Original Paper Plant Tissue Culture Letters, **2** (1), 14–21 (1985)

Carbohydrate Utilization and Activities of Various Glycosidases in Cultured Japanese Morning-Glory Callus

Shigeru HISAJIMA and Trevor A. THORPE[a]

Institute of Applied Biochemistry, University of Tsukuba, Ibaraki, Japan 305
[a]Plant Physiology Research Group, Department of Biology, University of Calgary, Calgary, Alberta, Canada T2N 1N4

(Received October 1, 1984)
(Accepted December 3, 1984)

The capacity of various carbohydrates to support growth of Japanese morning-glory callus in the dark over a 14-day period was examined. Sucrose was the most effective compound, but glucose, fructose, trehalose, maltose, cellobiose, raffinose and soluble starch supported significant growth. The callus remained alive in the presence of inulin, mannitol, inositol, methyl-α-glucoside and glycerol; while cells grown on galactose, mannose, sorbose, xylose, arabinose, melibiose, lactose, dextran, carboxy-methyl cellulose, sorbitol, galactitol, ethylene glycol were necrotic. Examination of the effects of these various carbon sources on the cell wall and cytoplasmic activities of acid invertase, trehalase, maltase, cellobiase, melibiase and lactase could not be correlated with the growth-promoting activity of their substrates. Extracellular hydrolysis of sucrose, trehalose, maltose, cellobiose, lactose, raffinose, and inulin occurred as a consequence of the presence of cell wall hydrolases in the morning-glory callus, and hydrolytic products could be detected in the medium.

Plant cells in culture require an exogenous carbon/energy source for growth and differentiation. Although sucrose, the sugar of transport in plants, is the most widely used carbohydrate, other classes of carbohydrates are able to fulfill this role.[1–3] The biochemical mechanism for utilization of carbohydrate from the medium has not been thoroughly examined for sugars other than sucrose and glucose.[3,4] We have been examining sucrose metabolism in tobacco[5,6] and Japanese morning-glory callus.[4,7–10] While in tobacco extracellular hydrolysis of sucrose is apparently not of major significance in its utilization, in Japanese morning-glory cell wall bound invertase plays a key role in sucrose utilization.[4,7,11] Such differences in the pattern of sucrose utilization have been observed with other tissues.[3]

Our interests in carbohydrate utilization in vitro has led us to use other systems (e.g. soybean and persimon callus[12]) as well as to use other carbon sources. In this paper, we examined the effects of various carbohydrates on the growth of Japanese morning-glory callus, and on the activity of several glycosidases, which could be involved in carbohydrate hydrolysis.

Materials and Methods

Plant material and tissue culture conditions. Callus of morning-glory (*Pharbitis nil* L. var. Violet) was cultured at 27°C in the dark on medium containing Murashige & Skoog[13] basal salts 0.3% yeast extract, 0.75% agar and 3% sucrose. The medium was adjusted at pH 5.8 before autoclaving at 121°C for 5–8 min. For experimentation about 150 mg of friable cells were inoculated in 50 ml medium containing 175 mM of hexose monosaccharide, 87.5 mM of

disaccharide, 58.5 mM of trisaccharide or 3% (w/v) of other carbohydrate in the dark at 27°C. Preliminary experiments showed that no breakdown sugars could be detected in the media after autoclaving. After 14 days in culture, cells were harvested and fresh weight measured.

Enzyme preparation. Callus (2 g) was homogenized with 5 ml of 100 mM glycine buffer (pH 8.5) in a Teflon homogenizer. The homogenate was centrifuged at 10,000×*g* for 15 min. The extraction was repeated thrice and supernatants were collected. The solution was dialysed against 6 *l* of 5 mM glycine buffer (pH 8.5) overnight, and the dialysate was centrifuged at 15,000×*g* for 20 min. The supernatant was used as the cytoplasmic fraction. The precipitate was washed with cold water by centrifugation and suspended in 4-ml cold water. The suspension was used as the cell wall fraction.

