Purification and characterization of 6-phosphogluconate dehydrogenase (6-PGD) from grass carp (Ctenopharyngodon idella) hepatopancreas

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6-Phosphogluconate dehydrogenase (6-PGD, E.C.: 1.1.1.44) was purified and characterized from the hepatopancreas of grass carp (Ctenopharyngodon idella) for the first time. Grass carp represents the second largest aquaculture industry in the world after silver carp, constituting 14.7% of the world aquaculture production, with an average annual increase of 14% in China, mainly as a source of food. The purification procedure involved a single 2’, 5’-ADP-Sepharose 4B affinity chromatographic step by using different elution buffers. The enzyme was purified 309-fold with a specific activity of 5.259 U/mg protein and yield of 68%. The purity and subunit molecular weights of the 6-PGD were checked on SDS-PAGE and purified enzyme showed a single band on the gel. The subunit molecular mass was 57 kDa, with an optimum pH, temperature and ionic strength at 7.96, 50°C and 100 mM Tris-HCl, respectively. The Km values of 6-PGA and NADP+ were 0.019 and 0.0052 mM, respectively, while Vm of 6-PGA and NADP+ was 0.69 U/ml. Dissociation constants (Ki) for 6-PGA and NADP+ were 2.05 and 0.12 mM, respectively. NADPH inhibited the enzyme in a competitive manner and its Ki value was 0.032 mM. The Cu2+, Zn2+, Cd2+ and Al3+ showed inhibitory effects on the enzyme with IC50 values of 0.293, 0.099, 0.045 and 1.526 mM, respectively. All tested metals inhibited the enzyme in a competitive manner, indicating that these metals might be toxic even at low concentrations for the 6-PGD. As the fish is one of valuable foodstuff of animal sources for human consumption, under certain environmental conditions, metal ions accumulated in fish up to a lethal concentration may be harmful for human health. Therefore, it is impending to reduce the concentration of metal ions in contaminated lakes and rivers for fishery and also for human health.

Keywords: Ctenopharyngodon idella, Grass carp, 6-Phosphogluconate dehydrogenase, Purification, Kinetic behavior, Metal ions inhibition.

6-Phosphogluconate dehydrogenase (6-PGD, EC 1.1.1.44) catalyzes the reversible oxidative decarboxylation of 6-phosphogluconate (6PG) to D-ribulose 5-phosphate and CO2 in the presence of NADP+ with the concomitant generation of NADPH2. It is a key enzyme of pentose phosphate metabolic pathway (PPP) and the product NADPH is a co-enzyme participating in the synthesis of a number of biomolecules, such as fatty acids, steroids and amino acids. NADPH also has very important functions in the protection of the cell against oxidative agents by transferring its reductive power to glutathione disulfide (GSSG) via glutathione disulfide reductase3,6.

6-PGD is widely distributed among microorganisms, plants and in different tissues of animals4-9. Due to the important role of the enzyme in the PPP metabolic pathway, 6-PGD has been purified from different sources by gel filtration, ion-exchange and/or affinity chromatography and preparative gel electrophoresis3-16 and has been studied for kinetic properties and mechanistic investigation12,13,17,18. However, in fish, to our best knowledge, study on kinetic parameters is only limited in rainbow trout19, and information on other fish species is very scarce.

In the recent years, due to a dramatic increase in environmental poisoning by pollutants as a consequence of industrial, agricultural and anthropogenic activities, aquatic organisms have been exposed to a significant amount of these pollutants20. Among the pollutants, metals are in particular important since some of them are found in the active sites of some enzymes and can be beneficial at small amounts for metabolic pathways. However, if found in high concentrations in living metabolism, they can cause toxicological effects.
Due to these vital metabolic factors, toxicology studies about the effects of metals on various enzyme activities are becoming increasingly important. Such studies provide precious data about the contaminating concentrations of metal ions and also give important information about the mechanism of actions of these metals in the organisms. At present, to our knowledge, little information is reported on the in vitro effect of metal ions on 6-PGD activity in fish, although in vivo study has shown that tissue 6-PGD is potential target for metal ions and functions to protect against metal toxicity.

