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Characterization of Three Enzymatic Forms of Glucose-6-Phosphate Dehydrogenase from *Aspergillus oryzae*

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Three forms (I, II and III) of glucose-6-phosphate dehydrogenase were isolated from mycelium of *Aspergillus oryzae* grown on ribose as the carbon source, by ion-exchange chromatography. The Km values determined for the three forms with respect to glucose-6-phosphate were nearly identical; however the K_m for $NADP^+$ were different and the V_{max} for the isoenzymatic form II was higher than those for I and III. Inhibition by NADPH was competitive with respect to $NADP^+$, isoenzyme II showing the highest K_i. The optimum pH for forms I, II and III were 9.0, 8.0 and 8.5, respectively, and form I was more thermostable than the others. The apparent molecular weights, determined by gel filtration, were 92,000, 117,500 and 141,000 for forms I, II and III, respectively.

Key words: Glucose-6-phosphate dehydrogenase, Isoenzymes, *Aspergillus oryzae*.

Since the existence of multiple forms of glucose-6-phosphate-dehydrogenase (G-6-PDH) was first described by Tsao (22), several studies concerning the characterization of such isoenzymes from different sources have been reported (6, 7, 10-12). However, although the enzyme has been purified from *Candida utilis* (5) and *Penicillium* (14), very little is known about the occurrence of multiple enzymatic forms of G-6-PDH in fungi. The existence of three isoenzymes of G-6-PDH in *Aspergillus oryzae* has been described by Muiño Blanco *et al* (16). In this case, three different forms of the enzyme were isolated from mycelium grown on ribose, but only two isoenzymes were identified when the mycelium was grown on glucose as carbon source. These results showed that ribose acts as a nutritional inducer of one of the isoenzymes (type II).

The present work deals with the kinetic characterization of these three different isoenzymes in order to obtain further information about their physiological role. The results obtained suggest that isoenzyme II kinetic behaviour compensates

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for the NADPH biosynthesis requirements, when the cellular glucose pool should disminish in mycelium grown on ribose.

Materials and Methods

The growth of the mycelium of *Aspergillus oryzae* (University of Salamanca [Spain], Department of Microbiology), the preparation of cell-free extracts, and the purification of the isoenzymes of G-6-PDH, by chromatography through CM-Sephadex C-50 of a dialysed pellet obtained by 85 % saturation of $(NH_4)_2SO_4$ of a mycelium homogenate, were carried out as previously described (16).

Protein concentration was measured by the method of LOWRY *et al* (13) and G-6-PDH assay and thermal inactivation were done as described previously (16).

Molecular weights were determined according to the method of ANDREWS (1). A column (2.5 × 40 cm) of Sephadex G-200 equilibrated with 0.05 M imidazole-HCl, pH 7. The samples were applied in a volume of 1 ml, and a constant flow rate of 20 ml/h was used for elution. The column was calibrated with the following standards (Mw): cytochrome c (12,000), myoglobin (17,800), egg-albumin (45,000), serum-albumin (67,000) and aldolase (147,000). Distribution coefficients (kd) were calculated from the basic equation given by GELOTTE (8).

Electrophoresis was performed in a polyacrylamide gel system (18). For analytical electrophoresis, 7 % (w/w) gels were run in tris-glycine buffer, pH 8.4. G-6-PDH activity was detected by the method of BREWER and ASHWORTH (3).

Results and Discussion

Purification of the isoenzymes.— The purification process is summarized in table I. Three peaks of enzymatic activity were obtained. Isoenzyme I was eluted unadsorbed, and isoenzymes II and III were eluted in a NaCl gradient of 0-0.5 M (16). The three forms appeared to be homogeneus in analytical gel polyacrylamide electrophoresis (fig. 1) under the experimental conditions used. The most predominant isoenzymatic form corresponds to type II. Assuming that recovery during the purification process is the same for each isoenzyme, type II accounts for 66 % of the total G-6-PDH activity present in the mycelium grown on ribose as the sole carbon source.

Kinetic constants.— The values of the apparent Michaelis constants (K_m) and maximun velocity (V_{max}) were determined from Lineweaver-Burk plots. The K_m values for glucose-6-phosphate (0.3 mM)

Table I. *Isolation of glucose-6-phosphate dehydrogenase isoenzymes from A. oryzae.*

Fraction	Volume (ml)	Total activity (IU)	Total protein (mg)	Specific activity (mIU/mg)
Cell-free extract	67	185	569.5	325
30 % satd $(NH_4)_2SO_4$ supernatant	61	184	427	433
60 % satd $(NH_4)_2SO_4$ pelled dialyzed	12	158	280	565
1st peak CM-Sephadex	48	9.4	26	361
2nd peak CM-Sephadex	66	60.9	39.6	1,534
3rd peak CM-Sephadex	45	3.7	9	412