Enzyme assay. In a standard assay, a reaction mixture containing substrate 1 μmol, buffer (optimum pH) 8 μmol, and 30 μl of enzyme preparation in a total volume of 100 μl was incubated for 0, 15 or 30 min. After the reaction period, enzyme activity was stopped by heating at 100°C for 1 min. The reducing sugars formed were assayed by Nelson's method or the glucose oxidase method.[14] Natural substrates were used, i.e., sucrose for invertase (β-D-fructofuranosidase, EC 3.2.1.26), maltose for maltase (α-D-glucosidase, EC 3.2.1.20), cellobiose for cellobiase (β-D-glucosidase, EC 3.2.1.21), melibiose for melibiase (α-D-galactosidase, EC 3.2.1.22), lactose for lactase (β-galactosidase, EC 3.2.1.23) and trehalose for α,α-trehalase (EC 3.2.1.28). One unit of each enzyme is defined as the amount of the enzyme which hydrolyzed 1 μmol of substrate per min in the standard assay condition. The average enzyme activity was calculated from the initial velocities of duplicate enzyme preparations.

Sugar identification. After the culture period, medium sugars were extracted with hot 80% methanol according to the method previously described.[7,15] The sugars were separated by cellulose thin layer chromatography with Avicel S. F. using *n*-butanol–pyridine–water (6: 4: 3) or *n*-butanol–acetic acid–water (4: 1: 1) as solvents and identified by using urea-HCl or alkaline-$AgNO_3$ as spray reagents.

Results and Discussion

Carbohydrate nutrition

The effects of various carbohydrates, mono-, di-, tri- and poly-saccharides on growth of

Table 1. Growth of dark-grown Japanese morning-glory callus after 14 days in culture on various carbon sources.

Monosaccharides		Oligo- and polysaccharides		Sugar alcohols	
Compound	%FW	Compound	%FW	Compound	%FW
glucose	84	trehalose	87	sorbitol	6.6
fructose	57	maltose	66	mannitol	10
galactose	2.5	cellobiose	76	galactitol	7
mannose	3.6	melibiose	8	inositol	15
sorbose	3.3	lactose	23	methyl-α-glucoside	11
xylose	2.5	raffinose	44	glycerol	10
arabinose	3.6	inulin	12	ethylene-glycol	3.6
		dextran	7.3		
no sugar	5.3	sol. starch	87		
		CMC	9		

Data expressed as a % of that obtained on sucrose; for monosaccharides, 2.00 g FW/culture =100%; for oligosaccharides 1.51 g FW/culture=100%, and for sugar alcohols, 1.48 g FW/culture=100%. CMC=carboxymethyl cellulose.

Japanese morning-glory callus were determined quantitatively during log phase growth for the sucrose-grown cultures (**Table 1**). Sucrose, as is generally found, was the most effective carbon source among the carbohydrates tested. The growth condition of cells could be classified into three groups based on their appearance after 2 weeks: (1) actively growing, (2) alive but not growing and (3) necrotic. The first group contained cells grown on sucrose, glucose, fructose, trehalose, maltose, cellobiose, raffinose and soluble starch. The fresh weights of these cells varied from 44–87% of those obtained when the tissues were grown on sucrose. These cells were yellow or light yellow in colour and were friable. The second group contained cells grown on inulin, mannitol, inositol, methyl α-glucoside, and glycerol. These cells were yellow in colour and compact. The fresh weights of these cells were between 10–15% of those grown on sucrose. These cells maintained the size of original inoculum (or the fresh weights of the original inocula). This suggests that these cells (or at least some of the cells) were still alive. Maintenance may well have been a result of sucrose carry over from the inoculum as was suggested for sorbitol-grown callus of tobacco.[16] The third group contains cells grown in galactose, mannose, sorbose, xylose, arabinose, melibiose, lactose, dextran, carboxymethyl cellulose (CMC), sorbitol, galactitol, ethylene glycol or no carbohydrate. These cells were characterized by being in part black, compact and showing varying degrees necrosis. The fresh weights of the cells grown on these last group of carbohydrates were generally lower than those grown on no carbohydrate, indicating that probably after these cells had used their cell reserves, the carbon sources had become toxic.

