Journal of Enzyme Inhibition and Medicinal Chemistry, October 2005; 20(5): 485–489

Effects of some drugs on the activity of glucose 6-phosphate dehydrogenase from rainbow trout (*Oncorhynchus mykiss*) erythrocytes *in vitro*

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(Received 10 April 2005; in final form 10 May 2005)

Abstract

Inhibitory effects of some drugs on glucose 6-phosphate dehydrogenase from the erythrocytes of rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) were investigated. The enzyme was purified 2488-fold in a yield of 76.8% using ammonium sulfate precipitation and 2′,5′-ADP Sepharose 4B affinity gel at 4°C. The drugs pental sodium, $MgSO_4$, vancomycin, metamizol, marcaine, and prilocaine all exhibited inhibitory effects on the enzyme. While $MgSO_4$ ($K_i = 12.119$ mM), vancomycin ($K_i = 1.466$ mM) and metamizol ($K_i = 0.392$ mM) showed competitive inhibition, pental sodium ($K_i = 0.748$ mM) and marcaine ($K_i = 0.0446$ mM) displayed noncompetitive inhibition.

Keywords: *G6PD enzyme, oncorhynchus mykiss, inhibition, drug, glucose 6-phosphate dehydrogenase*

Introduction

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ oxidoreductase EC 1.1.1.49; G6PD) is the first enzyme in the pentose phosphate pathway. The main physiological function of G6PD is to produce NADPH and ribose 5-phosphate, which are essential for reductive biosynthesis, nucleic acid and membrane lipids synthesis [1–3]. NADPH in erythrocytes regeneration of reduced glutathione, which prevents hemoglobin denaturation, preserves the integrity of red blood cell membrane sulfhydryl groups, and detoxifies hydrogen peroxide and oxygen radicals in and on the red blood cells [4,5]. When one molecule palmitate is synthesized in the biosynthesis of fatty acids, 14 NADPH molecules are used. Six molecules of NADPH are synthesized in the pentose phosphate pathway which is essentially more active in adipose tissue than muscle,is showing that the G6PD enzyme is very important in the biosynthesis of fatty acids [3,6]. Growth rate in fish is influenced by various factors such as dietary regime, and feeding competition and frequency [7]. Fish in general require more dietary protein than do other vertebrates and NADPH plays an important role in initiating protein synthesis [8]. NADPH is essential to growth and proliferation processes, serving as hydrogen and electron sources for a variety of reductive biosynthetic reactions, including the synthesis of fatty acids and cholesterol [9,10].

Although the effects of many drugs on rainbow trout, human and rat G6PD enzyme activity has been investigated [11–14], no reports could be found in the literature on the *in vitro* effects of pental sodium, $MgSO_4$, vancomycin, metamizol, marcaine, and prilocaine on rainbow trout erythrocyte G6PD. This study was aimed at purifying rainbow trout erythrocyte G6PD, and to determine the effects of some drugs on the enzyme.

Materials and methods

Chemicals

2′,5′-ADP Sepharose 4B was purchased from Pharmacia. $NADP^+$, glucose 6-phosphate, protein assay reagent, and chemicals for electrophoresis were

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ISSN 1475-6366 print/ISSN 1475-6374 online © 2005 Taylor & Francis
DOI: 10.1080/14756360500213256

purchased from Sigma. All other chemicals used were of analytical grade and were purchased from either Sigma or Merck.

Fish husbandry and maintenance

Fish samples were obtained from the rainbow trout farm of the Department of Aquaculture, Faculty of Agriculture, Ataturk University. The live rainbow trout (n = 5) used in this study were mature, healthy, 4–5 years old fish with an average weight of 1.6–2.2 kg. The average water temperature was 9 ± 2°C during the tests. At the time of sample collection fish were fed a commercial trout feed at 2% body weight twice per day. Prior to the experiment, fish in each group were kept in 1 × 1.2 m (wide-deep) fiber-glass tanks for one month. Tanks were supplied with fresh water at a flow rate of 0.01 l/min per kg of body weight. The water quality parameters were measured as $O_2 = 8.8$ ppm, pH = 8.1, $SO_4^{-2} = 0.33$ mg/l, PO_4^{-3} = trace, $NO_3^- = 3.45$ mg/l, NO_2^- = trace, and conductivity = 240 us/cm.

