HPLC Detection of Soluble Carbohydrates Involved in Mannitol and Trehalose Metabolism in the Edible Mushroom *Agaricus bisporus*

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A convenient and sensitive method was developed to separate and detect various types of carbohydrates (polyols, mono- and disaccharides, and phosphorylated sugars) simultaneously using high-performance liquid chromatography (HPLC). The method consists of a chromatographic separation on a CarboPac PA1 anion-exchange analytical column followed by pulsed amperometric detection. In a single run (43 min) 13 carbohydrates were readily resolved. Calibration plots were linear over the ranges of 5−25 μM to 1.0−1.5 mM. The reliable and fast analysis technique, avoiding derivatization steps and long run times, was used to determine the levels of carbohydrates involved in mannitol and trehalose metabolism in the edible mushroom *Agaricus bisporus*. Moreover, the method was used to study the trehalose phosphorylase reaction.

Keywords: *HPLC method; mannitol; trehalose; edible mushroom; Agaricus bisporus*

INTRODUCTION

Carbohydrates are present in fungi primarily as polysaccharides, in amounts varying between 16% and 85% of the dry weight (Blumenthal, 1976). Mono- and disaccharides are usually present in low concentrations in fungi, with the exception of certain storage carbohydrates. In the basidiomycete *Agaricus bisporus* the trehalose and mannitol contents may vary between 1−10% and 3−30% of the dry weight, respectively (Hammond and Nichols, 1975, 1976, 1979). Apparently these carbohydrates have important functions within this fungus. Mannitol probably acts as an osmoticum in the fruit bodies during active growth or as a postharvest substrate (Hammond and Nichols, 1976). Trehalose is supposed to function in translocation of carbon from mycelium to fruit bodies, at the onset of fructification (Hammond and Nichols, 1979; Wannet et al., 1999). Recently, the enzyme catalyzing both synthesis and degradation of trehalose in *A. bisporus*, trehalose phosphorylase, was purified to homogeneity from this fungus (Wannet et al., 1998). Besides trehalose and mannitol other carbohydrates might significantly contribute to the fungal metabolism and can be used for studying metabolic pathways. Studies on fungal carbohydrates have focused mainly on polyols (Witteveen et al., 1989; Witteveen and Visser, 1995; Hallsworth and Magan, 1997; Sánchez, 1998). Thus far the determination of other carbohydrate types in fungi has hardly received any attention, due to laborious and time-consuming analysis techniques (Bieleski, 1982; Beck and Hopf, 1990). For example, analysis of carbohydrates by gas chromatography is widely used but requires derivatization prior to determination (Knapp, 1979). Moreover, derivatization reduces the percentage recovery and involves handling carcinogenic compounds. As an alternative high-performance liquid chromatography (HPLC) columns have been used for the separation of various carbohydrates (for a recent review see LaCourse, 1997). With these columns, separation of various types of carbohydrates can be achieved, without the need to derivatize.

The present study was carried out to develop a sensitive and efficient HPLC protocol for the separation and detection of polyols, mono- and disaccharides, and phosphorylated sugars from the edible mushroom *A. bisporus*. The aim was to quantify these different types of carbohydrates in a single analysis without derivatization while using a minimal run time. Carbohydrates were analyzed in both mycelium and fruit bodies of this fungus. The HPLC method was also applied to monitor and quantify the carbohydrates involved in the metabolism of trehalose in *A. bisporus*.

MATERIALS AND METHODS

Materials. Mannitol, trehalose, fructose, glucose-1-phosphate, glucose-6-phosphate, mannitol-1-phosphate, mannose-6-phosphate, trehalose-6-phosphate, and fructose-6-phosphate were obtained from Sigma Chemical Co. (St. Louis, MO). Glucose, galactose, mannose, and sucrose were from Merck Biochemica (Darmstadt, FRG). NaOH (50% (w/w); analyzed reagent) and sodium acetate (analyzed reagent) were purchased from J.T. Baker (Deventer, NL). The CarboPac PA1 anion-exchange and guard columns were from Dionex (Sunnyvale, CA).

Organism and Enzyme Preparation. Fruit bodies and mycelium from the commercial mushroom *A. bisporus* (strain Horst U1) were obtainded from the Mushroom Experimental Station (Horst, NL). The fungus was cultured on compost as described before (Baars, 1996). Cell-free extracts (CFE) of *A. bisporus* fruit bodies and mycelium were prepared as described previously (Wannet et al., 1998, 1999). Carbohydrates were extracted from the CFE using a sonication apparatus (Fisons, Calgary, Canada) for 30 s at 30 W, followed by heating in a boiling water bath for 5 min. Subsequently, the sample was centrifuged (10 min at 10000*g*), and the supernatants were filtered through 0.45-μm Spartan 13 filters (Schleicher & Schull, Dassel, Germany) and analyzed for carbohydrates by HPLC.

