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"Substrate Inhibition" of Intestinal Glycosidases

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The influence of *substrate concentration* on the initial rate of hydrolysis of different glycosides by hog intestinal glycosidases has been studied.

In some cases marked "substrate inhibition" was observed at 0.139 M substrate concentration, which is the concentration previously used as a standard concentration for the determination of intestinal glycosidase activities. *Transglycosylation* plays an important role in the "substrate inhibition" but it is not the only factor involved.

The use of 0.028 M substrate concentration as a standard concentration for the determination of intestinal glycosidase activities, rather than 0.139 M, is recommended. The influence of this lower substrate concentration on the absolute and relative activities of some purified hog intestinal glycosidases, and of crude hog intestinal glycosidase preparations, on different substrates has been determined.

Methods for determination of intestinal glycosidase activities are described, and units for expressing intestinal glycosidase activities are defined.

For studies of glycosidases, 0.139 M substrate is commonly used as a standard concentration [1], and so was done by the present author in a recent investigation of hog intestinal α-glucosidases [2]. During this investigation it was observed, however, that some intestinal α-glucosidases show marked "substrate inhibition" at 0.139 M substrate concentration and that lowering the concentration of substrate resulted in an *increase* of glycosidase activity with these enzymes [3].

A similiar observation has recently been made with invertase from sheep rumen protozoa [4,5]. In that case the authors found 0.025 M sucrose to give maximal invertase activity.

In the present paper a detailed study on the influence of substrate concentration on hog intestinal glycosidase (α-glucosidase, β-galactosidase and β-glucosidase) activities will be reported and procedures for the quantitative determination of intestinal glycosidase activities will be described. Since the standard substrate concentration now recommended is lower than the concentration used earlier, the absolute and relative activities of crude and purified intestinal glycosidase preparations on different substrates have been determined at this new substrate concentration.

RESULTS AND DISCUSSION

Influence of substrate concentration on the activities of isolated intestinal glycosidases

A) α-Glucosidases

The different α-glucosidase activities exerted by extracts from hog intestinal mucosa are caused by a mixture of at least five separate enzymes with varying specificity [2]. Three of these enzymes have been isolated, namely *invertase* (= maltase I), *maltase III* and *trehalase.** The "substrate inhibition" has been studied with each of these enzymes separately.

1) *Invertase.* Purified hog intestinal invertase hydrolyzes *sucrose*, *maltose* and *turanose* [6]. The influence of substrate concentration upon these activities of purified invertase is seen in Fig. 1. V_{max} in the figure has been calculated from the v_{obs} (observed initial velocity of hydrolysis) at *low* substrate concentrations (below 0.06 M) by the graphical method of Lineweaver and Burk [7] as modified by Dixon [8].

When the substrate concentration exceeded 0.1 M, the initial velocity of hydrolysis of all three substrates was *lower* than could be predicted from the Michaelis-Menten equation [9]. The "substrate inhibition" was more marked with *turanose* than with the other two substrates. At 0.28 M substrate concentration the initial velocity of hydrolysis of turanose was 30 % lower than the value calculated from the Michaelis-Menten equation. With sucrose or maltose as substrates the initial velocity at 0.28 M substrate concentration was 10—

* Maltase II has been isolated too, but only in small amounts [3].

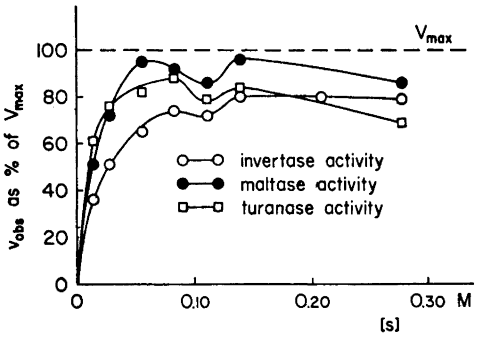

Fig. 1. Influence of substrate concentration, [s], on the invertase, maltase and turanase activities of purified intestinal invertase. Temp. 37°C, pH 6.5 (0.05 M maleate buffer). The observed initial rate of hydrolysis (v_{obs}) is expressed as per cent of V_{max} (calculated from the v_{obs} at low substrate concentrations as described in the text).

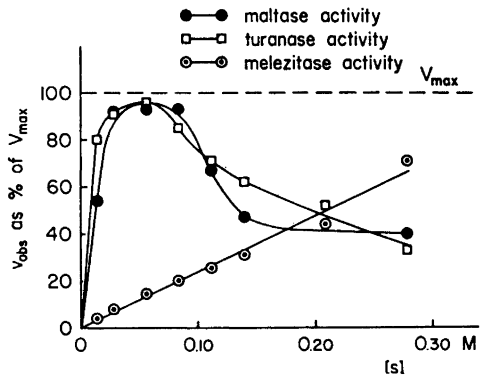

Fig. 2. Influence of substrate concentration on the maltase, turanase and melezitase activities of purified intestinal maltase III. Experimental conditions, see Fig. 1.