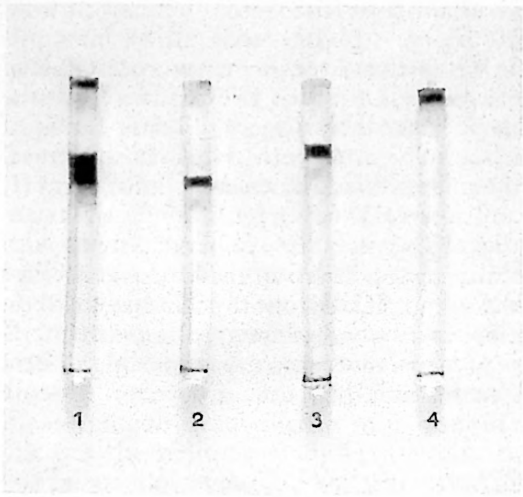

Fig. 1. *Analytical acrylamide-gel electrophoresis of glucose-6-phosphate dehydrogenase was performed at pH 8.3 in 7.5 % gels with 3 mA per tube for 60 min.*
The gels labelled 1, 2, 3 and 4 correspond to crude extract, isoenzyme I, II and III, respectively.

Table II. *Kinetic constants of glucose-6-phosphate dehydrogenase isoenzymes.*
Values are the average ± standard deviation of 6 different experiments.

Constant	Form I	Form II	Form III
K_m (G6P) mM	0.3±0.05	0.3±0.03	0.35±0.06
K_m ($NADP^+$) μM	25±2.5	30±3.0	15.0±1.8
V_{max} (mIU/mg)	480±28.0	1,895±92.0	526±34.0

were similar in each form, while the V_{max} was higher for the form II (table II). These values are similar to those given for the enzyme of elsewhere (19, 21).

Inhibition by NADPH.— The inhibition of G-6-PDH by NADPH was first described by NEGELEIN and HAAS (17) in yeast, and confirmed for the enzymes of different species by various authors (2, 9, 15, 20), becoming generally accepted that the activity of G-6-PDH is controlled by the cytosolic ratio (free $NADP^+$)/(free NADPH).

Table III. *Inhibitory effect (%) of NADPH on glucose-6-phosphate dehydrogenase isoenzymes activity.*
The $NADP^+$ was 0.1 mM, and NADPH was 0.1 mM (ratio 1), 0.2 mM (ratio 2) and 0.3 mM (ratio 3). Values are the average ± standard deviation of 4 different experiments.

NADPH/$NADP^+$	Form I	Form II	Form III
1	35±1.2	22±1	29±0.9
2	45±1.5	36±2	40±1.8
3	60±3	47±1.5	52±3
K_i (NADPH) μM	71±0.5	140±1.5	100±1.2

Our results are in agreement with the above data, since the isoenzymes of G-6-PDH of *A. oryzae* were inhibited by NADPH competitively with $NADP^+$, since the inhibition was greater as the NADPH/$NADP^+$ ratio increased (table III). The K_i values were different for each isoenzyme, form I being more strongly inhibited, where the inhibition approached 60 % when the ratio of nucleotides reached a value of 3 (table III).

Effect of pH.— The activity of the three enzymatic forms was measured in acetate buffer (pH = 5-5.5), citrate buffer (pH = 6), imidazole-HCl buffer (pH = 6.5-7.5), Tris-HCl buffer (pH = 8-8.5) and glycine-NaOH buffer (pH = 9-10). The pH-activity curves of the three forms were similar. The optimum pH for forms I, II and III are 9, 8 and 8.5, respectively. These results are in agreement with other authors (15). No differences were observed in the stability of the three forms over the range of pH studied (data not shown).

Heat inactivation.— The three enzymatic forms were incubated for 5 min at temperatures of 10-60° C, with a differ-

ence of 5° C between consecutive assays (fig. 2). Isoenzyme I only decreases its activity in 25 % after incubation at 50° C whereas the type II activity declines linearly from 40° C to 60° C, showing 58 % inhibition at 50° C. The isoenzyme III shows an intermediate pattern of heat stability, decreasing its activity 35 % after incubation at the same temperature of 50° C.

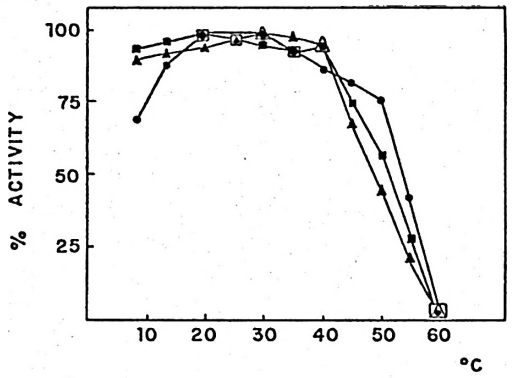

Fig. 2. *Heat inactivation of isoenzymes of glucose-6-phosphate dehydrogenase during 5 min.* Enzymatic extracts were incubated for 5 min at 20-60° C; then the activity was determined. ● Isoenzyme I; ▲ Isoenzyme II; ■ Isoenzyme III.