The purification of *A. bisporus* trehalose phosphorylase was carried out according to Wannet et al. (1998).

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10.1021/jf990596d CCC: $19.00
Published on Web 01/19/2000

Table 1. Wave Form Parameters for Analysis

time (s)	potential (mV)
0.00	+500
0.20	+500
0.40	+500
0.41	+600
0.60	+600
0.61	−150
1.00	−150

Enzyme Assays. Trehalose phosphorylase activity was assayed in both directions. For trehalose degradation the reaction mixture contained 100 mM trehalose in 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.0). After incubation at 30 °C for 0−180 min, the reaction was stopped by heating (5 min at 100 °C) and the reaction mixture was centrifuged (10 min at 10000*g*). The glucose and α-glucose-1-phosphate liberated were assayed by HPLC. For trehalose synthesis the reaction mixture contained 50 mM glucose and 10 mM α-glucose-1-phosphate in 100 mM MES-KOH buffer (pH 6.5). After incubation at 30 °C for 0−180 min the reaction was stopped by heating (5 min at 100 °C). After centrifugation (10 min at 10000*g*) the liberated trehalose was assayed by HPLC.

Prior to HPLC analyses the samples were diluted with milliQ (Waters, Molsheim, France) water to a volume of 470 μL. After addition of 30 μL of galactose (2.83 mM) as internal standard, the samples were filtered (0.45-μm Spartan filters) and used for analysis by HPLC as described in the next section.

Separation of Carbohydrates. Standard carbohydrate solutions in the range of 0.25−1.50 mM were prepared in milliQ water and all contained 0.17 mM galactose as the internal standard. The 12 carbohydrates used were mannitol, trehalose, glucose, mannose, fructose, sucrose, trehalose-6-phosphate, mannitol-1-phosphate, α-glucose-1-phosphate, glucose-6-phosphate, mannose-6-phosphate, and fructose-6-phosphate. Separation and quantification of the carbohydrates were achieved using a Hewlett-Packard (Cupertino, CA) 1050 Ti automated gradient system consisting of a gradient pump, an autosampler, a UV detector, and an AD converter (interface 35900), which translated the signal of the pulsed electrochemical detector (pulsed amperometric mode; Dionex) to the computer. The pulsed electrochemical detector was equipped with a gold electrode and a Ag/AgCl reference electrode. The waveform parameters are given in Table 1. The system was controlled by a HPLC ChemStation (Windows version). The HPLC system was equipped with a CarboPac PA1 guard column (Dionex P/N 43096; 4 × 50 mm) connected to a CarboPac PA1 anion-exchange analytical column (Dionex P/N 35391; 4 × 250 mm). The column material (capacity 100 μequiv) contained polystyrene/divinylbenzene substrate agglomerated with microbeads containing a quaternary amine functional group linked to the latex (polyacrylate) matrix.

The mobile phase was prepared by degassing milliQ water with helium for 1 h, and then NaOH (50% (w/w)) was added to give a final solution of 100 mM (pH 13.5). To prepare eluent C, ground solid sodium acetate was added to 100 mM NaOH to a final concentration of 1 M. The water was degassed to reduce contamination of the mobile phase with Na_2CO_3, which causes accelerated elution of carbohydrates from the column resulting in reduced peak resolution. The degassing of the eluents continued throughout the experiments. The flow-rate of the mobile phase was 1 mL min^{-1}, and the injection volume was set at 20 μL. Injections of the standard solutions were preceded by two injections of milliQ water.

Separation of the carbohydrates was performed by a complex gradient of NaOH and sodium acetate (Table 2). First, isocratic elution took place during 7 min with 10 mM NaOH. Hereafter, a linear gradient was formed in 13 min from 10 to 50 mM NaOH, followed by a linear gradient from 50 mM NaOH to 50 mM NaOH plus 0.4 M sodium acetate formed during the next 25 min of elution. At this point all the standard carbohydrates were eluted from the column. After a 25-min reequilibration step from 50 to 10 mM NaOH, respectively, the next sample could be injected. The compounds to be analyzed were detected with a Dionex pulsed amperometry detector set at 5000 μCoulomb full scale. The UV detector was set at a wavelength of 215 nm to monitor the formation of the gradient. Elution was performed at ambient temperature.