Grass carp (Ctenopharyngodon idella) represents the second largest aquaculture industry in the world after silver carp, constituting 14.7% of the world aquaculture production, with an average annual increase of 14% in China, mainly as a source of food. Recently, our laboratory has reported purification and characterization of glucose-6-phosphate dehydrogenase (G6PD) from grass carp. In this study, we report purification and characterization of 6-PGD, a key enzyme of PPP pathway, from hepatopancreas of grass carp.

**Materials and Methods**

**Chemicals**

2', 5'-ADP-Sepharose 4B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Protein markers were purchased from Tiangen Biotech (Beijing, China). 2-Mercaptoethanol (2-ME), NADP⁺, NADPH, 6-phosphogluconate acid (6-PGA) and all other chemicals used were obtained from Sigma-Aldrich Chemical Co., MO, USA.

**Fish husbandry and maintenance**

In the current study, 36 healthy grass carp (body weight 503.9 ± 60.6 g, body length 31.1 ± 0.7 cm) were obtained from a local fish dealer and they were kept in three indoors circular fiberglass tanks, with 12 fish for each tank for 14-day acclimatization. During acclimatization, they were provided with a commercial Haid feed at 2% of body weight daily and with continuous aeration to maintain the dissolved oxygen level above saturation. Fecal matter was removed before feeding.

The experiment was conducted at ambient temperature and natural photoperiod (approx. 12-h light: 12-h darkness during the experiment). The water quality parameters were measured in the morning twice a week: dissolved oxygen > 5.3 mg l⁻¹, pH 6.9-7.5, total ammonia-nitrogen 0.04-0.09 mg l⁻¹, water temperature 25 ± 3°C. All procedures used were approved by the Huazhong Agricultural University Animal Research Ethics Board and conformed to the principles of the China Council for Animal Care.

**Sampling**

At the end of the 2-wk experiment, fish were starved for 24 h before sampling. Then, they were killed by severing of the spinal cord. The hepatopancreas was isolated immediately using sterile forceps in ice, quickly frozen in liquid nitrogen and stored at -80°C (not longer than 2 weeks) for subsequent analysis.

**Purification of 6-PGD from hepatopancreas of grass carp**

6-PGD was purified from grass carp hepatopancreas according to the procedure described previously, with slight modification. The purification procedure after ultracentrifugation consisted of one step: 2', 5'-ADP-Sepharose 4B affinity chromatography. All the procedures were carried out at +4°C.

At first, the hepatopancreas was cut with scissors. Excess blood was removed from the samples after washing with ice-cold saline and homogenized in a glass-Teflon homogenizer with 3 vols of 10 mM Tris-HCl buffer containing 1 mM EDTA, 5 mM 2-ME, pH 7.42 (buffer A) on ice. The homogenate was centrifuged at 100,000 × g for 60 min at 4°C. The supernatant obtained was loaded on to 2', 5'-ADP-Sepharose 4B column (1.6 cm × 10 cm), pre-equilibrated with buffer A. The column was washed with 50 ml of buffer A and washing was continued until the final absorbance was lower than 0.01 at 280 nm. Then, the enzyme G6PD was eluted with 30 ml of 10 mM Tris-HCl + 5 mM 2-ME + 1 mM EDTA+0.177 mM NADP⁺, pH 7.42. After G6PD was washed, 6-PGD was eluted using buffer A containing 2 mM NADP⁺. The flow rate for washing and equilibration was adjusted to 16.2 ml/h. Purification scheme of 6-PGD from grass carp hepatopancreas is shown in Table 1.