Preparation of the hemolysate

Blood was sampled from the caudal vein using a 10 ml plastic heparinized syringe (5 IU/ml). It was then transferred to tubes and centrifuged (Hettich® Micro 22 R, refrigerated centrifuge) at 2,500 × g for 15 min. The plasma was removed by drip. After the residue of red cells was thrice washed with KC1 solution (0.16 M), the samples being centrifuged at 2,500 × g each time and supernatants removed. The erythrocytes were hemolyzed with 5 volumes of ice-cold water and centrifuged (+4°C, 10.000 × g) for 30 min to remove the ghosts and intact cells [15].

Ammonium sulphate fractionation and dialysis

The hemolysate was subjected to progressive precipitation by the slow addition of ammonium sulfate (10–20%, 20–30%, 30–40%, 40–50%, 50–60%, 60–70% and 70–80%). This mixture was centrifuged at 5,000 × g for 15 min and the precipitate was dissolved in 50 mM phosphate buffer (pH 7.0). For each respective precipitation, the enzyme activity was determined in both the supernatant and precipitate. The enzyme was observed to precipitate at 40–65% precipitation. It was then dialyzed at 4°C in 50 mM K-acetate/5 mM K-phosphate buffer (pH 7.0) for 2 h with 2 changes of buffer [15].

2′,5′-ADP sepharose 4B affinity chromatography

For 10 ml of bed volume, 2 g of dry 2′, 5′-ADP Sepharose 4B was washed several times in 400 ml of distilled water. After the removal of the air in the gel, it was resuspended in buffer (0.1 M K-acetate + 0.1 M K-phosphate, pH 6.0) at a ratio of 25% buffer to 75% gel and was packed in a column (1 × 10 cm). After settling of the gel, it was equilibrated with the same buffer by means of a peristaltic pump (flow rate: 50 ml/h). The dialyzed enzyme solution obtained previously was loaded on the column, and the flow rate was adjusted to 20 ml/h. The column was sequentially washed with 25 ml of 0.1 M K-acetate + 0.1 M K-phosphate (pH 6.0) and 25 ml of 0.1 M K-acetate + 0.1 M K-phosphate (pH 7.85). The washing with the latter was continued until the final absorbance difference became 0.05. Finally, the enzyme was eluted with a solution of 80 mM K-phosphate + 80 mM KC1 + 0.5 mM $NADP^+$ + 10 mM EDTA (pH 7.85). The enzyme activity was measured in the final fractions, and the activity-containing tubes were pooled and the protein content determined. During all procedures, the temperature was kept at +4°C [15–17].

Activity determination

Activity was measured by Beutler's method [18] by spectrophotometric measurement at 340 nm. One enzyme unit was defined as the amount of enzyme reducing 1 umol of $NADP^+$ per min at optimum pH.

Protein determination

Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method [19], with bovine serum albumin being used as a standard.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The control of enzyme purity, using Laemmli's procedure [20], was carried out in 3% and 8% acrylamide concentrations for running and stacking gel, respectively. To the gel solution was added 10% SDS. The gel was stabilized in a solution containing 50% propanol + 10% TCA + 40% distilled water for 30 min. Staining was performed for about 2 h in a solution of 0.1% Coommassie Brilliant Blue R-250 + 50% methanol + 10% acetic acid. Finally, washing was carried out in a solution of 50% methanol + 10% acetic acid +40% distilled water until the protein bands were clear.

Inhibitor studies

In the media with or without inhibitor, the substrate (G6P) concentrations were 0.012 mM, 0.024 mM, 0.036 mM, 0.048 mM, and 0.06 mM. Inhibitor (drugs) solutions were added to the reaction medium, using 3 different fixed concentrations of inhibitor in 1 ml of total reaction volume. K_i values were calculated from Lineweaver-Burk graphs [21].

Table I. Purification scheme for glucose-6-phosphate dehydrogenase from rainbow trout erythrocytes.

Purification step	Activity (U/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Haemolysate	0.241	50	38.4	1920	12.05	0.0062	100	1
Ammonium sulphate precipitation (40–65)%	0.754	14	1.41	11.28	10.55	0.534	87.5	86.13
2′, 5′-ADP Sepharose 4B affinity chromatography	2.315	4	0.15	0.6	9.26	15.43	76.8	2488

In order to determine I_{50} values, activities were calculated with a 0.60 mM constant substrate (G6P) and different inhibitor concentrations. Activity in the absence of drug was taken as 100%. Regression analysis graphs were drawn from inhibition % values by a statistical package (SPSS-for windows; version 10.0) and inhibitor concentrations causing up to 50% inhibition (I_{50}) were determined from the graphs.

