Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was purified from *Aspergillus aculeatus*, a filamentous fungus previously isolated from infected tongue of a patient. The enzyme, apparently homogeneous, had a specific activity of 220 units mg⁻¹, a molecular weight of 105,000 ± 5,000 Da by gel filtration and subunit size of 52,000 ± 1,100 Da by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The substrate specificity was extremely strict, with glucose 6-phosphate (G6P) being oxidized by nicotinamide adenine dinucleotide phosphate (NADP) only. At assay pH of 7.5, the enzyme had $K_m$ values of 6 µM and 75 µM for NADP and G6P respectively. The $k_{cat}$ was 83 s⁻¹. Steady-state kinetics at pH 7.5 produced converging linear Lineweaver-Burk plots as expected for ternary-complex mechanism. The patterns of product and dead-end inhibition suggested that the enzyme can bind NADP and G6P separately to form a binary complex, indicating a random-order mechanism. The enzyme was irreversibly inactivated by heat in a linear fashion, with G6P providing a degree of protection. Phosphoenolpyruvate (PEP), adenosinetriphosphate (ATP), and fructose 6-phosphate (F6P), in decreasing order, are effective inhibitors. Zinc and Cobalt ions were effective inhibitors although Cobalt ion was more potent; the two divalent metals were competitive inhibitors with respect to G6P, with $K_i$ values of 6.6 µM and 4.7 µM respectively. It is proposed that inhibition by divalent metal ions, at low NADPH /NADP ratio, is another means of controlling pentosephosphate pathway.

**Keywords:** *Aspergillus aculeatus*, Glucose 6-phosphate dehydrogenase, Inhibitors, Kinetics, Molecular weight, Purification
therefore focused on the G6PD in this fungus as part of the overall study of the biochemistry of this infectious organism.

**Experimental Procedures**

**Materials** *A. aculeatus* (Lizuka) which was kindly supplied by A. Ajaya (Department of Microbiology of this University) had previously been characterized. Substrates, nucleotides, reactive blue-2 agarose, molecular weight standards for SDS gel electrophoresis and gel filtration, and blue dextran were obtained from Sigma Chemical Co. (St. Louis, USA). All other reagents were of analytical grade.

**Growth of microorganism** The conidiophores of *A. aculeatus* grown on sabouraud dextrose were harvested and used to inoculate the growth medium, in aliquots of 400 ml, into separate 2.5 l cultures kept for 96 h at 25°C. After washing with 3.5 l of the same medium, the harvested mycelia were obtained from a 4 liter culture. The mycelia were dispersed into five different 2 l culture flasks. The cultures were kept at 25°C without agitation or bubbling. Mycelia were harvested by suction filtration and further extensively washed with deionized water before being blotted dry. An approximate 160 g of mycelia was obtained from a 4 liter culture.

**Extraction and purification of enzyme** The mycelia were homogenized in a porcelain mortar immersed in ice with acid-washed sand at a ratio 1:2 (mycelia to sand) in 0.02 M sodium phosphate buffer pH 6.8 containing 2 mM MgCl2, 1 mM EDTA, and 50 μM L-α-amino -n-caproic acid (buffer A). Approximately, the ratio of mycelia to homogenizing buffer A was 1:2 (v/v). The homogenate was centrifuged at 12,000 g for 20 min in MSE Mistral 6000 refrigerated centrifuge. The clear supernatant was brought to 15% ammonium sulphate saturation by the addition of solid ammonium sulphate (84 g/liter). The precipitate formed was collected by centrifugation for 20 min and was discarded. The supernatant was taken to 65% saturation by adding solid ammonium sulphate (335 g/liter). The precipitate that was formed was collected and suspended in a minimum volume of buffer A before being dialysed extensively for 24 h at 4°C against the same buffer, with several changes.

The dialysed enzyme was loaded on to a reactive blue-2 agarose column (1.5 × 5.7 cm) that was previously equilibrated with buffer A. After washing with the column with 100 ml of buffer A, at a flow rate of 20 ml/h, the enzyme was eluted by washing the column with another 100 ml of buffer A that contained 2 M NaCl and 2.5 μM NADP. The peak fractions containing enzyme activity were pooled and dialysed extensively against 50 mM triethanolamine buffer pH 7.5 for 96 h at 25°C. The mixture was shaken vigorously in a shaker at 25°C for 10 min before it was added to 3.5 l of the same medium. The growing mycelia were harvested by suction filtration and extensively washed with deionized water before being blotted dry. An approximate 160 g of mycelia was obtained from a 4 liter culture.