**Activity determination**

6-PGD activity assay was carried out at 25°C by following rates of NADP⁺ reduction at 340 nm according to Beutler. For spectrophotometric measurements, the reaction mixture contained 100 mM Tris-HCl buffer, pH 7.96, 10 mM MgCl₂, 0.1 mM NADP⁺ and a suitable amount of the enzyme. The assay was initiated by the addition of 6-PGA to
give a final concentration of 1.0 mM. The production of NADPH was measured every 10 s for 1 min at 340 nm at 25°C. Assays were carried out in duplicates. One unit of enzyme (U) activity was defined as the amount of enzyme that produced 1 µmol NADPH per min and expressed as units per mg of hepatopancreas soluble protein. Protein concentrations were estimated by Bradford method using bovine serum albumin as the standard.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

To determine the enzyme purity and apparent molecular mass of the subunit, SDS-PAGE was performed by Laemmli’s method using markers — Taq enzyme (94 kDa), bull serum albumin (66.2 kDa), chicken ovalbumin (45 kDa), thrombin (33 kDa), a new protein from TIANGEN (26 kDa), soybean trypsin inhibitor (20 kDa) and muramidase (14.4 kDa). The acrylamide concentration of stacking and running gels was 3% and 10% containing 1% sodium dodecyl sulfate (SDS), respectively. The gel was stabilized in a solution containing 50% propanol, 10% trichloroacetic acid, 40% acetic acid and 40% distilled water. The washing was carried out in the same solution without the dye until protein bands were cleared. The electrophoretic pattern was photographed.

Optimal pH, temperature and ionic strength determination

For optimal pH determination, the enzyme activity was measured in 100 mM Tris-HCl buffers within a pH of 6.35, 6.95, 7.56, 7.96, 8.52, 9.00, 9.91 and 10.46, respectively. For optimal temperature determination, the enzyme activity was measured at 5°C interval from 25°C up to 45°C and then at 10°C interval from 50°C to 70°C, respectively at optimal pH 7.96.

For determination of optimal ionic strength determination, 0-160 mM Tris-HCl buffers were used. The ionic strength was varied by adding Tris-HCl buffers at optimal pH for this purpose.

Kinetic studies

For $K_m$ and $V_{max}$ evaluation, Lineweaver-Burk curves were used. They were determined in 5 different concentrations of NADP$^+$ (0.01, 0.02, 0.05, 0.10 and 0.20 mM, respectively) with the constant concentration of 6-PGA. The same experiments were performed for 6-PGA (at 5 different concentrations; 0.10, 0.15, 0.20, 0.30 and 0.50 mM) with a fixed NADP$^+$ concentration. All kinetic studies were performed at 25°C and at pH 7.96. $K_m$ and $V_{max}$ values were determined from Lineweaver-Burk plots with the respective slope and intercept replots.

To determine $K_i$ values of inhibitor NADPH, 3 different NADPH concentrations (0.01, 0.025 and 0.05 mM) were tested. 6-PGA was used as substrate in 5 different concentrations (0.10, 0.15, 0.20, 0.30 and 0.50 mM, respectively). Activities were determined and Lineweaver-Burk graphs were drawn for determination of $K_i$ and inhibition type for NADPH.

Effects of metal ions in vitro

Cu (0, 0.05, 0.1, 0.2, 0.5, 0.6 mM), Zn (0, 0.05, 0.1, 0.2, 0.4, 0.5 mM), Cd (0, 0.01, 0.02, 0.05, 0.1 mM) and Al (0, 0.5, 1.0, 2.0, 3.5 mM) were used as inhibitors to determine the in vitro effect of metal ions on 6-PGD activity, respectively. Assays were carried out with varying concentrations of each metal ion. The activity of control cuvette in the absence of an inhibitor was taken as 100%. All metal ions were tested in triplicates at each concentration used. For each inhibitor, an activity % - [inhibition] graph was drawn. Metal ions concentrations that produced 50% inhibition (IC$_{50}$) were calculated from the regression graphs.

