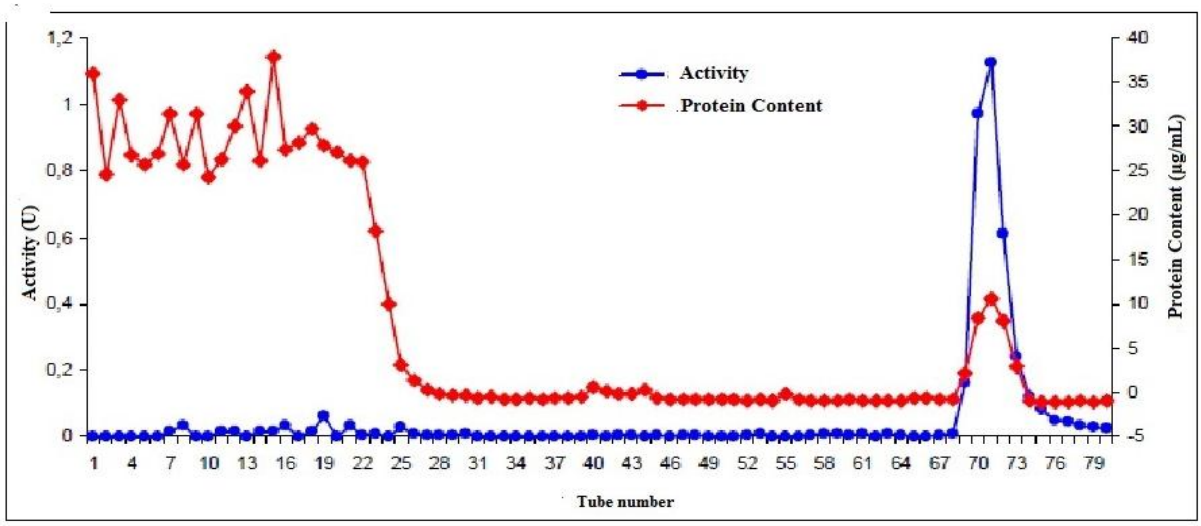

Figure 1. Purification of human erythrocyte glucose-6-phosphate dehydrogenase by affinity chromatography

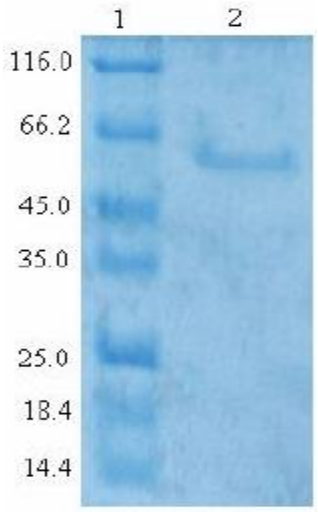

Figure 2. SDS-PAGE of purified human erythrocyte glucose-6-phosphate dehydrogenase. molecular weight standards (*β*-galactosidase,116 kDa; bovine serum albumin, 66.2 kDa; egg albumin, 45 kDa; lactate dehyrogenase, 35 kDa; Rease Bsp981 (*E.coli*), 25 kDa; *β*-lactoglobulin, 18.4 kDa; Lysozyme, 14.4 kDa

In literature review, no research has been found about the insecticides used in the present study effects on human erythrocyte G6PD enzyme's activities. However, there are researches about some pesticides and chemicals' effects on G6PD enzyme's activities. Özmen (2009). investigated in vitro effects of 2,4-dihidroxy-5fluoropyrimidine and sodium 2-sulfaniletansulfanot, which are cytotoxic chemical, on human erythrocyte G6PD enzyme; and, found out that these substances inhibit the enzyme [25]. Hopa et al. (2011) investigated in vitro effects of glyphosate and 2.4-dichlorophenoxy acetic acid (2,4-D) pesticides on human erythrocyte G6PD enzyme. Glyphosate's inhibition type is identified as semi competitive; its Ki value is identified as 13.45 mM; its IC_{50} value is identified as 32.35 mM; and, 2.4-D's inhibition type is identified as competitive; its Ki value is identified as 8.45 mM; its IC_{50} value is identified as 38.34 [21]. The effects of deltamethrin, cypermethrin, and propoxur on G6PD enzyme, which was purified from the blood of rainbow trout by Şentürk et al. (2009), were investigated. Deltamethrin's Ki value is identified as 1.84+-0.33 mM, IC_{50} value is identified as 0.63 mM, and inhibition type is