15 % too low. These findings suggest that *transglycosylation* may be the cause of the "substrate inhibition", since the invertase and maltase activities were measured with reducing sugar methods, which measure both the glycon and the aglycon liberated, while the turanase activity was measured with a glucose oxidase reagent, which measures only the glycon liberated. If transglycosylation is the cause of the "substrate inhibition" the amount of glycon liberated will be lower than the amount of aglycon liberated.

To elucidate whether transglycosylation was the cause of the "substrate inhibition" the amounts of glucose (glycon) and fructose (aglycon) liberated during the hydrolysis of turanose were measured separately by quantitative paper chromatography [10]. The amount of glucose liberated at 0.28 M substrate concentration was 31 % lower than the amount of fructose liberated. This indicates that transglycosylation is the main factor responsible for the "substrate inhibition" of this enzyme.

An irregularity of the shape of the curve between 0.08 and 0.15 M substrate concentration was observed with all three activities (Fig. 1). The cause of the irregularity is not known. It may indicate that more than one factor is involved in the "substrate inhibition" of intestinal invertase.

2) *Maltase III.* Purified hog intestinal maltase III hydrolyses *maltose, isomaltose, turanose, phenyl-α-D-glucopyranoside* and *melezitose* [2,3,11]. The influence of substrate concentration upon the maltase, turanase and melezitase activities of this enzyme is seen in Fig. 2.

With this enzyme the "substrate inhibition" of the maltase and turanase activities was considerably more marked than with the activities exerted by invertase (= maltase I) (Fig. 1). When the substrate concentration exceeded 0.05 M, the initial velocity of hydrolysis of these substrates was lower than calculated from the Michaelis-Menten equation and at 0.28 M substrate concentration the initial velocity was about 60 % lower.

With the melezitase activity no "substrate inhibition" could be demonstrated. This fact is, however, readily explained by the low affinity of the enzyme for melezitose (see Fig. 2) [11,13].

As was done in the experiments with purified invertase described above, the amounts of glucose and fructose liberated from turanose by maltase III were measured separately. At 0.28 M substrate concentration the amount of glucose liberated was 35 % lower than the amount of fructose liberated. This indicates that the transglycosylase reaction also with maltase III plays an important role in the "substrate inhibition", although it can explain only a part of the inhibition.

3) *Trehalase.* Purified hog intestinal trehalase differs from the two enzymes described above by that it hydrolyzes only one single substrate, namely *α,α-trehalose* [12]. The influence of substrate concentration upon trehalase activity is seen in Fig. 3. No "substrate inhibition" could be demonstrated with this enzyme. In other experiments substrate concentrations as high as 0.8 M (= 30 % trehalose) were used without any decrease of trehalase activity. It is known that intestinal trehalase, in contrast to other intestinal glycosidases, has no transglycosylase activity [12].

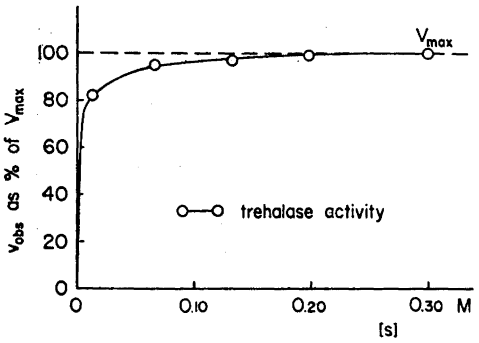

Fig. 3. Influence of substrate concentration on the activity of purified intestinal trehalase. Temp. 37°C, pH 6.0 (0.05 M maleate buffer).

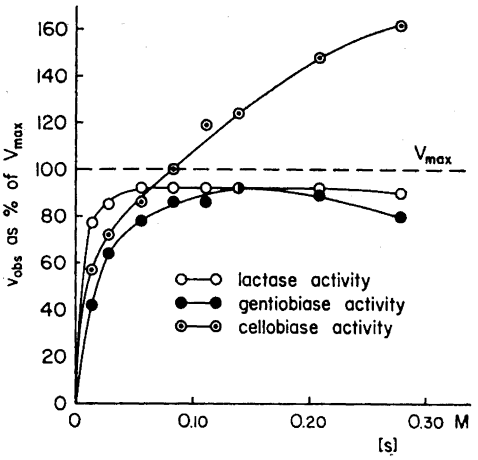

Fig. 4. Influence of substrate concentration on the lactase, cellobiase and gentiobiase activities of a crude hog intestinal glycosidase preparation. Temp. 37°C, pH 6.0 (0.05 M maleate buffer). When the substrate concentration exceeds 0.10 M, the observed initial rate of hydrolysis (v_{obs}) of cellobiose exceeds the V_{max} calculated from the v_{obs} at substrate concentrations below 0.06 M.