To determine $K_i$ values, three different inhibitor concentrations (0.1, 0.25 and 0.5 mM for Cu, 0.1, 0.25 and 0.5 mM for Zn and 0.01, 0.05 and 0.1 mM for Cd) were tested for each metal ion. In these experiments, 6-PGA was used as substrate at four concentrations (0.1, 0.2, 0.3 and 0.5 mM). All arrays were repeated three-times. Lineweaver-Burk graphs were drawn by using 1/V vs. 1/[S] values. $K_i$ and the inhibitor type were calculated from the graphs.

Results and Discussion

Purification of 6-PGD

In the present study, a rapid procedure using a single step 2’, 5’-ADP-Sepharose 4B affinity chromatography was employed to purify 6-PGD from grass carp hepatopancreas. 6-PGD was separated well from G6PD at the end of purification with different elution solution concentrations of NADP$^+$, similar to other reports. The 6-PGD was eluted using buffer A containing 2 mM NADP$^+$ (Fig. 1). The enzyme was purified 309-fold with a specific activity of 5.259 U/mg protein and yield of 68%. A summary of the purification is presented in Table 1.
Table 1—Purification scheme of 6-PGD from hepatopancreas of grass carp (C. idella)

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total volume (ml)</th>
<th>Activity (U/ml)</th>
<th>Total activity (U)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (U/mg protein)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>55</td>
<td>0.073</td>
<td>4.015</td>
<td>4.234</td>
<td>0.017</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>100,000 g, supernatant</td>
<td>35</td>
<td>0.107</td>
<td>3.745</td>
<td>3.049</td>
<td>0.035</td>
<td>93.275</td>
<td>2.064</td>
</tr>
<tr>
<td>2',5'-ADP-Sepharose 4B</td>
<td>9</td>
<td>0.305</td>
<td>2.745</td>
<td>0.058</td>
<td>5.259</td>
<td>68.369</td>
<td>309.331</td>
</tr>
</tbody>
</table>

The specific activity of 6-PGD was lower than those reported in rat small intestine (15 U/mg protein)\(^1\) and kidney (25 U/mg protein)\(^1\), but was similar to that in rat erythrocytes (5.15 U/mg protein)\(^1\) and higher than in rat liver and rat kidney cortex (0.207 and 0.056 U/mg, respectively)\(^13\) and *Trypanosoma brucei* (3.2 U/mg protein)\(^1\).

The differences observed in the specific activities of 6-PGD might be due to the different origin of the enzyme\(^12,14\) and different analytical conditions used in experiments, such as the type of buffer, pH [salt], NADP, RSH, mono- and divalent [cations]\(^3,10,12,34\).

Purified 6-PGD gave a single band on the SDS-PAGE (Fig. 2). For the standard proteins and 6-PGD, Rf values were calculated. Rf-logMr graph (Fig. 3) obtained according to Laemmli procedure\(^29\) showed the molecular mass of 6-PGD as 57 kDa, which was similar to 6-PGD from rat erythrocytes (59.57 kDa)\(^14\) and kidney (56 kDa)\(^16\), but was higher than 6-PGD obtained from sheep liver (51 kDa)\(^35\), rat liver (52 kDa)\(^8\), rabbit mammary gland (52 kDa)\(^9\), human erythrocytes (52 kDa)\(^36\), rat small intestine (52 kDa)\(^16\) and pig liver (42 kDa)\(^10\).
Optimum pH and temperature of 6-PGD

The optimum and stable pH conditions of the enzyme have an important role for kinetic studies, because each enzyme in different tissues could have a specific optimum pH for the enzyme activity and stability\(^{33}\). In this study, the optimum pH of the 6-GPD using 100 M Tris-HCl buffer was found to be 7.96 (Fig. 4A), which was slightly higher than that in rat erythrocytes (7.0)\(^{14}\), rat small intestine (7.35)\(^{16}\), pig liver (7.5)\(^{10}\), but was similar to 6-PGD from Drosophila melanogaster (7.8)\(^{17}\) and rat liver and kidney (8.0)\(^{33}\).

