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CARBOHYDRATE CATABOLISM IN HARVESTED MUSHROOMS

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Key Word Index—*Agaricus bisporus*; Basidiomycetes; mushroom; glucose-6-phosphate dehydrogenase; phosphofructokinase; glucosephosphate isomerase; mannitol dehydrogenase; NADPH oxidase; product specific labelling.

Abstract—The activities of five enzymes of carbohydrate catabolism were assayed in mushrooms stored for up to four days at 18°. Glucose-6-phosphate dehydrogenase decreased rapidly during storage. Phosphofructokinase, glucosephosphate isomerase and mannitol dehydrogenase showed smaller decreases. NADPH oxidase activity showed an overall increase in storage. Glucosephosphate isomerase showed a peak in activity 2–3 days after harvest. Radiorespirometry and product specific labelling experiments indicated a reduction in pentose phosphate pathway activity during storage. It was concluded that there is a decline in pentose phosphate pathway activity after harvest.

INTRODUCTION

Mannitol is the major soluble carbohydrate constituent of the cultivated mushroom (*Agaricus bisporus*), making up to 50% of the dry weight [1]. In a previous paper it was shown that a decrease in mannitol levels in harvested mushrooms could account for about half of the post-harvest CO_2 production over a 4-day period [2]. An NADP-dependent mannitol dehydrogenase which synthesizes mannitol from fructose is present in *A. bisporus* [3]; oxidation of mannitol is thought to occur by a reversal of the reaction [4]. Mannitol breakdown may normally be restricted because of the high level of hexose oxidation by the pentose phosphate pathway, leading to a high NADPH:NADP ratio [4, 5, 6]. Mannitol turnover in the growing mushroom is low [7].

In the harvested mushroom it is possible that a reduction in the relative participation of the pentose phosphate pathway in hexose oxidation could allow mannitol oxidation via the Embden–Meyerhof pathway, thus bypassing the restriction. This paper reports the changes in activity after harvest of some enzymes involved in carbohydrate breakdown, and the results of ^{14}C labelling and radiorespirometry experiments used to determine the flux through the glycolytic pathways *in vivo*.

RESULTS

Changes in enzyme activity

Glucose-6-phosphate dehydrogenase activity decreased rapidly after harvest, showing a mean fall of 84 ± 5% (s.e.m.) over 4 days storage (Fig. 1). In one experiment no activity could be detected on the third or fourth day. The values for glucose-6-phosphate dehydrogenase are not corrected for 6-phosphogluconate dehydrogenase activity; the contribution of the latter was determined in a separate experiment (Table 1). Both activities decreased during storage. Added mannitol had no effect on glucose-6-phosphate dehydrogenase activity; thus changes in mannitol level in the undialysed extracts could not influence enzyme assays.

Phosphofructokinase levels decreased by a mean value of 30 ± 11% over 4 days storage (Fig. 1). The ratio

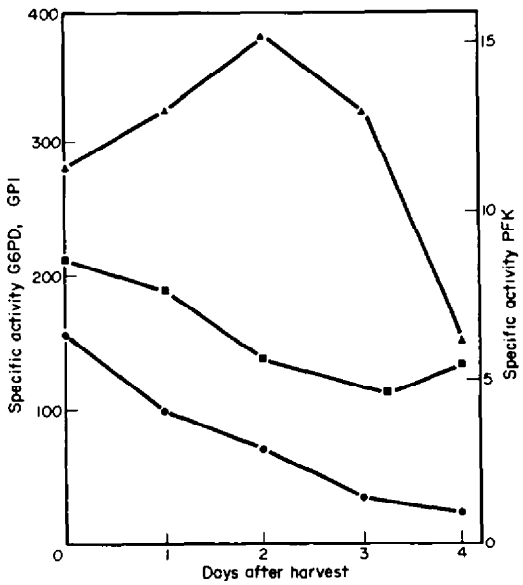

Fig. 1. Specific activity of glucose-6-phosphate dehydrogenase (G6PD) (●), glucosephosphate isomerase (GPI) (▲) and phosphofructokinase (PFK) (■) in mushrooms during storage. Values for G6PD and GPI are means of 6 experiments, and PFK for 5 experiments. Units mg protein^{-1}.

