

# Modified Substrates and Modified Products as Inducers of Carbohydrases

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Cellobiose and isomaltose are both inducers and repressors of cellulase and dextranase, respectively. The repression can be avoided by supplying the disaccharide slowly. This has been done by use of palmitate and acetate esters which are hydrolyzed by esterases of the growing organism to yield the inducer. Sucrase yields, also, are greatly increased (to 80 times) by substituting sucrose monopalmitate for sucrose in the culture medium.

Substrates are the usual inducers of the enzymes which catalyze their hydrolysis, but *products* of enzyme action appear to be the agents which stimulate the production of the hydrolases of various polymers. Thus, cellobiose, the dimeric product of cellulose hydrolysis, induces cellulase (endo- $\beta$ -1 $\rightarrow$ 4 glucanase) in fungi (4) and in bacteria (K. Nisizawa, K. Yamane, and H. Suzuki, Amer. Chem. Soc. Monogr. Ser., *in press*); maltose, the dimeric product of amylose hydrolysis, induces  $\alpha$ -amylase (2). For inducing enzymes which hydrolyze more complex polysaccharides, e.g. pneumococcal polysaccharides (9), the product which induces best is often larger than a dimer and contains within it the linkage for which the enzyme is specific. Even products lacking the glycosidic bond, i.e. monomers, have been claimed to be inducers [e.g. D-galacturonic acid, etc. (5)]. However, in view of our finding that a trace amount of impurity (sophorose) accounts for the induction of cellulase by reagent grade glucose (3), we would suggest reexamination of the role of monomer.

However, at high concentrations, soluble inducers repress the inductive effect (4). Thus, it becomes necessary to supply the inducer at low concentrations over a long period of time. We have previously accomplished this by using a modified inducer, capable of being converted slowly into inducer by action of the growing organism (4). The present paper describes experiments using palmitate esters of inducing disaccharides to produce three different enzyme systems.

## MATERIALS AND METHODS

**Preparation of palmitate esters.** The palmitate esters of sucrose were prepared by transesterification be-

tween methyl palmitate and sucrose (V. D'Amato, U.S. Patent 3,054,789, 1962). The monopalmitate contained traces of the di- and triesters; the dipalmitate contained some triester, and a trace of monopalmitate.

The palmitates of isomaltose and of cellobiose were made by reacting palmitoyl chloride with the disaccharide (1). The monopalmitates of isomaltose were essentially free of higher esters; the dipalmitate was made up of two isomers free of monopalmitate and the x-palmitate contained higher esters, but only traces of the dipalmitate.

**Enzyme production.** Microorganisms were grown on a mineral salts solution (4) containing the test compound (0.5%). The nonionic surfactant, Tween 80, previously shown to stimulate enzyme production (8), was added at 0.1%. The cultures were grown on a reciprocal shaker at 29 C for 2 to 3 weeks, during which time samples were withdrawn for estimation of enzyme activity, by procedures previously described (4, 6, 7). One unit equals one microequivalent of glycosidic bonds hydrolyzed per minute at 50 C, except for sucrose where the hydrolysis was at 35 C.

## RESULTS

**Induction of endo-glucanases by products and modified products (of endo-glucanase action): cellulase.** The best yields of cellulase were obtained using cellulose as substrate (Table 1). Cellobiose, under the test conditions, is not a good inducer of cellulase in fungi; cellobiose esters are much better inducers. Of the palmitates, cellobiose monopalmitate tends to inhibit growth. It was particularly toxic to *Basidiomycete* QM 806 (even at 0.1%) and to *Chrysosporium pruinatum* QM 826, and less toxic to the others. The dipalmitate and multipalmitate esters were insoluble and nontoxic. For all four fungi, the dipalmitate was the best of the palmitate esters as an inducer of cellulase, giving yields of enzyme 4- to 70-fold greater than those obtained