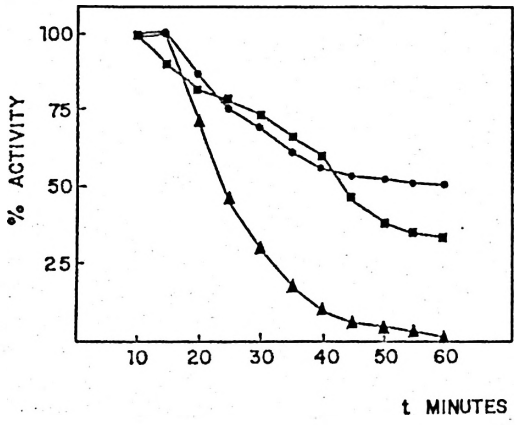

Fig. 3. *Heat inactivation of isoenzymes of glucose-6-phosphate dehydrogenase at 45° C.* Enzymatic extracts were incubated a 45° C for 10-60 min and then the remaining activity was determined. ● Isoenzyme I; ▲ Isoenzyme II; ■ Isoenzyme III.

When the three forms were incubated for different times at 45° C, form I was the most heat stable (fig. 3), losing 50 % of its activity in 60 min, whereas this treatment leads to total inactivation of form II, and form III retains only 35 % of its activity. These results are in agreement with those obtained from crude extracts since the homogenate from mycelium grown on ribose, in which activity of isoenzyme II is predominant, was more sensible to heat inactivation than that grown on glucose (16).

Determination of molecular weight.— Isoenzymes of G-6-PDH were found to have an apparent molecular weight in the same range as that of the enzyme from *Candida*, with a value of 104,000 (5) (fig. 4). The values were 92,000, 117,500 and 141,000 for isoenzymes I, II and III, respectively.

Isoenzymes I and III appear to be the two most related ones since they have similar pH optimum, thermal stability and kinetic parameters. Since both forms correspond to the whole enzymatic activity present in mycelium grown on glucose as the sole carbon source, further investiga-

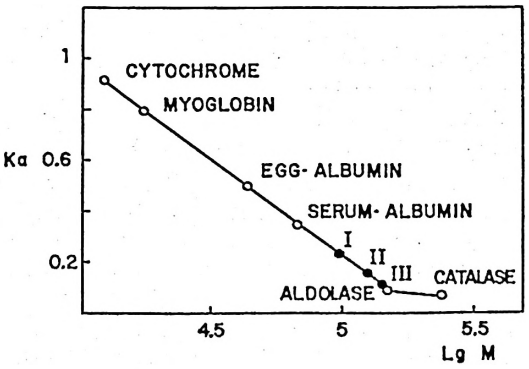

Fig. 4. *Molecular weight determination by Sephadex G-200 column (2.5 × 40 cm) equilibrated with 0.05 M imidazole HCl-buffer pH 7.*

tion should be required to find out if the apparent differences in the molecular weight determinations, carried out in this work, could be due to different subunits content, as it has been described in erythrocytes G-6-PDH (4).

Isoenzyme II shows clear differences in respect to the other two enzymatic forms, specially recognised in its thermal stability and kinetic behaviour. This isoenzyme has the highest K_m for $NADP^+$, K_i for NADPH and maximal activity, being the most predominant one when the mycelium is grown on ribose. These results clearly suggest that this form is responsible for the major metabolism of glucose via pentose phosphate pathway under this metabolic condition. It is remarkable that G-6-PDH activity highly increases when mycelium is grown in the presence of ribose (14), and that such increased activity is due to an inducible isoenzyme (type II), less regulated by NADPH and with the highest maximal activity. These facts suggest that the kinetic behaviour of this inducible enzyme represents compensation mechanisms, by which the cellular pool of glucose could be more efficiently oxidised by the G-6-PDH isoenzyme II. This would provide the NADPH needed for the biosynthetic activities during growth, under conditions in which the cell glucose and NADPH/$NADP^+$ ratio should decrease, in respect to those of mycelium grown on glucose.

Resumen

Se separan tres formas de glucosa-6-fosfato-deshidrogenasa (I, II y III), mediante cromatografía de intercambio iónico, a partir de micelios de *Aspergillus oryzae* desarrollados sobre ribosa como única fuente de carbono. Los valores de K_m respecto a la glucosa-6-fosfato son casi idénticos para las 3 formas; sin embargo, las K_m para $NADP^+$ son diferentes y la V_{max} de la isoenzima II es mayor que las obtenidas para las isoenzimas I y III. El NADPH inhibe a las tres formas competitivamente respecto al $NADP^+$, siendo la enzima II la de mayor K_i. Los valores de pH óptimos son 9,0, 8,0 y 8,5, para las formas I, II y III, respectivamente, siendo la forma I la de mayor estabilidad térmica. Los pesos moleculares aparentes, determinados por gel filtración son 92.000, 117.500 y 141.000 para las formas I, II y III, respectivamente.

Palabras clave: Glucosa-6-fosfato deshidrogenasa, Isoenzimas, *Aspergillus oryzae*.

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