Table 1. 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase activities during storage

Day storage	Enzyme activity (nmol mg protein^{-1} min^{-1}) Glucose-6-phosphate dehydrogenase	6-phosphogluconate dehydrogenase
0	120	38
1	87	26
2	73	9
3	28	0
4	22	0

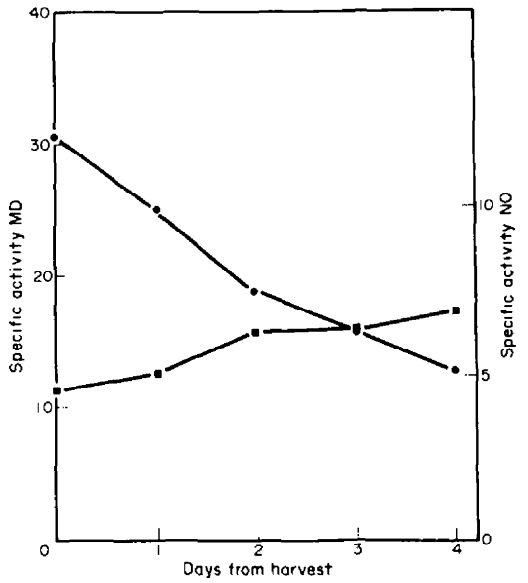

Fig. 2. Specific activity of mannitol dehydrogenase (MD) (●) and NADPH oxidase (NO) (■) in mushrooms during storage. Values are means of 6 experiments; units mg protein $^{-1}$.

of phosphofructokinase activity to that of glucose-6-phosphate dehydrogenase increased from 0.054 in fresh to 0.220 in 4-day-old mushrooms. Glucosephosphate isomerase activity increased to a peak 40 ± 12% greater than the day 0 value 2 to 3 days after harvest (Fig. 1). Following this a drop in activity to day 4 was observed, the overall fall in activity being 52 ± 8%.

Mannitol dehydrogenase showed a fall of 65 ± 6% from the initial value at the end of storage (Fig. 2). The decrease was fairly regular and there were no peaks comparable with those in glucosephosphate isomerase activity.

NADPH oxidase activity was present in the dialysed extracts. Mushroom phenol oxidase can oxidize NADH [8]. However, the observed activity was unaffected by treatment of the extract with phenylhydrazine, which is reported to be a specific inhibitor of mushroom phenol oxidase [9]. In addition a distinct band of NADPH oxidizing activity, which was separate from those of phenol oxidase, was observed after polyacrylamide gel electrophoresis. The level of NADPH oxidase varied considerably between experiments, but in all cases there was an increase after harvest (Fig. 2).

Table 2. Values for relative participation of the pentose phosphate pathway in hexose oxidation determined by (a) radiorespirometry, (b) product specific labelling

(a)	Days storage	% participation
	0	22
	1	10
	Means of 4 experiments	
(b)	Days storage	% participation
	0	65
	2	44
	4	32
	Means of 3 experiments	

Radiorespirometry

Results from the radiorespirometry experiments were calculated using the formula of Wang [10]: $G_p = G_1 - G_6/1 - G_6$, where G_p is the relative contribution of the pentose phosphate pathway; G_1 and G_6 are the activities of $^{14}CO_2$ produced from [1-^{14}C] or [6-^{14}C] glucose. G_1 and G_6 are expressed as fractions of the quantity of active glucose applied at the start of the experiment. Values for percentage pentose phosphate pathway oxidation of glucose from freshly harvested mushrooms and those stored for 1 day showed a decrease of *ca* 50% (Table 2a).

Specific activity determinations

The relative contributions of the two pathways of carbohydrate oxidation were also assessed by the product specific activity method [11]. The specific activity of alanine, which is rapidly labelled after glucose-[^{14}C] feeding of mushrooms [7], was determined. Tissue from fresh mushrooms gave values for pentose phosphate pathway activity of between 57 and 79%, while those stored for 4 days showed levels between 9 and 59% (Table 2b). In all 3 experiments a decrease of 25% or more in pentose phosphate pathway activity was observed over the storage period.