TABLE 1. Increased yields through growth on modified inducers

Enzyme	Carbon-sources (0.5%)	Max enzyme yields (units/ml) <sup>a</sup>			
		TV	Basid.	Chrys.	Pest.
Cellulase (endo- $\beta$ -1 $\rightarrow$ 4 glucanase, E.C. 3.2.1.4)	Cellulose	0.2	0.4	0.4	0.2
	Cellulose monopalmitate	1.3	NG <sup>b</sup>	0.9	5.9
	Cellulose dipalmitate	4.8	1.6	1.5	13.5
	Cellulose-"x"-palmitate	0.2	0.7	0.2	0.7
	Cellulose octaacetate	0.2	13.4	1.5	20.1
	Lactose	3.1	5.3	0.7	0.4
	Cellulose	22.5	42.1	29.3	35.9
Dextranase (endo- $\alpha$ -1 $\rightarrow$ 6 glucanase, E.C. 3.2.1.11)	Isomaltose	1.5	0.2	0.2	
	Isomaltose monopalmitate	823.5	6.0	3.3	
	Isomaltose dipalmitate	1,098.0	11.0	102.5	
	Isomaltose-x-palmitate	153.7	2.2	62.6	
	Sucrose monopalmitate	0.2	0	0.2	
	Dextran	1,079.7	18.7	46.9	
	Starch	0.4	0.2	0.2	
Sucrase ( $\beta$ -fructofuranosidase, E.C. 3.2.1.26)	Sucrose	1.3	1.5	0.4	0
	Sucrose monopalmitate	108.0	9.0	6.2	0.5
	Sucrose dipalmitate	4.9	0.5	1.5	0.7
	Sucrose octaacetate	1.5	0.4	0.2	0.4
	Raffinose	2.2	2.4	0.4	0

<sup>a</sup> Abbreviations: *Trichoderma viride* QM 9123 (TV); Basidiomycete QM 806 (Basid.); *Chrysosporium* (= *Sporotrichum*) *pruinatum* QM 826 (Chrys.); *Pestalotiopsis westerdijkii* QM 381 (Pest.); *Penicillium funiculosum* QM 474 (PF); *Spicaria violacea* QM 1031 (SV); *P. lilacinum* QM 4e (PL); *Pullularia pullulans* QM 72c (PP 72c); *Candida utilis* QM 824 (CU); *Aspergillus niger* QM 877 (AN).

<sup>b</sup> No growth.

from unmodified cellobiose. The octaacetate of cellobiose is also a good inducer of fungal cellulase. Lactose, which can be considered as cellobiose modified at the C<sub>4</sub> position of the nonreducing glucose, is often a better inducer of cellulase than is cellobiose. In no instance to date have we been able to obtain yields equaling those produced on cellulose itself.

**Dextranase.** Three known dextranase-producing fungi were grown on a variety of substrates (Table 1) for 3 weeks. Yields of enzyme were low on starch and isomaltose and high on the palmitates of isomaltose and on dextran. On a branched dextran (35% 1 $\rightarrow$ 3 links, B1355 Fr S from A. Jeanes), yields (not shown) were appreciable but less than those on the unbranched dextran. Yields of all three fungi were higher on the dipalmitate of isomaltose, than on the other palmitates. Indeed, two fungi grown on this substrate gave yields equal to or greater than those obtained on dextran itself. Almost no dextranase was produced during growth on sucrose mono-

palmitate. This control was required since sucrose monopalmitate often increases enzyme yields because of its surfactant effect (8; Table 2).

**Production of glycosidases by growth on substrates and modified substrates (of the glycosidase): sucrase (invertase).** Sucrose (at 0.5%) is a rather poor carbon source for sucrase production in the organisms studied (Table 1). Raffinose is slightly better. Sucrose monopalmitate, the most soluble of the esters, is by far the best inducer tested. The dipalmitates and octaacetate of sucrose are less effective. Melezitose, inulin, and  $\beta$ -2 $\rightarrow$ 6 fructan, all containing  $\beta$ -fructosyl groups, were no better than sucrose and raffinose as inducers for the two *Pullularia* strains tested.

