Significance of the Non-Oxidative Pentose Phosphate Pathway in *Aspergillus oryzae* Grown on Different Carbon Sources

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Specific enzyme activities of the non-oxidative pentose phosphate pathway in *Aspergillus oryzae* mycelia grown on different carbon sources were determined. Mycelia grown on glucose, mannitol and ribose show the highest specific activities, ribose 5-phosphate isomerase being specially very enhanced. Moreover, transketolase, transaldolase, ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase were determined in different developmental stages of mycelia grown on glucose, mannitol and ribose. The non-oxidative pentose phosphate pathway is more active during conidiogenesis, except for ribulose 5-phosphate 3-epimerase, suggesting a fundamental role of this pathway during that stage to supply pentoses for nucleic acids biosynthesis. A general decrease of the enzyme activities was found in sporulated mycelia. Arabinose 5-phosphate was tested as metabolite of the pentose pathway. This pentose phosphate was not converted into hexose phosphates or triose phosphates and inhibits significantly the ribose 5-phosphate utilization, being therefore unappropriate to support the *Aspergillus oryzae* growth.

Introduction

The Aspergilli use a wide range of organic compounds as source of carbon and energy, and the growth yield is quite different for hexoses, pentoses and pentitols [1]. Many of them support growth, after an obvious time lag, revealing an adaptive metabolism to particular environmental conditions. The differences in growth on different carbohydrates could be due to regulation of key catabolic enzymes or to specific uptake systems [2–4].

Fungi generally utilize aldo-pentoses by reduction to pentitols, which are interconverted through keto-pentoses and other pentitols to D-xylulose, the only phosphorylated pentose to enter the pentose phosphate pathway, as was proposed by Lewis and Smith [5] and confirmed by Hankinson [6] in *Aspergillus nidulans*.

The occurrence of the pentose phosphate pathway in filamentous fungi, including species of *Aspergillus,* was documented by Blumenthal in 1965 [7]. The specific activities of all the enzymes of the pathway, with the exception of epimerase, have been reported in *Candida utilis* [8] and in *A. nidulans* [9] in which the specific activities and content of transaldolase isoenzymes of various strains were also reported [10].

The pentose phosphate pathway plays different roles during *Aspergillus* differentiation, and one of the main changes in enzyme profiles, associated with conidiogenesis in filamentous fungi, is the stimulation of this pathway. The increased capacity of the pathway is consistent with an increased demand of NADPH for biosynthesis.

In fungi, most of the studies on the interference of different nutritional conditions with the pentose phosphate pathway basically concern the oxidative phase, and especially glucose 6-phosphate dehydrogenase [11, 12]. This step, together with the transketolase activity are the two principal enzymes controlling the two branches of the pathway. The control of the non-oxidative branch at the transketolase step has been less studied and, to date, there have been no reports of allosteric regulation of transketolase or transaldolase.

This paper is intended to provide information on the four enzymes of the non-oxidative branch of the pentose phosphate pathway during the growth of *Aspergillus oryzae* on different carbon sources.

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Materials and Methods

Enzymes and chemicals

Enzymes, sugar phosphates and coenzymes were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). All other chemicals and solvents were of analytical reagent grade.

Growth of mycelium

The Aspergilli were grown with different substrates as sole carbon source, as previously described [12]. Conidial suspensions were prepared in sterile distilled water. A small volume of the concentrated conidial suspension was added to 100 ml of Mulder's medium in a Roux flask. The flasks were maintained without rotation and the temperature was controlled at 30 °C. The mycelia were harvested, washed and stored at −20 °C for 1−2 weeks. Crude extracts were prepared using a glass homogenizer.

Analytical procedure

Enzyme assays. All enzyme assays were performed at 25 °C. The assay for transketolase was performed as described by Simcox *et al.* [13]. Transaldolase was assayed as described by Novello and McLean [14]. For ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase, the assays proposed by Kiely *et al.* [15] were used.