DISCUSSION

Previous work has shown that considerable development of the gill tissue occurs after harvest of the mushroom and that this is supported by an increase in respiration [2]. The enzyme changes reported here imply that post-harvest development is accompanied by a change in the relative participation of the pentose phosphate and Embden–Meyerhof pathways in carbohydrate catabolism. Growing mushrooms oxidize a relatively large proportion of hexose by the pentose phosphate pathway [5, 6]. The marked reduction seen in glucose-6-phosphate dehydrogenase activity after harvest was much greater than that observed for phosphofructokinase or glucose-phosphate isomerase, the first two enzymes of the Embden-Meyerhof pathway. A decrease in relative participation of the pentose phosphate pathway during storage was also indicated by radiorespirometry and product specific labelling experiments. The former method shows a drop of about 50% in pentose phosphate pathway activity after 1 day, while the latter showed a similar fall after 4 days' storage.

The variation in extent of decrease of pentose phosphate pathway activity seen between product specific labelling experiments and between these and the radiorespirometry experiments may be due to natural variation between crops of mushrooms. Variation between experiments in absolute values for enzyme activity was also considerable. This may also partly explain the large difference in absolute values for pentose phosphate pathway activity found using the different methods of determination since the experiments were conducted with different material. The specific labelling method may be expected to give larger values for pentose phosphate pathway activity [11], but the discrepancy between these and the radiorespirometric results appears too large to be due simply to the use of different assumptions in the calculation of results.

The post-harvest fall in pentose phosphate pathway activity may facilitate mannitol oxidation by reducing NADPH production. The increase in NADPH oxidase

activity could be a further factor in reducing the NADPH: NADP ratio during mannitol oxidation. There is some evidence that similar changes in glucose-6-phosphate dehydrogenase, glucosephosphate isomerase and NADPH oxidase activities occur during maturation of the growing mushroom (Hammond, unpublished results). Thus a loss of pentose phosphate pathway activity may be a normal part of mushroom ageing which is merely accelerated after harvest.

Mannitol dehydrogenase levels decreased during storage. However, if NADPH levels were low this would not create an obstacle to mannitol oxidation due to the large quantities of mannitol and small amount of fructose present [2], and to the excess of enzyme present even at the end of the storage period.

The changes in glucosephosphate isomerase show an interesting parallel with the changes in rate of CO_2 production, which rises to a peak one to two days after harvest [2]. It seems likely that the increase in enzyme activity is connected with the need for greater substrate utilization during gill expansion. The increased glucosephosphate isomerase activity could allow faster transfer of hexose derived from trehalose and polysaccharide breakdown into the Embden–Meyerhof pathway. Work is in progress to gain further insight into these processes.

EXPERIMENTAL

Mushrooms (*Agaricus bisporus* (Lange) Sing.; strain 'Darlington's 649') grown under commercial conditions were harvested as 'buttons' (Stage 2 [1]) and stored at 18° in a humidified airflow.

Enzyme assays. In each experiment a bulk sample of 4 mushrooms was taken daily. Half of each mushroom was sliced into liquid N_2, an Me_2CO powder was made from the other halves.

For glucose-6-phosphate dehydrogenase (EC 1.1.1.49-D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase) and glucosephosphate isomerase (EC 5.3.1.9-D-glucose-6-phosphate ketol-isomerase) assays the frozen tissue was homogenized in cold Tris–HCl buffer (0.1 M, pH 7.7) containing 50 mM 2-mercaptoethanol and 1 mM EDTA. After centrifugation for 10 min at 6000 *g* the supernatant was used for glucose-6-phosphate dehydrogenase assays. The same supernatant was dialysed overnight before assaying for glucosephosphate isomerase. Phosphofructokinase (EC 2.7.1.11-ATP: D-fructose-6-phosphate 1-phosphotransferase) was assayed in the supernatant after homogenizing frozen tissue in KH_2PO_4–NaOH buffer (50 mM, pH 6.5) containing 5 mM 2-mercaptoethanol, and centrifuging For mannitol dehydrogenase (EC 1.1.1.138-D-mannitol: $NADP^+$ 2-oxidoreductase) and NADPH oxidase determinations the Me_2CO powder was extracted with cold Tris-HCl buffer (0.1 M, pH 7.7) for 2 hr. After centrifugation the supernatant was dialysed overnight.