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**Molecular weight determination** The molecular weight of the native enzyme was determined by gel filtration in a 1.5 × 96 cm Sephacryl S-200 cm column using buffer A containing 25 μM NADP. The void volume of the column was determined with Blue Dextran. The standard proteins used in calibrating the column were ovalbumin (45,000), bovine serum albumin (67,000), hexokinase (100,000), bovine gamma globulin (150,000) and pyruvate kinase (230,000). A stock solution of G6PD was diluted to a concentration of 20 μg/ml in buffer A. The solution was concentrated by ultrafiltration and was dialysed extensively against 70% saturated ammonium sulphate in buffer A. The precipitate obtained in dialysed extensively against 70% saturated ammonium sulphate in buffer A. The precipitate obtained was suspended in a minimum volume of buffer A before being dialysed extensively for 24 h at 4°C against the same buffer, with several changes.

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**Assaying proteins and enzyme activity** Protein concentrations were determined using the method of Lowry et al. (1957), using bovine serum albumin as standard. G6PD was assayed, using Cecil CE 595 double beam digital spectrophotometer, by measuring the absorbance at 340 nm. All assays were conducted in duplicate at 25°C according to the method of Lohr and Walker (1974), in a total reaction volume of 3 ml and were initiated by the addition of NADPH to the reaction mixture. The enzyme, before it was assayed, was exhaustively dialysed against the assay buffer in order to remove any bound NADP. In a typical assay, the reaction mixture contained 30 μM triethanolamine buffer pH 7.5, 0.083 mM NADP and 0.833 mM glucose 6-phosphate (G6P). All solutions of G6P and NADP were appropriately buffered for all kinetic assays that were carried out.

**Measurement of steady-state kinetic parameters** The purified enzyme was dialysed extensively against 50 mM triethanolamine buffer pH 7.5. First, steady-state kinetic studies were carried out by measuring reaction rates at varying concentrations of G6P for a series of constant concentrations of NADP. The concentration of G6P was varied between 0.05 mM and 0.833 mM while constant concentration of NADP was between 0.02 mM and 0.5 mM. The experiment was repeated by varying the concentration of NADP between 0.02 mM and 0.05 mM at constant concentration of G6P which was between 0.05 mM and 0.833 mM. The Michaelis-Menten equation for the two-substrate reaction catalysed by G6PD, using the nomenclature of Cleland (1970) is

\[ V = \frac{V_{max} [A][B]}{K_m + [A][B] + K_A + [B]} \]
where A and B are NADP and G6P respectively.

The data that were obtained were analysed in accordance with data analysis devised by Cleland (1970).

**Effect of temperature** The effect of temperature on the reaction rate was studied between 25 and 55°C. The energy of activation ($E_a$) was calculated from the slopes of Arrhenius plot, which equals $-E_a/2.303R$ (Lehrer and Barker, 1970; Low et al., 1973). The heat stability of the enzyme was determined by incubating it at 55°C at a protein concentration of 60 μg/ml in the assay buffer. Aliquots were removed at time intervals and inserted immediately in ice before each residual activity was determined at 25°C.

**Effect of divalent cations** We investigated the effect of transition metal ions such as Co$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, and Mn$^{2+}$ on *A. aculeatus* G6PD catalysed reaction in order to find out if the phenomenon of divalent transition metal ion-induced inactivation is a common one. Assays were carried out under standard conditions with varying concentration of each metal. The inhibition of the enzyme by Zn$^{2+}$ and Co$^{2+}$ was further examined by varying G6P concentration (50-500 μM) at a fixed NADP concentration (83 μM) and at five different constant concentrations of each metal ion.

**Regulatory ligands** Several ligands, which are neither substrates nor products of G6PD catalysed reaction, have been found to affect the enzyme *in-vitro*. Many useful conclusions have been drawn from the results of such studies in the past. We tested the effects of many nucleotides, phosphoenolpyruvate (PEP), hexose phosphates and glycerol phosphate at a fixed concentration of each compound on the enzyme.

**Product inhibition studies** The inhibition of *A. aculeatus* glucose-6-phosphate dehydrogenase by NAPDH was determined by measuring initial rates for a series of NADPH concentrations (0-33 μM) with 0.417 mM G6P and NADP concentrations varied from 20 μM to 500 μM. A similar experiment was carried out at varying concentrations of G6P (30-300 μM) at a fixed concentration of NADP (83 μM) and NADPH concentrations, again, being varied from 0-33 μM. Inhibition constants were calculated using the equations for product inhibition in a bireactant enzyme system (Fromm 1975; Kanji et al., 1976).