The fresh weight of the cells cultured on lactose increased slightly. However, the culture turned black and compact, suggesting necrosis. Taking into account that morning-glory cells hydrolyzed medium lactose (**Table 5**), could utilize glucose (**Table 1**), and that galactose added in the medium inhibited cell growth (**Table 1**), galactose formed from lactose in the medium probably inhibited cell growth. It should be noted that some cells were still alive, because it is from such cultures that we previously obtained a line of morning-glory callus that became adapted to lactose on repeated subculture on that carbon source.[17] The lactose-adapted line had the same morphological and growth characteristics as the original line and grew better on lactose than sucrose and could also grow on galactose. Raffinose contains galactose. In this study cells grown on raffinose were not inhibited (**Table 1**). Medium sugar analysis showed that raffinose was hydrolyzed into fructose and melibiose (**Table 5**). The cells did not assimilate melibiose. Thus these cells utilized fructose to grow.

Morning-glory cells could not utilize any sugar alcohol. In general plant cells cannot utilize sugar alcohols as sole carbon sources, except members of the Rosaceae, in which these compounds are important natural carbohydrates.[18]

Effect of carbohydrates on activities of glycosidases

On the basis of studies on sucrose metabolism in Japanese morning-glory callus, it was suggested that sucrose added in the medium was hydrolyzed by cell wall-bound invertase and then incorporated into the cultured cells as monosaccharides.[7] Also in these morning-glory cells, glucose derived from sucrose was preferentially taken up into the cells over fructose, the other moiety of sucrose. Several disaccharidases beside invertase have also been detected in both cytoplasmic and cell wall fractions of cultured morning-glory cells.[15] In those experiments disaccharides were used as substrates. The disaccharidases are thought to play a role in the corresponding disaccharide nutrition. The effects of carbohydrates on activities of glycosidases, except for invertase, have not generally been studied in plant tissue cultures. In addition, cellular conditions, for instance, active growth, inactive growth and incipient necrosis, may reflect disaccharidase activities, which can be correlated with nutrition. Therefore, we

Table 2. Effect of monosaccharides in activities of glycosidases in cytoplasmic (A) and cell wall (B) fractions of Japanese morning-glory callus.

Carbohydrate	acid invertase	Trehalase	Maltase	Cellobiase	Melibiase	Lactase
			A			
Sucrose Act.	(71)	(196)	(7.1)	(7.3)	(5.4)	(52)
	100%	100	100	100	100	100
Glucose	150	136	100	159	133	115
Fructose	180	122	96	82	111	130
Galactose	53	17	+	91	89	25
Mannose	35	45	+	120	+	50
Sorbose	61	27	+	127	+	5
Xylose	61	14	+	184	+	+
Arabinose	57	13	+	269	94	29
No sugar	66	11	+	118	67	8
			B			
Sucrose Act.	(154)	(90)	(4.6)	(10)	+	(6.3)
	100%	100	100	100	+	100
Glucose	124	100	120	97	+	114
Fructose	113	82	113	87	+	124
Galactose	160	71	49	47	+	42
Mannose	147	73	48	47	+	
Sorbose	102	74	45	48	+	27
Xylose	108	75	41	69	+	53
Arabinose	97	76	38	58	+	37
No sugar	312	83	41	63	+	0

Activity (Act.)=10^{-3} unit·g^{-1} FW. Activity in sucrose-grown tissue (in parentheses) =100% for each enzyme. Activities of enzymes on other carbon sources expressed as a % of that on sucrose. +=trace activity.

selected several glycosidases and determined their activities in both the cytoplasmic and cell wall fractions in 2 week-old cultures (**Tables 2–4**).

Cells grown on sucrose or no carbohydrate were employed as the reference standards; however the data are presented as a percentage of the particular activity of the sucrose-grown cells. From these data, it is hard to pick out any specific trends. However, several characteristics are observed. (1) The activities of all disaccharidases in both fractions of the cells grown on glucose or fructose were generally higher than in tissue grown on sucrose (**Table 2**). It is also interesting that invertase activity in cells grown on sucrose is lower than those grown on glucose or fructose. It is not easy to explain this phenomenon, because it is reasonable that morning-glory cells grown on sucrose should have more invertase activity than those grown on its component sugars. However, high invertase activity has been found in tobacco callus under these conditions, but the role of invertase in that tissue is not clear.[6] (2) Living but not growing, or necrotic cells gave low activities of the cytoplasmic glycosidases except cellobiase (**Tables 3, 4**) and melibiase (**Table 4**) in some cases in comparison with the activities of corresponding glycosidases in sucrose-grown cells. Induction of disaccharidase activity by the corresponding disaccharide did not appear to occur, judging from the lack of increase in glycosidase activity observed (**Table 3**). It should be noted that the enzymes were assayed using natural substrates and thus the activity observed should have functional significance. However, in some respects the measurable enzyme activity appeared to be independent of any obvious function.