**Other glycosidases** (Table 2). Production of other enzymes was stimulated by growth on sucrose monopalmitate, but this is a surfactant effect rather than an inducing action (8).  $\alpha$ -Galactosidase (melibiase) production was not stimulated by growing the organisms on  $\alpha$ -galactosyl-containing compounds (melibiose,

TABLE 2. Effect of sucrose monopalmitate as carbon-source on production of  $\alpha$ -galactosidase and of cellobiase

Enzyme	Carbon-source (growth)	Enzyme yields (units/ml) <sup>a</sup>			
		PP 72c	PP 5752	AF 45h	AF 328
Melibiase ( $\alpha$ -galactosidase, E.C. 3.2.1.22)	Melibiose	0.2	0.2	0	
	Melibiose octaacetate	0.2	0	0	
	Sucrose monopalmitate	1.1	0.9	0.2	
	Sucrose	0.2	0	—	
	Raffinose	0.2	0.2	0	
		AN 877	PP 1878	Pest. 381	
Cellobiase ( $\beta$ -glucosidase, E.C. 3.2.1.21)	Sucrose	2.7	0.9	0.2	0.2
	Sucrose monopalmitate	0.4	0.7	1.3	0.9
	Sucrose monostearate	0.4	0.7	—	—

<sup>a</sup> Abbreviations: (see Table 1): *Pullularia pullulans* QM 5752 (PP 5752); *Aspergillus fumigatus* QM 45h (AF 45h); *Penicillium parvum* QM 1878 (PP 1878); *A. foetidus* QM 328 (AF 328).

melibiose octaacetate, raffinose). The amount of enzyme produced on these was no more than that produced on sucrose. The interesting fact is that yields were much better on sucrose monopalmitate.

$\beta$ -Glucosidase (cellobiase) yields were increased by growth on sucrose monopalmitate in those organisms that produce very little enzyme when grown on sucrose [*Pestalotiopsis*, *Aspergillus foetidus*, and *A. miyakoensis* (not shown)]. This is essentially the case also with  $\alpha$ -galactosidase, but where the organism normally produces an appreciable amount of cellobiase when growing on sucrose, there is a decrease in yield on the sucrose derivatives.

#### DISCUSSION

One of our long-term aims is to determine the conditions under which an organism will produce and secrete a maximum amount of enzyme. It is well known that the use of suitable inducers greatly increases enzyme production. Recently it has been shown that further increases can be achieved by the use of nonionic surfactants (8). Modified substrates (7, 8) and modified products (3, 4) frequently give much higher enzyme yields than do the unmodified compounds. In some cases, these modified compounds function both as inducers and as surface active agents (8).

Normally enzymes are induced by growing the organism on the substrate of the enzyme. When the substrate is a small soluble molecule (sucrose), it may also be the inducer. However, when the substrate is very large (dextran) or insoluble (cellulose), it cannot enter the cell and so cannot act as inducer, despite the fact that growth on these polymers does result in production of high yields of enzyme. The true inducers of enzymes

produced on such compounds are the dimeric products of enzyme action (isomaltose, cellobiose). However, when the organisms are grown on these dimers, yields of enzyme are usually much lower than those obtained on the polymeric substrate, because the soluble sugars are very rapidly metabolized and repress enzyme formation. [Similar repressions can be obtained by the use of metabolites that are not inducers (4)]. High levels of enzyme can be obtained on these soluble inducers only when repression is avoided. One way to achieve this is to supply the inducer continuously at a low level (4). Another way is to retard metabolism by use of unfavorable growing conditions so that the inducer is consumed slowly (4). The third way is to supply a compound that releases the inducing disaccharide. This may be through use of the polymeric substrate itself (dextran, cellulose) or of a modified soluble substrate (sucrose ester) or a modified product (isomaltose ester, cellobiose ester) that can be broken down by the organism to release the inducer slowly.

We find that for polysaccharides the order of inducing ability is frequently: polymeric substrate > modified product >> product; for soluble substrates, the order is modified substrate >> substrate. In addition to the examples cited, we have shown that nucleosidases can best be produced by growing organisms on the polymer nucleic acid, or on nucleoside phosphate (nucleotide), both of which yield nucleoside as a result of enzymatic hydrolysis, whereas very little enzyme is produced when the nucleoside itself is used as carbon-source (6).

We have been only partially successful in our

attempts to use modified inducer to obtain great increases in enzyme yield (unless we consider that the polymeric glucans are essentially modified inducers). The most spectacular results have been with sucrase, dextranase, and nucleosidase production. From 10- to 100-fold increases in enzyme are not uncommon. The difficult problem is to prepare a modified inducer which the organism can act on at just the right rate to supply inducer slowly over a long period of time. Since organisms differ in the nature of the enzymes they produce, they will differ in their response to the variously modified compounds. The data show such species differences, thus making the prediction of an ideal inducer for any particular organism extremely hazardous.

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