### Results and Discussion

#### Enzyme purification

A summary of the purification procedure adopted in this report is presented in Table 1. The enzyme was purified approximately 230 fold with a 9% yield and a specific activity of 220 units/mg protein. The 15-65% ammonium sulphate fraction contained more than 80% of the active enzyme in the crude extract. However, a considerable amount of the enzyme was lost along with other proteins on the Reactive Blue-2 Agarose column.

**Molecular weight** The molecular weight of the native enzyme was estimated by gel filtration to be 105,000 ± 5,000. The SDS-polyacrylamide gel electrophoresis revealed the presence of only one protein band which had a size of 52,000 ± 1000 daltons. The enzyme, apparently, thus appeared to be homogenous. From the foregoing, *A. aculeatus* G6PD would seem to be a dimer made up of two structurally equivalent subunits. Available reports indicate that functional glucose-6-phosphate dehydrogenase in fungi, like other organisms (Levy, 1979), occurs either as dimers as in *Periconia duponti* (Malcolm and Shepherd, 1972) or as tetramers in *A. parasitica* (Niehaus and Dils, 1984). However, an unusual functional trimeric G6PD has been reported for *A. niger* and *A. nidulans* (Wennekes et al., 1993). There has not been any report on free, functional, monomeric species of the enzyme.

The exact explanation for these discrepancies in the value of molecular weight of fungal G6PD is not available at the moment. In our investigation, ε-amino-n-caproic acid was added to the extraction buffer to prevent degradation by proteases, which have been reported to be present in fungi (Olutiola and Nwaogwugwu, 1982). Besides, a limited interaction of G6PD with gel filtration matrix during gel permeation chromatography, which would lead to retardation, thus yielding a falsely low value of molecular weight, could not be ruled out (Jagannathan et al., 1956).

**Substrate and coenzyme specificity** The specificity of *A. aculeatus* G6PD with respect to the pyridine nucleotides and sugar phosphate substrates was determined. In so doing, the nucleotides and substrate analogues were tested at concentrations that were ten times the $K_m$ values for NADP and G6P respectively. No detectable activity could be measured with 0.833 mM glucose, 0.833 M glucosamine 6-phosphate and 0.5 mM NAD respectively.

#### Steady state kinetics

The results of the initial velocity

### Table 1. Purification of *A. aculeatus* glucose-6-phosphate dehydrogenase

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total enzyme unit (μmol/min)</th>
<th>Total protein (mg)</th>
<th>Specific activity (μmol/min/mg protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>2820</td>
<td>2930</td>
<td>0.96</td>
<td>100</td>
</tr>
<tr>
<td>15-65% (NH$_4$)$_2$SO$_4$ Fraction</td>
<td>2410</td>
<td>710</td>
<td>3.4</td>
<td>85</td>
</tr>
<tr>
<td>Reactive Blue-2</td>
<td>860</td>
<td>11</td>
<td>78</td>
<td>30</td>
</tr>
<tr>
<td>Agarose Affinity</td>
<td>620</td>
<td>1.2</td>
<td>220</td>
<td>9</td>
</tr>
<tr>
<td>Gel Filtration chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
studies for each of the two substrates indicated that the enzyme displayed a hyperbolic saturation curve. The same results gave linear double-reciprocal plots which converge at a point above the abscissa, an indication that $K_{i\text{NA}}$ should be larger than $K_m\text{NADP}$ (Cleland 1970); and a situation which suggests that the binding of the fixed substrate lowers the apparent $K_m$ for the varied substrate. Indeed, the fact that $K_m\text{NADP}$ is smaller than $K_{i\text{NA}}\text{NADP}$ (see Table 2) is an indication that G6P enhances the affinity of the enzyme for the coenzyme. A representative double reciprocal plot is shown in Fig. 1.

Secondary plots of these results (insets of Fig. 1) were linear and yielded the kinetic constants which are summarized in Table 2. The linear and intersecting patterns of the double reciprocal plots rule out a ping-pong kinetic mechanism. However, the plots are consistent with a sequential mechanism in which both substrates must bind to the enzyme simultaneously before product formation can occur (Fromm, 1975). The turnover number, $k_{cat}$, was calculated to be 83 sec$^{-1}$ while the specificity constants $k_{cat}/K_m\text{NADP}$ and $k_{cat}/K_m\text{G6P}$ for the two substrates were $1.4 \times 10^7$ M$^{-1}$ s$^{-1}$ and $1.1 \times 10^6$ M$^{-1}$ s$^{-1}$ respectively.