Table 3. Effect of oligo- and polysaccharides on activities of glycosidases in cytoplasmic (A) and cell wall (B) fractions of Japanese morning-glory callus.

Carbohydrate	acid invertase	Trehalase	Maltase	Cellobiase	Melibiase	Lactase
			A			
Sucrose Act.	(62.5)	(210)	(5.8)	(9.0)	(30)	(30.5)
	100%	100	100	100	100	100
Trehalose	76	91	30	73	80	97
Maltose	76	94	64	69	94	84
Cellobiose	77	76	50	73	120	93
Melibiose	36	11	+	76	160	21
Lactose	73	138	77	176	167	90
Raffinose	55	80	60	125	75	109
Inulin	83	14	33	257	0	41
Dextran	35	11	31	171	100	81
Soluble starch	36	49	65	63	100	62
CMC	36	13	33	214	160	56
			B			
Sucrose Act.	(156)	(73)	(2.7)	(29)	+	(6.1)
	100%	100	100	100	+	100
Trehalose	116	133	67	102	+	140
Maltose	57	73	113	89	+	48
Cellobiose	114	129	67	109	+	90
Melibiose	162	85	87	84	+	100
Lactose	122	128	133	134	+	73
Raffinose	150	107	100	70	+	81
Inulin	300	81	117	93	+	55
Dextran	320	73	78	59	+	110
Soluble starch	65	74	111	83	+	64
CMC	488	105	22	10	+	18

Activity (Act.)=10^{-3} unit·g^{-1} FW. Activity in sucrose-grown tissue (in parentheses)=100% for each enzyme. Activities of enzymes on other carbon sources expressed as a % of that on sucrose. +=trace activity, CMC=carboxymethyl cellulose.

Sugar components in culture medium

Sugar components in the culture medium after the 14 day culture period were determined (**Table 5**). Sucrose, trehalose, cellobiose, lactose and raffinose supplied in the medium were apparently hydrolyzed into their component sugars. Medium sucrose was hydrolyzed in carrot,[19] and as indicated earlier, similar hydrolysis by cell wall-bound invertase, was followed by incorporation of the monosaccharides into morning-glory.[7] It appears that trehalose, maltose and cellobiose were hydrolyzed by the corresponding disaccharidases. However the precise mechanisms are as yet unknown. Raffinose in the medium was changed into fructose and melibiose (**Table 5**). This suggests that raffinose was hydrolyzed by β-fructofuranosidase but not by α-galactosidase. In the inulin-containing medium, fructose and inulooligosaccharides were present. It is not yet known whether inulin was hydrolyzed by inulinase or β-fructofuranosidase in the morning-glory cells. In the starch medium, glucose, maltose and maltooligosaccharides were detected (**Table 5**). Utilization of starch by tissue culture has been shown to be dependent on the secretion of amylases into the medium, e.g., in *Juniperus*[20] and in sugarcane.[2] In a preliminary experiment, the same level of α-amylase activity was detected in liquid media supplied with sucrose or starch after suspension culture of morning-glory cells. The result suggests that α-amylase was not induced by starch.

Table 4. Effect of sugar alcohols on activities of glycosidases in cytoplasmic (A) and cell wall (B) fractions of Japanese morning-glory callus.