The optimum temperature of 6-PGD was found to be 50°C (Fig. 4B), which was similar to the enzyme in rat small intestine (49.4°C)\(^{16}\), but was higher than that in rat erythrocytes (45°C)\(^{14}\), Drosophila melanogaster (37°C)\(^{17}\) and lower than rat liver and kidney (55°C)\(^{33}\). In our experiments, we preferred to study at physiological temperature, 25°C. The effect of ionic strength on 6-GPD is depicted in Fig. 4C. Maximum enzyme activity was obtained at 100 mM Tris-HCl.

Kinetic parameters

Lineweaver-Burk double reciprocal plots obtained for 6-PGA as varied substrate at different fixed NADP\(^+\) concentrations are shown in Fig. 5A. \(1/v\) vs. \(1/[\text{NADP}^+]\) plots at different fixed 6-PGA concentrations are depicted in Fig. 6A. In the figures, the intersection points of the family of the lines were above the horizontal axis. The values could not
Fig. 6—(A) Primary plots: Double-reciprocal plot of initial velocity against NADP+ as that increases at different fixed 6-PGA concentrations for the reaction catalyzed by 6-PGD from grass carp hepatopancreas; (B) Secondary plots: variations of the intercept of the primary plots with the reciprocals of the 6-PGA concentration. The velocities were determined at 25°C in 100 mM Tris-HCl buffer and pH 7.96 be calculated directly, thus secondary plots \(1/v \) vertical axis intercept vs. \(1/[\text{NADP}^+]\) plots were used to determine the values (Fig. 5B) and \(1/v \) vertical axis intercept vs. \(1/[6\text{-PGA}]\) plots were used to determine the values (Fig. 6B). \(K_{m6\text{-PGA}}\) and \(K_{m\text{NADP}^+}\) are Michaelis constants for 6-PGA and NADP+, respectively. \(K_{i6\text{-PGA}}\) is the dissociation constant for 6-PGA. In the Figs 5A and 6A, the intersection points of the family of the lines were above the horizontal axis, indicating that the reaction catalyzed by 6-PGD proceeded by a sequential mechanism. In the present study, the \(K_m\) values for 6-PGA and NADP+ and \(V_m\) were calculated as 0.019 mM, 0.0052 mM and 0.69 U/ml, respectively (Table 2). The higher \(K_m\) value for 6-PGA than for NADP+ suggested the lower affinity of 6-PGD to 6-PGA, when compared with NADP+ and was in agreement with that of rat erythrocytes. In contrast, higher \(K_m\) value is reported for NADPH than those for 6-PGA in rat liver and kidney cortex.

The \(K_i\) values for NADP+, 6-PGA and NADPH calculated from Lineweaver-Burk graph were 0.12, 2.05 and 0.032 mM, respectively and NADPH inhibited 6-PGD competitively (Fig. 7). Similarly, earlier study, has reported that NADPH is the competitive inhibitor for 6-PGD with a \(K_i\) of 0.0319 mM. The \(K_i\) values of 2.500, 0.052 and 0.070 mM are also reported for ATP, NADPH and NADH, respectively, but NADPH inhibits it in a non-competitive manner in rat erythrocytes. The \(K_i\) for NADPH obtained in this study (0.032 mM) was similar to that reported for human erythrocytes (0.03 mM), rat small intestine (0.032 µM), Corynebacterium glutamicum (0.03 mM), but was lower than for rat erythrocytes (0.052 mM) and sheep liver (0.05 mM). The relatively lower \(K_i\) value for NADPH indicated the importance of NADPH in the regulation of pentose phosphate pathway (PPP) through 6-PGD. Thus, the effect of inhibition of NADPH on 6-PGD activity was noteworthy for the important metabolic states related to the PPP. On the other hand, another study has suggested that 6-PGD activity is subjected to a two-way regulation — NADPH, which regulates the PPP, inhibits the enzyme, while 6-PGA, whose concentration would increase when NADPH inhibition is removed, acts as an activator ensuring that 6PGA is rapidly removed.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_{m6\text{-PGA}}) (mM)</td>
<td>0.019 ± 0.006</td>
</tr>
<tr>
<td>(K_{m\text{NADP}^+}) (mM)</td>
<td>0.0052 ± 0.0001</td>
</tr>
<tr>
<td>(V_m) (U/ml)</td>
<td>0.69 ± 0.04</td>
</tr>
<tr>
<td>(K_{i6\text{-PGA}}) (mM)</td>
<td>0.12 ± 0.06</td>
</tr>
<tr>
<td>(K_{i6\text{-PGA}}) (mM)</td>
<td>2.05 ± 0.5</td>
</tr>
<tr>
<td>(K_{i\text{NADPH}}) (mM)</td>
<td>0.032 ± 0.01</td>
</tr>
</tbody>
</table>