A possible arabinose 5-phosphate 2-epimerase was tested using the following incubation mixture: 40 mM glycylglycine/KOH, pH 7.4; 0.6 mM arabinose 5-phosphate; 0.13 mM xylulose 5-phosphate; 20 μM TPP; 5 mM $MgCl_2$; 0.3 units transketolase; 4 units triose phosphate isomerase; 1 unit *a*-glycerol phosphate dehydrogenase and 0.1 mM NADH. A suitable dilution of the enzyme preparation was added. Distilled water was added to a final volume of 1 ml after the addition of the sample. The oxidation of NADH was followed at 340 nm.

Aldolase (EC 4.1.2.13) was assayed by the method described by Hankinson and Cove [9] for aldolase in *Aspergillus nidulans*.

Triose and hexose phosphate formation from pentose phosphate was measured with the method described by Williams *et al.* [16] based on Horekker *et al.* [17]. Pentose phosphates were incubated with dialyzed preparations of *Aspergillus oryzae* and, at different incubation times, aliquots were withdrawn, and triose phosphate and hexose phosphate formation were measured.

Protein determination

The protein measurement was performed using the method described by Lowry *et al.* [18].

Results

Table I shows the enzyme activities of the non-oxidative pentose phosphate pathway determined in crude extracts prepared from mycelia of *Aspergillus oryzae* grown on different carbon sources and collected at the vegetative growth phase. Ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase, when detected, showed higher specific activities than transketolase and transaldolase. Glucose and mannitol-grown mycelia presented very similar specific activities for all enzymes assayed.

Table I. Specific enzyme activities of the non-oxidative pentose phosphate pathway in mycelia grown in different carbon sources. *Aspergillus oryzae* was grown in 6% (w/v) carbon source, and enzyme activities were determined in the crude extracts. The results are means of three determinations and the standard deviations were less than 10%.

	Transketolase [mU/mg]	Transaldolase [mU/mg]	R 5 P isomerase [mU/mg]	Ru 5 P 3-epimerase [mU/mg]
Glucose	111	200	1.105	369
Mannitol	98	227	1205	365
Ribose	227	312	788	367
Arabinose	104	95	250	122
Fructose	52	272	n.d.	n.d.
Sorbitol	164	120	n.d.	n.d.

n.d., not detected.

However, *Aspergillus oryzae* grown on ribose shows a decrease of the ribose 5-phosphate isomerase activity whereas the transketolase and transaldolase increased their activity in comparison with those of the glucose or mannitol-grown mycelia. Growth on arabinose causes a relative decrease of almost all enzyme activities. When growth was carried out in the presence of either fructose or sorbitol, neither ribose 5-phosphate isomerase nor ribulose 5-phosphate 3-epimerase activities were detected.

In order to obtain further information on the activities found when the growth occurred in arabinose-containing media, a series of experiments was carried out in which pentose-dependent hexose and triose phosphates formation by ribose-grown mycelium extracts were studied. The results obtained are shown in Fig. 1 and 2. Ribose 5-phosphate was converted into hexose and triose phosphates but arabinose 5-phosphate was not. When both pentose phosphates were present in the incubation mixture the ribose-dependent hexose phosphates formation was also significantly inhibited. However, arabinose only slightly inhibited formation of triose phosphates from ribose, and in fact a tendency to accumulation was observed at longer incubation periods (Fig. 2). The triose and hexose formation from the mentioned pentose phosphates were also measured in crude extracts from mycelia grown on arabinose as sole carbon source. The maximum hexose and triose phosphate formation from ribose 5-phosphate is 2 μM and 64 μM respectively. Neither hexose phosphates nor triose phosphates formation were detected when only arabinose 5-phosphate was present in the incubation mixture. When mycelia grown on arabinose were used, very small amounts of hexose phosphates and triose phosphates were detected as formed from ribose phosphate. When arabinose 5-phosphate was present, neither triose phosphates nor hexose phosphates were found at all.