B) β-Galactosidase and β-Glucosidase

Hog intestinal mucosa preparations hydrolyze *lactose*, which is a β-galactoside, and a number of β-glucosides as *cellobiose*, *gentiobiose* and *phenyl-β-D-glucopyranoside*. The relation between these activities is under current investigation, but preliminary results indicate that the β-galactosidase and β-glucosidase activities of hog intestinal mucosa are caused by one single enzyme, intestinal lactase. The activities on these substrates therefore were studied with crude hog intestinal glycosidase preparations, obtained as described earlier [2].

The influence of substrate concentration upon the *lactase*, *cellobiase* and *gentiobiase* activities of a crude hog intestinal glycosidase preparation is seen in Fig. 4. The "substrate inhibition" of the lactase and gentiobiase activities is relatively weak. Up to 0.1 M substrate concentration these activities followed the Michaelis-Menten relationship. At 0.28 M substrate concentration the lactase activity was 10 % and the gentiobiase activity 15 % lower than calculated. The velocity of hydrolysis of these substrates was measured with the glucose oxidase reagent, and therefore with lactose as substrate only the *aglycon* (glucose) liberated was measured. The *glycon* (galactose) does not react with this reagent. This means that even if the transglycosylase reaction may contribute to the "substrate inhibition" of lactase, it is not the only factor

involved, for in that case the rate of liberation of the aglycon would not have decreased.

The *cellobiase* activity shows a behaviour quite different from that of the other activities studied in the present paper. It shows "substrate *activation*" rather than "substrate innibition". At low substrate concentration (0.01—0.06 M) the cellobiase activity seemed to follow the Michaelis-Menten equation, but when the substrate concentration was further increased, the velocity of hydrolysis became considerably greater than the V_{max} calculated from the values obtained at low substrate concentrations (Fig. 4). This phenomenon appeared in repeated experiments. At present no explanation can be given for this behaviour of the cellobiase activity.

The choice of a standard substrate concentration

As was pointed out in the introduction of the present paper, 0.139 M is commonly used as a standard substrate concentration for glycosidase activity determinations. From the results described above it appears, however, that some of the intestinal glycosidases show marked "substrate inhibition" at that concentration. In studies of intestinal glycosidases it seems preferable therefore, to use 0.028 M substrate as a standard concentration. At that substrate concentration all the activities hitherto studied (with the exception of melezitase, which has an extremely high K_s value [11]) will yield a velocity of hydrolysis which is more than 50 % of V_{max}.

There is one additional advantage in using the more dilute substrate as a standard concentration. The low solubilities of heteroglycosides (*e.g.* phenylglycosides) make it difficult to prepare reaction mixtures with 0.139 M concentration of these substrates [3]. The preparation of a reaction mixture with 0.028 M substrate will, however, not be difficult in these cases.

If 0.028 M substrate is selected as a standard concentration for the determination of intestinal glycosidase activities, the figures earlier reported [2,3] for the relative activities on different substrates of crude intestinal glycosidase preparations and of purified intestinal glycosidases will have to be recalculated. A comparison therefore was made of the relative activities of intestinal glycosidases on different substrates at 0.139 M and at 0.028 M substrate concentration.

Relative activities at 0.139 M and at 0.028 M substrate concentration

The lowering of the standard substrate concentration for determination of intestinal glycosidase activities to 0.028 M will result in the following alterations of the absolute and relative rates of hydrolysis of different substrates.

A. Isolated enzymes

1) *Invertase* (= *Maltase I*). The *invertase* activity will *decrease* by 40 %, the *maltase* activity by 30 % and the *turanase* activity by about 10 % (Table 1). The *maltase/invertase activity quotient* of purified invertase (maltase I)

Table 1. Relative activity of purified hog intestinal invertase (= maltase I) on different substrates at 0.139 M and 0.028 M substrate concentration in the presence of 0.05 M maleate buffer pH 6.5 and at temperature 37°C. The maltase activity of the enzyme at 0.139 M substrate concentration has been arbitrarily set at 100.