**Product and dead-end inhibition studies** A careful analysis of the patterns of product inhibition studies can indicate the order of addition of substrates in sequential mechanism (Cleland, 1970). In inhibition studies obtained at different fixed concentrations of NADPH, when the concentrations of NADP and G6P are varied respectively, the intersection of the double reciprocal plots on the vertical axis indicated competitive inhibition with respect to NADP and non-competitive inhibition with respect to G6P (data not shown). However, the inhibition constants obtained from the figures, using the equations for bi-reactant enzymic systems (Fromm 1975; Kanji et al., 1976), are shown in Table 2. The patterns of inhibition studies are consistent with sequential mechanism that can be either compulsory-order mechanism in which NADP is bound first or a rapid-equilibrium random mechanism with a dead-end enzyme-G6P-NADPH complex. Different kinetic mechanisms have been reported for G6PD from various sources. While Soldin and Balinsky (1969), Olive et al. (1971) and Aklanyan (1972) have described the mechanism of the enzyme as a sequential ordered one, Malcolm and Shepherd (1972), Levy and Cook (1991), Ragunathan and Levy (1994) and Wang et al. (2002) have proposed a random mechanism.

Glucosamine-6-phosphate reacts differently with different G6PDs. For some, it can serve as a weak substrate (Levy, 1979) while for some it is an inhibitor (Levy, 1979; Levy and Cook, 1991; Wang et al., 2002). On the other hand, and interestingly enough, G6P has been found to be an activator of G6PD from Azotobacter vinelandii (Anderson et al., 1997). In preliminary assay, we found that G6P could not be oxidized by A. aculeatus G6PD. As an analogue of G6P, G6P was therefore chosen as a dead-end inhibitor. The use of dead-end inhibitor can assist in the differentiation of sequential order from random order (Fromm, 1975; Levy and Cook, 1991; Wang et al., 2002). Glucosamine-6-phosphate was found to be a competitive inhibitor to G6P (Fig. 2) ($K_i = 21 \pm 1$ mM) and a non-competitive with respect to NADP ($K_{i\text{NA}} = 4.2 \pm 0.1$ mM; $K_{i\text{NA}} = 21 \pm 1$ mM). The inference from

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### Table 2. Kinetic parameters of A. aculeatus glucose-6-phosphate dehydrogenase

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Values obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m\text{NADP}$</td>
<td>$6 \pm 1$ µM</td>
</tr>
<tr>
<td>$K_m\text{G6P}$</td>
<td>$75 \pm 6$ µM</td>
</tr>
<tr>
<td>$K_m\text{NADP}$</td>
<td>$23 \pm 3$ µM</td>
</tr>
<tr>
<td>$K_i\text{NADP}$ (NADP varied)</td>
<td>$20 \pm 1$ µM</td>
</tr>
<tr>
<td>$K_i\text{G6P}$ (G6P varied)</td>
<td>$41 \pm 1$ µM</td>
</tr>
<tr>
<td>$K_i\text{G6P}$ (G6P varied)</td>
<td>$43 \pm 1$ µM</td>
</tr>
</tbody>
</table>

---

**Fig. 1.** Double-reciprocal plot for initial velocity versus glucose 6-phosphate concentration at pH 7.5. Concentrations of NADP$^+$ were 27 µM (+), 33 µM (◆), 50 µM (▼), 67 µM (×), 83 µM (■), 167 µM (●) and 500 µM (▲). The inserts show (A) intercepts replot and (B) slopes replot, versus NADP$^+$ concentration respectively.
these inhibition studies is that the dead-end inhibitor is capable of binding to both the free enzyme and G6PD-NADP complex. Since glucosamine 6-phosphate is an analogue of G6P, it is probable that the substrate (G6P) too can bind to both the free enzyme and enzyme-NADP complex. Thus, the inhibition studies seem to indicate that G6P and NADP can bind to the free enzyme. The recent information about the direct formation of crystal G6PD-G6P complex with human red cell G6PD (Wang et al., 2002) is also consistent with this suggestion. It therefore seems that, on the basis of limited inhibition studies, the kinetic mechanism of Aspergillus aculeatus G6PD may be random. Perhaps it should be emphasized that the dividing line between compulsory-order and random mechanisms, through kinetic experiments, could be fuzzy. In a random order, an enzyme may bind either substrate first, but it is not likely that both routes will be equally probable.