\(K_{i6\text{-PGA}}\) is the dissociation constant of enzyme-NADP+ complex. \(K_{i6\text{-PGA}}\) is the dissociation constant of enzyme-6-PGA complex.
Fig. 8—Activity (%) vs metals regression analysis graphs of 6-PGD from hepatopancreas of grass carp in the presence of different metals concentrations (A) Cu$^{2+}$, (B) Zn$^{2+}$, (C) Cd$^{2+}$ and (D) Al$^{3+}$.

Fig. 9—Double-reciprocal plots of the inhibition of 6-PGD in grass carp hepatopancreas by (A) Cu$^{2+}$, (B) Zn$^{2+}$, and (C) Cd$^{2+}$ at three different concentrations for determination of $K_i$ [The controls show reactions with no inhibitor present]

Table 3—The dissociation constant ($K_i$) and values of 50% inhibitor (IC$_{50}$) of 6-PGD from grass carp hepatopancreas in the presence of different metal ion concentrations

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>IC$_{50}$ (mM)</th>
<th>$K_i$(mM)</th>
<th>Inhibition type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu$^{2+}$</td>
<td>0.293</td>
<td>0.084</td>
<td>Competitive</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>0.099</td>
<td>0.144</td>
<td>Competitive</td>
</tr>
<tr>
<td>Cd$^{2+}$</td>
<td>0.045</td>
<td>0.0285</td>
<td>Competitive</td>
</tr>
<tr>
<td>Al$^{3+}$</td>
<td>1.526</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Inhibition assays in vitro

At present, little information is available on the effect of metal ion in vitro on 6-PGD in fish. In our study, IC$_{50}$ values for Cu$^{2+}$, Zn$^{2+}$, Cd$^{2+}$ and Al$^{3+}$ were 0.293, 0.099, 0.045 and 1.526 mM, respectively (Table 3). In addition, $K_i$ values (inhibition types) were determined as 0.084, 0.144 and 0.0285 for Cu$^{2+}$, Zn$^{2+}$ and Cd$^{2+}$, respectively and they were all competitive-type (Fig. 8, Fig. 9). According to
$K_i$ values, Cd$^{2+}$ was the potent inhibitor for grass carp hepatopancreas 6-PGD. The inhibition of 6-PGD by Cu$^{2+}$, Zn$^{2+}$ and Cd$^{2+}$ might cause some important physiological changes, such as reducing production of NADPH, which plays important role in the regeneration of reduced glutathione (GSH)\textsuperscript{38,39} and, therefore, overwhelming antioxidant defense mechanisms. On the other hand, the fish is one of valuable foodstuff of animal sources for human consumption. Under certain environmental conditions, metal ions accumulated in fish up to a lethal concentration may be harmful for human health. Therefore, it is impending to reduce the concentration of metal ions in contaminated lakes and rivers for fishery and also for human health.

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