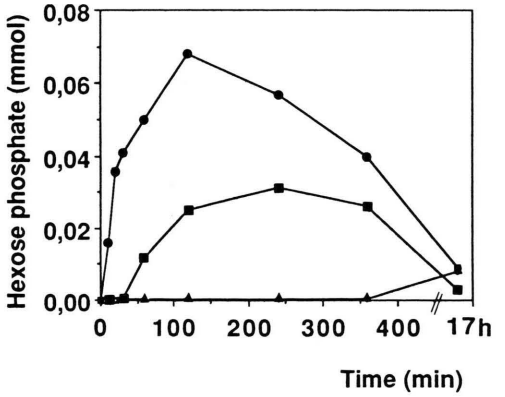

Fig. 1. Hexose phosphate formation by extracts of mycelium grown on ribose-containing media as carbon source; the extracts were incubated in the presence of 2 μmol of ribose 5-phosphate (●), ribose 5-phosphate and arabinose 5-phosphate (■), arabinose 5-phosphate (▲).

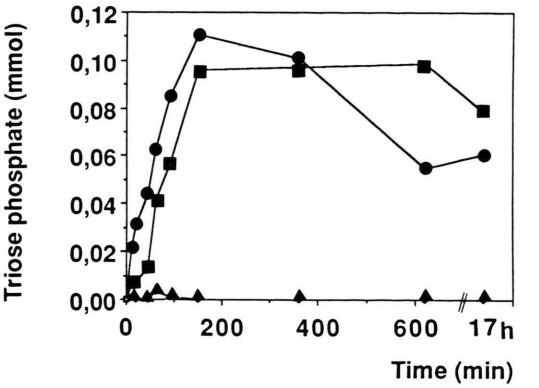

Fig. 2. Triose phosphate formation by extracts of mycelium grown on ribose-containing media as carbon source; the extracts were incubated in the presence of 2 μmol of ribose 5-phosphate (●), ribose 5-phosphate and arabinose 5-phosphate (■), arabinose 5-phosphate (▲).

Finally, the specific activities of the non-oxidative pentose phosphate pathway enzymes were studied during the fungal development in the presence of the best three carbon sources which induce the non-oxidative pentose phosphate pathway activities (glucose, mannitol and ribose). Three stages were considered: vegetative growth, conidial differentiation (conidiogenesis) and fungal spore formation. Table II shows that the non-oxidative pentose phosphate pathway is in general more active during conidiogenesis except for ribulose 5-phosphate 3-epimerase, which decreases in this stage, with respect to the value found in the hyphal differentiation. The ribose 5-phosphate isomerase activity enhancement observed during the conidia formation is noteworthy. Finally, a general decrease of the enzyme activities was found in sporulated mycelia.

Mycelia grown on ribose, glucose, mannitol and arabinose were tested for arabinose 5-phosphate 2-epimerase activity. With the assay described in

Table II. Non-oxidative pentose phosphate pathway enzymes in different development stages in *Aspergillus oryzae*. Specific activities of the enzymes were determined in crude extracts from mycelia grown in different media (9% carbon source) and in different development stages: vegetative growth, conidiogenesis and sporulate mycelia (standard deviations less than 10%).

		Vegetative growth	Conidiogenesis	Sporulated
Transketolase (mU/mg)	glucose	102	72	0
	mannitol	55	116	26
	ribose	87	208	48
Transaldolase (mU/mg)	glucose	263	158	17
	mannitol	50	175	84
	ribose	267	303	102
Ribose 5-P isomerase (mU/mg)	glucose	123	976	252
	mannitol	62	1323	170
	ribose	130	850	570
Ribulose 5-P 3-epimerase (mU/mg)	glucose	484	148	191
	mannitol	120	61	50
	ribose	422	575	170

Materials and Methods no activity was found in any of the crude extracts tested.