Results

G6PD was purified 2488-fold in a yield of 76.8% using ammonium sulfate precipitation and 2′, 5′-ADP Sepharose 4B affinity gel (Table I). SDS polyacrylamide gel electrophoresis was performed after the purification of the enzyme (Figure 1). For each drug the Lineweaver-Burk graphs were drawn and that for the most potent inhibitor graph is shown in Figure 2. The K_i constant and inhibition type was determined as 0.748 ± 0.11, 12.119 ± 0.73, 1.466 ± 0.40, 0.392 ± 0.10 and 0.0446 ± 0.012 mM from the graphs for pental sodium (noncompetitive), $MgSO_4$ (competitive), vancomycin (competitive), metamizol (competitive), and marcaine (noncompetitive) respectively (Table II).

In addition, [Drug] *vs.* % activity graphs were drawn for the drugs and that for the most potent inhibitor graph is shown in Figure 3. I_{50} values were calculated as 1.247, 85.555, 4.789, 1.167, 0.0329 and 0.392 mM from the graphs for pental sodium, $MgSO_4$, vancomycin, metamizol, marcaine and prilocaine respectively. I_{50} values, K_i constants and inhibition type are summarised in Table II.

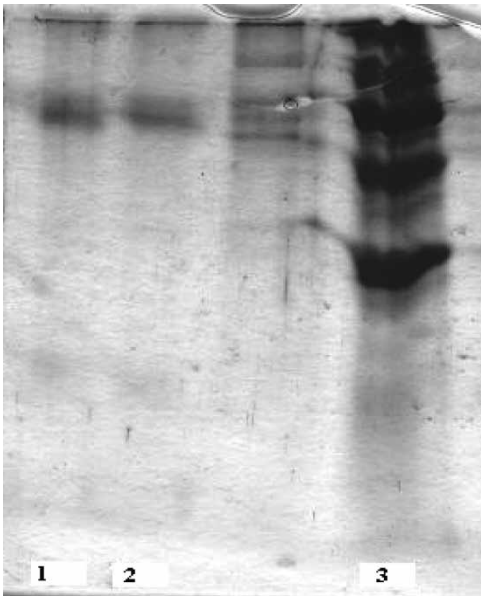

Figure 1. SDS-PAGE bands of G6PD. Lane 1: Rainbow trout erythrocytes G6PD. Lane 2: Chicken erythrocytes G6PD. Lane 3: Standards: *E.Coli* (galactosidase (116,000), rabbit phosphorylase B (97,400), bovine albumin (66,000), chicken ovalbumin (45,000), and bovine carbonic anhydrase (29,000).

Discussion

The importance of G6PD in metabolism has been well known for many years. GSH is used by antioxidant defense mechanisms and its production requires NADPH to be synthesized in the pentose phosphate metabolic pathway in which G6PD and 6PGD participate. For this reason, G6PD and 6PGD have been considered as antioxidant enzymes [22]. In the present study, the *in vitro* effects of some drugs on G6PD have been investigated.

The easier purification method for the enzyme described here makes it possible that investigations

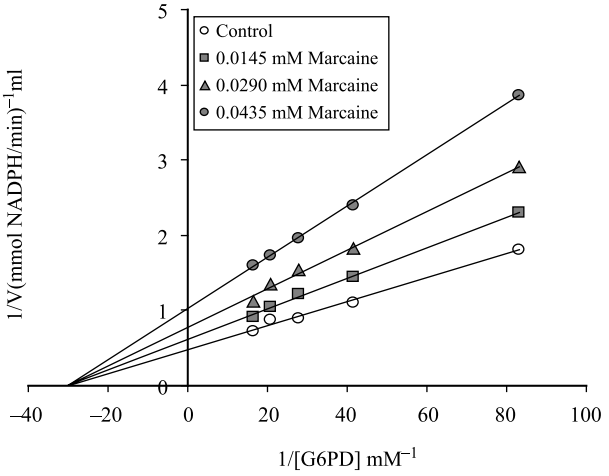

Figure 2. Lineweaver-Burk graph in 5 different substrat (G6P) concentrations and 3 different marcaine concentrations for determination of K_i.

Table II. K_i values obtained from Lineweaver-Burk graphs for G6PD 3 for different drugs.