identified as noncompetitive; cypermethrin's Ki value is identified as 2.69+-0.18mM, IC_{50} value is identified as 1.02 mM, and inhibition type is identified as noncompetitive; propoxur's Ki value is identified as 16.55+-0.35 mM, IC_{50} value is identified as 12 mM, and inhibition type is identified as noncompetitive[8]. Pençe et al. (2000) applied ceftriaxone disodium trisesquihydrate, penicillin G potassium and amoxicillin sodium antibiotics on human erythrocyte G6PD enzyme. Medicines' IC_{50} values are respectively 0.71 mM, 56 mM and 138 mM and at the end of the research, it is found out that these medicines show inhibition effect on G6PD enzyme [26]. Erdoğan et al. (2004) investigated the inhibition effects of tiamphenicol, amicasin, gentamicin, and netilmicin antibiotics on G6PD enzyme, which was purified from rainbow trout. As a result of the study, while tiamphenicol and amicasin show competitive inhibition, gentamicin and netilmicin show noncompetitive inhibition [27]. Haghighi and Ilghari (2004) analyzed the inhibition effect of Cd(II), Al(III) and Ni(II) on human erythrocyte G6PD enzyme. As a result, it is found out that metal ions alternately inhibit G6PD enzyme [28].

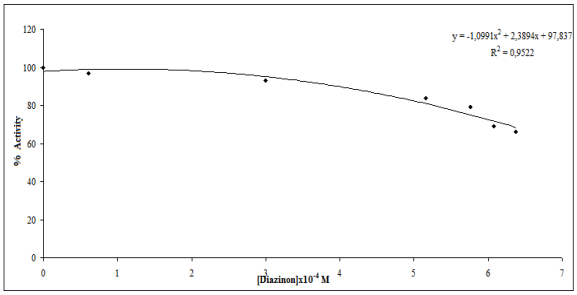

Figure 3. Activity % curve of G6PD in different diazinon concentrations

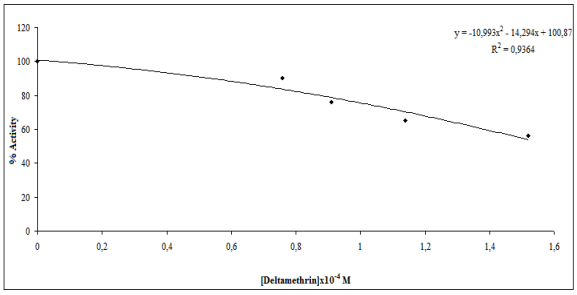

Figure 4. Activity % curve of G6PD in different deltamethrin concentrations

In conclusion, while the effects of insecticides named *0,0*-diethyl *0*-2-isopropyl-6-methylprimidin-4-il phosphorothioate (Diazinon), (*S*)-α- cyano-3-phenoxybenzil (1*R*,3*R*)-3-(2,2-dibromovinil)-2,2dimetilsiklopropan carboxylate (Deltamethrin) on human erythrocyte G6PD enzyme's activity were analyzed, DMSO was used in the solving process because they are insoluble in water. Since there is turbidity in the solution when deltamethrin's more concentrated solution than $1.52x10^{-4}$ M was prepared and transferred into 1 ml. quartz vessel, IC_{50} value could not be found. When diazinon's more concentrated solution than $6.380x10^{-4}$ M was added into 1 ml. vessel, turbidity occurred in the solution like it occurred in deltamethrin. It is identified that in low concentrations, deltamethrin and diazinon insecticides reduce enzyme activity respectively to 56% and 66% (Figure 3 and Figure 4). In accordance with the acquired data, it is observed that these two pesticides show important inhibition effect on G6PD enzyme. Therefore, in their use with agricultural aims, dose adjustment should be paid attention

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