Table 2. Elution Program for Separation of Carbohydrates on the CarboPac PA1 Anion-Exchange Column

	eluents[a]		
time (min)	A (%)	B (%)	C (%)
0	10	90	0
7	10	90	0
20	50	50	0
45	10	50	40
45.1	50	50	0
60	50	50	0
60.1	10	90	0
70	10	90	0

[a] Solutions: A = 100 mM NaOH, B = milliQ water, C = 100 mM NaOH + 1 M sodium acetate. All solutions were made in milliQ water and degassed thoroughly with helium before and during the separations. After 45 min all carbohydrates were eluted; the next 25 min was used for the removal of sodium acetate and to return the column to the starting conditions for the next analysis.

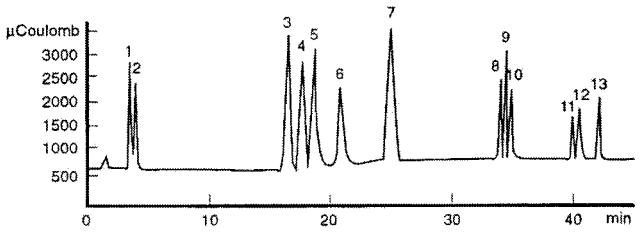

Figure 1. HPLC of standard carbohydrates. Peaks arose from: (1) mannitol, (2) trehalose, (3) galactose, (4) glucose, (5) mannose, (6) fructose, (7) sucrose, (8) trehalose-6-phosphate, (9) mannitol-1-phosphate, (10) α-glucose-1-phosphate, (11) glucose-6-phosphate, (12) mannose-6-phosphate, and (13) fructose-6-phosphate. Standards were present at a concentration between 0.25 and 1.50 mM (20-μL injection volume).

Sensitivity of Detection. Standard carbohydrate solutions (in the range of 0.25−1.50 mM) were made using the highest available purity grades. The solutions (20 μL) were injected onto the HLPC system, and peak areas were obtained with the Hewlett-Packard 3390A integrator. Calibration graphs were constructed by plotting the area against the amount (external standard) or by plotting the area ratio against the amount ratio (internal standard).

Protein Determination. Protein concentrations were determined with the BioRad protein micro assay kit (BioRad Laboratories, Richmond, CA) using γ-globulin as a standard.

RESULTS

Separation of Carbohydrates. The HPLC chromatogram of a standard mixture of pure carbohydrates obtained by anion-exchange chromatography on the CarboPac PA1 column, detected by pulsed amperometry, is shown in Figure 1. All standard carbohydrates could be separated in a single run. The affinity of the carbohydrates toward the stationary phase increased in the following order: sugar alcohol < monosaccharides < disaccharides < phosphorylated sugars. The retention times of the individual components were rather constant, though slightly decreased resolution was observed when ambient temperature was above 25 °C. This effect was more pronounced for the disaccharides and the phosphorylated sugars. It was possible to distinguish between the α- and β-anomers of glucose-1-phosphate, the latter having a significantly increased retention time (not shown).

Table 3. Retention Time and Calibration Plot Statistics of Carbohydrates on the CarboPac PA1 Column

		line equations of calibration curves			
		external standard method[c]		internal standard method[d]	
compound[a]	t_R[b] (min)	equation	correlation coefficient	equation	correlation coefficient
mannitol	3.4	$y = 1676x$	0.9945	$y = 2749x$	0.9997
trehalose	4.0	$y = 7096x$	0.9970	$y = 3611x$	0.9995
galactose	16.5	*e*		*e*	
glucose	17.6	$y = 8346x$	0.9764	$y = 3919x$	0.9967
mannose	18.6	$y = 5819x$	0.9979	$y = 3349x$	0.9997
fructose	20.6	$y = 11275x$	0.9980	$y = 4537x$	0.9995
sucrose	25.1	$y = 9959x$	0.9989	$y = 4125x$	0.9999
trehalose-6-phosphate	34.0	$y = 2582x$	0.9999	$y = 559x$	0.9979
α-glucose-1-phosphate	34.6	$y = 1645x$	0.9987	$y = 1317x$	0.9945
mannitol-1-phosphate	35.0	$y = 5182x$	0.9997	$y = 681x$	0.9967
glucose-6-phosphate	40.0	$y = 1851x$	0.9991	$y = 769x$	0.9970
mannose-6-phosphate	40.6	$y = 3604x$	0.9970	$y = 1488x$	0.9936
fructose-6-phosphate	42.2	$y = 3162x$	0.9997	$y = 1816x$	0.9972

[a] Limit of detection 1−5 pmol/injection. The area responses at the detection limits show a variation of about 10%. [b] The standard deviation in the retention time (t_R) was in all cases ±0.05 min or lower. [c] Amounts injected (up to 30 nmol) were plotted against the area response of the pulsed amperometric detector. [d] The amount ratio (compound/internal standard) was plotted against the area ratio (compound/internal standard). [e] Galactose was used as an internal standard at 3.3 nmol/injection.