Glucose-6-phosphate dehydrogenase, glucosephosphate isomerase and mannitol dehydrogenase were assayed as described previously [6, 7]. The contribution of 6-phosphogluconate dehydrogenase activity in glucose-6-phosphate dehydrogenase assays was determined in a separate experiment. The difference in the rate of NADP reduction with 5.3 mM 6-phosphogluconate and with 5.3 mM 6-phosphogluconate and 5.3 mM glucose-6-phosphate was taken as the activity due to glucose-6-phosphate dehydrogenase [12]. The effect of mannitol on glucose-6-phosphate dehydrogenase activity was determined after addition of mannitol to a final concn of 6.7 or 13.3 mM in the assay mixture. The phosphofructokinase assay was adapted from the method for the yeast enzyme [13]. The assay mixture contained 4 mM fructose-6-phosphate, 0.1 mM ATP, 5 mM $MgCl_2$, 0.15 mM NADH, 5 mM 2-mercaptoethanol, 0.6 units aldolase, 3 units glycerophosphate dehydrogenase, 9 units triosephosphate isomerase and enzyme prepn in 3 ml Tris–maleate buffer (50 mM, pH 7.0). The absorbance of the mixture at 340 nm was followed. A blank without ATP was run. NADPH oxidase was assayed by monitoring the change in *A* at 340 nm of 3 ml Tris–HCl buffer (0.1 M, pH 7.1) containing 0.67 mH NADPH and enzyme prepn. Inhibition of activity by phenylhydrazine was tested for after pre-incubating the extract with 1 mM phenylhydrazine chloride for 30 min at 25° [8]. Polyacrylamide gel electrophoresis of extracts was carried out on 7.5% gels using Tris–glycine (5 mM, pH 8.5) running buffer. Gels were stained for NADPH oxidase activity by incubation in the dark in a soln of 3 mg NADPH and 3 mg iodonitro-tetrazolium violet in 10 ml Pi buffer (0.1 M, pH 7.2), and for phenol oxidase by incubation in a soln of tyrosine or DOPA (0.5 mg ml^{-1}) in Tris–HCl buffer (0.1 M, pH 8.5).

Assay conditions for all enzymes were optimized for the extracts used. Apparent activity changes due to interfering materials in the extracts were checked for by assaying mixtures of extracts from different samples; no evidence was found for such effects.

Protein determinations were carried out by the method of Lowry *et al.* [14] using bovine serum albumin standards. Units of activity are expressed as: nmol NADPH oxidized min^{-1} for mannitol dehydrogenase and NADPH oxidase; nmol fructose-6-phosphate produced min^{-1} for glucosephosphate isomerase; nmol fructose-1,6-biphosphate produced min^{-1} for phosphofructokinase and nmol NADP reduced min^{-1} for glucose-6-phosphate dehydrogenase.

Radiorespirometry. Radiorespirometry was carried out as described previously [6] on 1–2 mm thick slices of pileus tissue.

Specific activity determinations. Slices of pileus 1–2 mm thick were placed in Tris–HCl buffer (20 mM, pH 7.1) containing 5 μCi of glucose-[1-^{14}C] or glucose-[U-^{14}C]. After incubation for 30 min at 20° the slices were dropped into hot 75% EtOH and boiled for 15 min. After filtration the filtrate was passed down a column of Zerolit 225 (H^+) cation exchange resin. After washing the resin with 75% EtOH, the amino acid fraction was eluted with 2 M NH_4OH in 75% EtOH. The eluate was taken to dryness after addition of norleucine internal standard. *N*-dimethylaminomethylene propyl esters of amino acids were formed by the method of Thenot and Horning [15] using dimethylformamide dipropyl acetal in acetonitrile. Analysis was carried out by GLC with column and equipment as described previously [2], using a programme of 4 min at 100° followed by a rise of 4° min^{-1} to 280°. The detector heater was set at 270°. Radioactivity in peaks was detected and counted with a radiogas detector attached to the chromatograph.

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