Carbohydrate acid		invertase	Trehalase	Maltase	Cellobiase	Melibiase	Lactase
				A			
Sucrose	Act.	(77)	(198)	(12.5)	(6.7)	(4.8)	(42)
		100%	100	100	100	100	100
Sorbitol		36	13	33	188	100	56
Mannitol		33	16	42	198	100	76
Galactitol		44	63	83	100	138	106
Inositol		31	20	56	163	100	62
Methyl α-glucoside		54	14	37	221	125	41
Glycerol		30	11	37	218	113	62
Ethylene glycol		24	9	34	205	40	41
				B			
Sucrose	Act.	(148)	(76)	(5.4)	(73)	+	(6.6)
		100%	100	100	100	+	100
Sorbitol		150	107	100	73	+	81
Mannitol		94	93	39	97	+	145
Galactitol		100	112	56	67	+	177
Inositol		317	50	56	7	+	73
Methyl α-glucoside		367	76	78	93	+	55
Glycerol		450	72	100	110	+	127
Ethylene glycol		125	67	44	60	+	90

Activity (Act.)=10^{-3} unit·g^{-1} FW. Activity in sucrose-grown tissue (in parentheses)=100% for each enzyme. Activities of enzymes on other carbon sources expressed as a % of that on sucrose. +=trace activity.

Table 5. Sugars present in the culture medium after growth of Japanese morning-glory callus for 14 days on different carbon sources.

Sugars added to the medium	Sugars after the culture period
Sucrose	glucose, fructose, sucrose
Trehalose	glucose, trehalose
Maltose	glucose, maltose
Cellobiose	glucose, cellobiose
Melibiose	melibiose
Lactose	glucose, galactose, lactose
Methyl α-glucoside	methyl α-glucoside
Raffinose	fructose, melibiose, raffinose
Inulin	fructose, fructooligosaccharides
Dextran	dextran
Starch	glucose, maltose, maltooligosaccharides
Carboxymethyl cellulose	carboxymethyl cellulose
Control (no sugar added)	none

In conclusion, Japanese morning-glory cells are useful for studies on carbohydrate nutrition and metabolism. The tissue shows a wide range of responses to medium carbohydrates and it can be used to produce specific carbohydrate adapted cells, as we showed earlier for lactose.[17] Furthermore the callus contains a large number of cell wall-bound glycosidases. On the basis

of our use of natural substrates it would seem that these hydrolases should have functional significance. However they do not appear to be substrate-induced, and their activity is independent of the growth effects of their substrates. Consequently the question remains as to why so many of these glycosidases should be present in the morning-glory cell walls and in the cytoplasm, when the tissue does not normally come into contact with their specific carbohydrate substrate. The possibility exists that these glycosidases may be non-specific and their presence in some cases may be unrelated to any physiological role. The fact that cultured tissues show great plasticity in the use of various carbon sources could be a consequence of the presence of low (constitutive?) levels of such glycosidases, which show increased activity (amplification?) during tissue adaption to a new carbon source. This view is not unreasonable as only in the case of glycerol-adapted cells has it been shown that the adaptation is a result of a mutation.[22)]

This work was supported by the research project fund of the University of Tsukuba, Japan to S.H., and by NSERC of Canada Grant No. A-6467 to T.A.T. S.H. was a Visiting Scientist at the University of Calgary during 1983-84.

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《和文要約》

アサガオカルス細胞培養系における炭素源代謝と数種グリコシダーゼ活性について

久島　繁・Trevor A. Thorpe*

筑波大学応用生物化学系
*キャルガリー大学生物学科

植物組織培養系における炭素源代謝研究の一環として，多糖から単糖に至る種々の糖の炭素源としての有効性，炭素源と細胞壁ならびに細胞質画分における数種糖加水分解酵素活性の相関等を検討した．

炭素源としての有効性から糖は三群に識別された．(i) 培養組織を生育させる糖，(ii) 生存させるが，生育を促さない糖，および(iii) 培養組織にネクロシスを起こさせる糖，であった．

炭素源の種類と検討したいずれの酵素活性，すなわち，インベルターゼ，トレハラーゼ，マルターゼ，セロビアーゼ，メリビア，ゼーラクターゼ活性の間にも相関性は認められなかった．

培養後，ショ糖，トレハロース，マルトース，セロビオース，ラクトース，ラフィノース，イヌリンの培地中に，それぞれの水解物が検出された．

実験結果に基づき，培地糖の代謝，炭素源適応などにつき論議した．