Table 4. Levels of Carbohydrates in Fruit Bodies and Mycelium of Compost-Grown *A. bisporus*, Measured by Pulsed Amperometric Detection[a]

growth stage of fruit body	mannitol (mM)	trehalose (mM)	fructose (mM)
1	85 ± 3.2	13 ± 0.4	1.6 ± 0.1
2	204 ± 3.8	15 ± 0.3	1.8 ± 0.1
3	264 ± 6.2	18 ± 0.4	1.8 ± 0.1
4	287 ± 6.2	15 ± 0.5	1.5 ± 0.1
5	302 ± 6.5	16 ± 0.5	1.9 ± 0.2
mycelium[b]	14 ± 0.5	38 ± 1.5	1.0 ± 0.1
mycelium[c]	12 ± 0.3	15 ± 0.6	0.8 ± 0.1

[a] Each value represents the mean ± SD (n = 3). [b] Mycelium harvested between periods of fruiting. [c]Mycelium harvested during fruiting.

Sensitivity of Detection. Retention times and calibration plot statistics of carbohydrates are compiled in Table 3. Data are given of both external and internal standard methods. The linear regression coefficients of the standard curves were calculated for each compound to determine the uniformity of the detector response over the range of the concentrations. Regression coefficients varried between 0.9936 and 0.9999. Up to at least 15−30 nmol of the various compounds can be accurately determined. The sensitivity of the individual components toward pulsed amperometric detection was higher for the mono- and disaccharides compared to the phosphorylated sugars. Detection limits were in the order of 1−5 pmol, which equals 50−250 nM in the sample when 20 μL is injected.

Levels of Carbohydrates in Fruit Bodies and Mycelium of *A. bisporus*. Table 4 shows the levels of the major soluble carbohydrates in cell-free extracts from compost-grown *A. bisporus* fruit bodies and mycelium, respectively. The fruit bodies contain large amounts of mannitol (up to 300 mM) next to smaller quantities of trehalose and fructose. The concentration of mannitol in the fruit bodies increased during growth, whereas the levels of trehalose and fructose almost remained constant. The low levels of fructose imply a fast turnover of this carbohydrate for mannitol synthesis. Mannitol concentrations in the different parts of the fruit body at growth stage 2 (Hammond and Nichols, 1976) were 192 ± 4.1, 212 ± 4.3, and 64 ± 1.2 mM for stipe, pileus, and lamellae, respectively (not shown). In the mycelium the levels of trehalose varied depending on the moment of harvesting, i.e., between or during periods of fruiting.

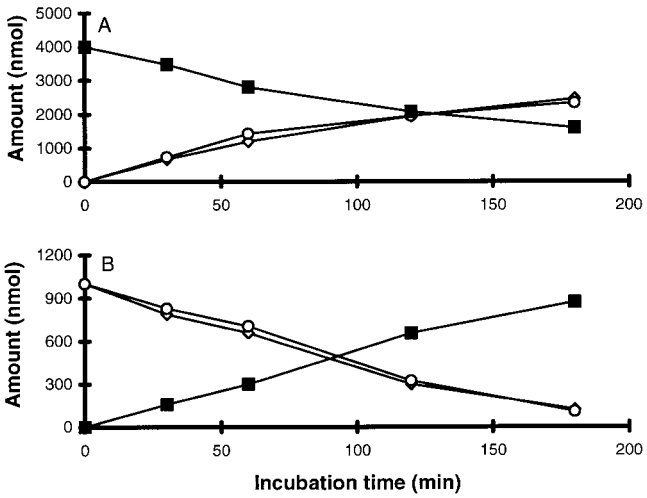

Figure 2. Degradation (A) and synthesis (B) of trehalose by trehalose phosphorylase from *A. bisporus*. The amounts of trehalose (■), glucose (○), and glucose-1-phosphate (◆) are plotted against time. Measurents were performed in duplicate.