Drug	I_{50} (mM)	K_i values (mM)	Mean K_i values (mM)	Inhibition type
Pental sodium	1.247	0.664	0.748 ± 0.11	Noncompetitive
		0.874		
		0.706		
$MgSO_4$	85.555	11.446	12.119 ± 0.73	Competitive
		12.014		
		12.897		
Vancomycin	4.789	1.855	1.466 ± 0.40	Competitive
		1.492		
		1.052		
Metamizol	1.167	0.284	0.392 ± 0.10	Competitive
		0.389		
		0.503		
Marcaine	0.0329	0.0493	0.0446 ± 0.012	Noncompetitive
		0.0465		
		0.0382		
Prilocaine	0.392			

G6PD can be easily performed (Table I) since a high purity for the enzyme was also obtained (Figure 1).

It is known that many drugs have adverse effects on organisms when used for therapeutic or other purposes [23] and that these effects may be dramatic and systemic [11]. A good example of this is that in 1926 pamaquine used for malaria treatment caused severe adverse effects in patients within a few days, resulting in black urination, hyperbilirubinemia, a dramatic decrease in blood Hb levels, and finally death, which occurred in cases of severe G6PD deficiency [6]. Similarly, acetazolamide inhibits carbonic anhydrase (CA), giving rise to severe diuresis [24].

However, the inhibitory effects of the drugs considered here on G6PD in fish have not been studied their effects on enzymatic activities in other animal species and human beings have been reported [11,12].

As shown in Table II, the K_i values were 0.748 ± 0.11, 12.119 ± 0.73, 1.466 ± 0.40,0.392 ± 0.10a-nd 0.0446 ± 0.012 mM for pental sodium, $MgSO_4$, vancomycin, metamizol, and marcaine respectively and the corresponding I_{50} values were 1.247, 85.555, 4.789, 1.167, 0.0329 and 0.392 mM respectively. K_i values and I_{50} values show that marcaine was the most potent inhibitor followed by metamizol, pental sodium, vancomycin and $MgSO_4$, respectively. K_i and I_{50} values for metamizol and $MgSO_4$ were similar to those obtained for the G6PD enzyme of human erythrocytes [12]. In another study, it was shown that prilocaine (between 0.38–6.08 mM) did not inhibit human erythrocyte G6PD enzyme activity in *in vitro* [25]. On the contrary, in this study we found that G6PD enzyme activity was inhibited by prilocaine (between 0.0775–0.775 mM) in *in vitro*. It has been reported that thiamphenicol, amikacin, gentamicin, netilmicin, chloramine-T and $CuSO_4$ inhibit rainbow trout red cells G6PD *in vitro* [13,14]. However no studies have been reported in the literature on pental sodium, vancomycin, and marcaine inhibiting G6PD.

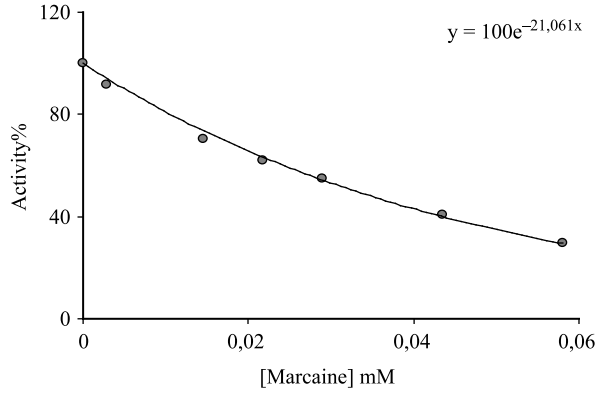

Figure 3. Activity % *vs* [marcaine] regression analysis graphs for Rainbow trout erythrocytes G6PD in the presence of 6 different marcaine concentrations.

In this investigation, these drugs showed potent inhibitory effects on the G6PD enzyme activity of trout erythrocytes. From a knowledge of the the obtained K_i and I_{50} values, undesirable side effects on G6PD activity and body metabolism and fatty acid synthesis can be reduced.

It should be noted that if it is necessary to give these drugs to fish, their dosage should be carefully determined to reduce their hemolytic side effects, since these drugs may be damaging to the health of fish and lead to fatal results.

Today, the number of fish farmers has increased in the world and many antibiotics and drugs are used frequently for bacterial and other diseases of fish, so that human consumers are taking the metabolic products of these drugs into their food chains which could cause side effects. If it is required to give these drugs to fish, then their half lives of drugs and metabolites should be carefully considered.

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