Discussion

The present reported estimation of enzyme activities of the non-oxidative pentose phosphate pathway clearly shows (Table I) that the metabolic utilization of glucose by this pathway is a consequence of the NADPH requirement for biosynthesis, supplied by glucose 6-phosphate dehydrogenase. In this case, high specific activities of all enzymes were found. Likewise, a similar enzymatic pattern was found when growth occurred in the presence of mannitol, a nutrient which is rapidly metabolized by the cell and converted into glucose (Roberts, [19]). Ribose-induced transketolase and transaldolase enhancement strongly suggests that glucose formation from this pentose plays an important metabolic role, in agreement with previous results of Muiño-Blanco *et al.* [20] and Cebrian-Pérez *et al.* [21] showing the induction in ribose-grown mycelium of a specific glucose 6-phosphate dehydrogenase isoenzyme, to compensate for NADPH biosynthetic requirements in such a glucose limiting situation. In this case, the decrease of the ribose 5-phosphate isomerase activity can be easily explained by lower requirements of ribose biosynthesis since this sugar is exogenously supplied.

On the other hand, it seems very likely that mycelia grown on fructose only require some transketolase and transaldolase activities (Table I) to supply ribose for nucleic acid biosynthesis by reversing the metabolic flow and forming pentoses from phosphorylated fructose derivatives. Therefore, metabolic production alternative to glucose 6-phosphate dehydrogenase might occur. The same metabolic performance was observed when the carbon source was sorbitol, which is well established to be rapidly metabolized into fructose [22].

However, arabinose-supported mycelia showed a striking enzyme level profile, in which all activities diminished (Table I). *In vitro* incubations carried out in this work show that arabinose 5-phosphate significantly inhibits ribose-dependent hexose phosphate formation (Fig. 1) while triose phosphates production is only slightly delayed (Fig. 2). These results can be explained as a consequence of the competitive inhibition of aldolase, transketolase and transaldolase by arabinose 5-phosphate, described by Muiño-Blanco *et al.* [20]. This could induce a delayed *in vivo* accumulation of non-oxidative pentose phosphate pathway metabolites, which finally inhibit the entire pathway.

On the other hand, Williams *et al.* [16] proposed that arabinose was involved in a putative pentose phosphate pathway, the so-called L-type [23], an

alternative recycling system of pentoses to hexoses. The results presented here rule out the existence of such a cycle in *Aspergillus oryzae* since no hexoses were formed from arabinose (Fig. 1) by extracts of mycelium grown on ribose. If arabinose were a normal intermediate of the non-oxidative pentose phosphate pathway, it should at least partially promote hexose formation. In any case, the metabolic inhibition by arabinose of the pentose phosphate pathway makes this sugar unappropriate as a carbon source for *Aspergillus oryzae* growth. Furthermore no arabinose 5-phosphate 2-epimerase activity was detected, and this enzyme was the one proposed by Williams [23] as being responsible for arabinose 5-phosphate incorporation into the pentose phosphate pathway.

The fundamental role of the non-oxidative pentose phosphate pathway during the conidiogenesis of *Aspergillus oryzae* is emphasized by the increase of the specific activities of the related enzymes (Table II) in respect to that observed in the hyphal differentiation. Especially remarkable is the enhancement of the ribose 5-phosphate isomerase activity, which suggests that, during this stage, pentose supply for nucleic acids biosynthesis accounts for a high potentiation of the metabolic flow of the pathway. The sole decrease of specific activity observed in this stage was in ribulose 5-phosphate 3-epimerase, which can be also explained as a mechanism of saving hexose formation to enhance ribose production. During sporulation, the pathway activity is low.

The development changes of the non-oxidative pentose phosphate pathway are very similar when *Aspergillus oryzae* grows in the presence of glucose, mannitol or ribose. In the three cases the pathway plays a definitive biological role which is fundamental for the rapid growth of the mycelium in these carbon sources and accounting for ribose formation and NADPH production, compensated in the case of ribose-dependent growth by the induction of a specific glucose 6-phosphate dehydrogenase [21].

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