This was also observed in a previous study on the carbohydrate metabolism of *A. bisporus* (Wannet et al., 1999). Besides trehalose, the mycelium contained small quantities of mannitol and fructose. Low concentrations of glucose (range 0.03−0.8 mM) and in some cases also low concentrations of sucrose (range 0.01−0.2 mM) were found both in fruit bodies and mycelium. Mannose appeared only in fruit body aggregates (stage 1) in concentrations between 0.01 and 0.3 mM. The retention times for the various carbohydrates from the extracts corresponded closely to those of the standard solutions (SD = 0.05 min).

Activity of Trehalose Phosphorylase. To test a further application of the HPLC method, both synthesis and degradation of trehalose by purified trehalose phosphorylase from *A. bisporus* were monitored. The results clearly show that both glucose and α-glucose-1-phosphate are produced at the expense of trehalose and that synthesis of trehalose takes place by using glucose and α-glucose-1-phosphate. From Figure 2A it can be calculated that in 180 min of incubation the trehalose content was decreased at a rate of 13.4 ± 0.15 nmol/min. Concomitantly glucose and α-glucose-1-phosphate were produced at a rate of 13.7 ± 0.18 and 13.1 ± 0.17

nmol/min, respectively. Thus, the amounts of trehalose converted equalled the amounts of glucose and α-glucose-1-phosphate produced. No glucose and α-glucose-1-phosphate were formed when trehalose was omitted from the enzyme reaction. From Figure 2B it can be calculated that in 180 min of incubation the glucose and α-glucose-1-phosphate contents decreased at a rate of 4.9 ± 0.10 and 5.0 ± 0.12 nmol/min, respectively. In the same period trehalose was produced at a rate of 4.9 ± 0.12 nmol/min. No trehalose was synthesized when glucose or α-glucose-1-phosphate was omitted from the enzyme reaction.

DISCUSSION

Anion-exchange chromatography can be used for the separation of different types of carbohydrates. Because the p*K* values of carbohydrates lie between 12 and 14 (Weiss, 1995), they can be converted into their anionic form in an alkaline environment and may be separated on a strongly basic anion-exchanger in the hydroxide form. Separation occurs on the basis of charge and conformation, and a compound can be identified by its retention time in the chromatogram. In the present study, this technique was used to determine and quantify carbohydrates present in mycelium and fruit bodies of the basidiomycete fungus *A. bisporus*.

With the applied HPLC method it was possible to analyze 13 different carbohydrates, including phosphorylated ones, in a single run within 43 min. Separation of carbohydrates was as effective for *A. bisporus* cell-free extracts as for standard solutions. From the regression coefficients of the standard compounds and from the low detection limits, it can be concluded that this mode of analysis is exceptionally accurate and represents a highly sensitive level of detection. The experiments show that the HPLC protocol can be used for accurately studying carbohydrate fluxes, on both a qualitatively and a quantitatively reliable basis.

HPLC analysis of carbohydrates is superior to nuclear magnetic resonance (NMR) and gas chromatography (GC). Pulsed electrochemical detection using a gold electrode is more sensitive than other modes of HPLC detection of carbohydrates (Weiss, 1995). Although it is possible to simultaneously determine polyols and trehalose by NMR (Hocking, 1986; Meikle et al., 1991; Van Eck et al., 1993), this technique is not as sensitive as HPLC and large samples are needed to perform accurate analyses (Van Eck et al., 1993). GC analyses have been used frequently in studies of fungal carbohydrates (Al-Hamdani and Cooke, 1978; Bidochka et al., 1990; Bhajekar and Kulkarni, 1992; Aoki et al., 1993). However, no GC protocol appears to be available for the simultaneous determination of different types of carbohydrates (Witteveen et al., 1995). Besides this, certain carbohydrates have to be derivatized prior to GC analysis (Pfyffer and Rast, 1980; Adler et al., 1982; Beck and Hopf, 1990).

Therefore, the proposed HPLC method has clear advantages over alternative methods for the identification of carbohydrates in biological samples. First of all, it is a highly sensitive procedure (Table 3), and the assay itself is completed within 43 min. Moreover, it is convenient in using low-hazard and inexpensive reagents and in requiring automated equipment. Hence, the method is suited for batch measurements of carbohydrates in biological tissues, as well as to monitor carbohydrate formation and degradation in enzymatic reactions.

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Received for review June 2, 1999. Revised manuscript received September 22, 1999. Accepted November 18, 1999.

JF990596D