### Effect of possible regulatory ligands

Several compounds were tested as possible regulatory effectors of the enzyme. The data presented in Table 3 are the results of such experiments. Phosphoenolpyruvate has been reported to be a potent inhibitor of G6PDs from several sources (Levy, 1979; Levy and Cook, 1991). However, the Aspergillus aculeatus G6PD, which is NADP specific, was not significantly inhibited under the conditions of our experiments. The enzyme, in this respect, is similar to the NADP-specific G6PD from Acetobacter hansenii which, also, was not inhibited by ADP and AMP (Levy and Cook, 1991).

### Activation energy and thermostability studies

The effect of temperature on the reaction rate of A. aculeatus G6PD was analysed using Arrhenius equation. A linear curve was obtained (Fig. 3), and from its slope, activation energy of 13
Aspergillus aculeatus Glucose 6-Phosphate Dehydrogenase

kcal mol⁻¹ was obtained. The thermo-stability study of the enzyme at 55°C revealed that it was rapidly inactivated (Fig. 3). The inactivation was an irreversible linear process with a rate constant of 0.22 min⁻¹ and a half life of 3.1 min. While 100 µM NADP did not offer any protection against heat-inactivation (half life = 3.2 min), 77 µM G6P offered a slight degree of protection with a half life of 7.5 min. The protection against heat-inactivation offered by G6P seems to be a phenomenon unique to fungal G6PDs in view of the report of Malcolm and Shepherd (1972) on penicillium G6PD that displayed a similar irreversible linear inactivation profile but was protected by glucose 6-phosphate.

**Effect of divalent metal ions** We found that *A. aculeatus* G6DP was inhibited by Zn²⁺ and other transition metal ions such as Ni²⁺, Co²⁺ and Cd²⁺. The inhibitions by Zn²⁺ and Co²⁺ ions were competitive with respect to G6P (Fig. 4) with a Kᵢ values of 6.6 ± 0.1 µM and 4.7 ± 0.1 µM respectively. Of all the divalent metals tested, Co²⁺ was the most potent inhibitor (Table 4). At 10 µM, Co²⁺ inhibited the enzyme by 60% compared with Zn²⁺ which caused 40% inhibition. Transition metal ions such as Zn²⁺ and Co²⁺ have been shown to be inhibitors of glucose-6-phosphate dehydrogenase from a number of sources and, in each case, the inhibition by zinc ion was also linear competitive with respect to glucose-6-phosphate (Niehaus and Dilts, 1984).

The probable significance of inhibition of *A. aculeatus* G6PD by zinc can be assessed from the fact that, in imperfect fungi, zinc ion has been shown to stimulate the production of polyketides and also to cause the inhibition of mannitol dehydrogenase (Hult and Gatenbeck, 1978; Niehaus and Dilts, 1982). Generally, polyketide formation is favoured by limiting the production of NADPH that is generated by the catalytic activities of fungal dehydrogenases such as G6PD (Niehaus and Dilts, 1984), 6-phosphogluconic dehydrogenase (Niehaus et al., 1996) and mannitol dehydrogenase (Hult and Gatenbeck, 1978; Niehaus and Dilts, 1982). In other words a low NADPH/NADP ratio is favoured by zinc ion. The relationship between the NADPH/NADP ratio and the regulation of G6PD activity has long been recognized (Afolayan, 1972; Eggleston and Krebs, 1974). The inhibition of *Aspergillus aculeatus* G6PD by the transition metal ions is perhaps another means of controlling the pentosephosphate pathway at low NADPH/NADP ratio that is known to favour the operation of the mannitol cycle in many imperfect filamentous fungi, including Aspergillus species. For, the operation of mannitol cycle, in the presence of zinc ion, leads to a stimulated synthesis of mycoxin versicolorin A which is a precursor of polyketide aflatoxin (Niehaus and Dilts, 1982). But not all *Aspergillus* species produce polyketides (Foreman and Niehaus, 1985). The full significance of the inhibition of *A. aculeatus* glucose-6-phosphate dehydrogenase activity by zinc and other transition metal ions may be strengthened by the outcome of future investigation into the synthesis, or otherwise, of polyketide mycotoxins in this infectious filamentous fungus.

**Table 4. Inhibition of *A. aculeatus* glucose-6-phosphate dehydrogenase activity by divalent cations.**

<table>
<thead>
<tr>
<th>Ion*</th>
<th>Activity remaining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>100</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>40</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>60</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>83</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>79</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>72</td>
</tr>
</tbody>
</table>

Enzyme assays were carried out under standard assay condition (section on experimental procedures). The final concentration of each cation in the assay mixture was 10 µM.

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Acknowledgments This research was supported partly by research grant Nos 1425EJ, 1425EV and 1425RE from the University Research Committee of the Senate of Obafemi Awolowo University, Ile-Ife.

References