Substrate	Relative activity at 0.139 M substrate concentration	Relative activity at 0.028 M substrate concentration
Maltose	100	75
Sucrose	170	110
Turanose	7.5	7.0

thus will increase from 0.6 to 0.7, and the *turanase/invertase activity quotient* from 0.04 to 0.06.

2) *Maltase II.* The influence of the substrate concentration upon the activities of purified maltase II has not been studied, but from the similiarity of this enzyme with maltase III may be expected that the dilution of the substrate will *increase* the rate of hydrolysis of the different substrates hydrolyzed by maltase II. This theory was confirmed by the alteration of the total maltase activity of a *crude* hog intestinal glycosidase preparation (Table 4).

3) *Maltase III.* The *maltase* activity of this enzyme will increase by 100 % (Table 2). Also the *isomaltase* and *turanase* activities of this enzyme will increase. The *phenyl-α-D-glucopyranosidase* activity will not be measurably affected. The *melezitase* activity, due to the low affinity of the enzyme for this substrate, will be lowered by 75 %.

The *isomaltase/maltase activity quotient* will decrease from 0.05 to 0.04, the *turanase/maltase activity quotient* from 0.06 to 0.05, the *phenyl-α-D-glucopyranosidase/maltase activity quotient* from 0.05 to 0.03 and the *melezitase/maltase activity quotient* from 0.0040 to 0.0005.

4) *Trehalase.* The *trehalse* activity will be lowered by about 10 %.

Table 2. Relative activity of purified hog intestinal maltase III on different substrates at 0.139 M and at 0.028 M substrate concentration in the presence of 0.05 M maleate buffer pH 6.5 and at temperature 37°C. The maltase activity of the enzyme at 0.139 M substrate concentration has been arbitrarily set at 100.

Substrate	Relative activity at 0.139 M substrate concentration	Relative activity at 0.028 M substrate concentration
Maltose	100	200
Isomaltose	5	7
Turanose	6	9
Phenyl-α-D-glucopyranoside	5	5
Melezitose	0.4	0.1

Table 3. Relative activity of intestinal lactase (?) on lactose and some β-glucosides at 0.139 M and at 0.028 M substrate concentration in the presence of 0.05 m maleate buffer pH 6.0 and at temperature 37°C. The lactase activity at 0.139 M substrate concentration has been arbitrarily set at 100.

Substrate	Relative activity at 0.139 M substrate concentration	Relative activity at 0.028 M substrate concentration
Lactose	100	90
Cellobiose	50	30
Gentiobiose	1.5	1
Phenyl-β-D-glucopyranoside	5.0	4.5

5) *"Specific" isomaltase and "specific" phenyl-α-D-glucopyranosidase* *. These enzymes have not been isolated, and the influence of the substrate concentration on their activities is not known.

6) *β-Galactosidase and β-glucosidase.* The *lactase* activity will decrease by 10 % and the *cellobiase* activity by 40 % (Table 3). The *cellobiase/lactase activity quotient* consequently will decrease from 0.5 to 0.3. The absolute and relative rates of hydrolysis of *gentiobiose* and *phenyl-β-D-glucopyranoside* will undergo only small alterations.

B. Crude intestinal glycosidase preparations

The relative activities on different substrates by a crude hog intestinal glycosidase preparation will of course be dependent on the relative amounts of the different intestinal glycosidases present in the preparation. Since many substrates are hydrolyzed by two or three different enzymes in the preparation, the relative activity on each of these substrates at 0.139 M and at 0.028 M substrate concentration will also vary with the composition of the enzyme mixture.

The variations in the relative amounts of the different glycosidases present in hog intestinal glycosidase preparations, obtained on different occasions, are, however, usually not great. It has been reported earlier that the *maltase/invertase activity quotient* of such preparations when measured at *0.139 M* substrate concentration usually is about 2, the *trehalase/invertase activity quotient* 0.5 (provided the preparation is obtained from the *upper* part of the small intestine [12]) and the *lactase/invertase activity quotient* 0.2 [15]. At this substrate concentration the three different maltases present (maltase I—III) account for about *one third each* of the total maltase activity [2,3].

The activities on different substrates at *0.028 M* substrate concentration by a preparation which the composition described above has been investigated, and the results are seen in Table 4. At 0.028 M substrate concentration the *maltase/invertase activity quotient* was 5, the *trehalase/invertase activity quotient*

* "Specific" isomaltase and "specific" phenyl-α-D-glucopyranosidase may be identical [8].

Table 4. Relative activities of a crude hog intestinal glycosidase preparation * on different substrates, measured in the presence of 0.05 M maleate buffer, at pH 6.0 for trehalase, β-galactosidase and β-glucosidase activities, and at pH 6.5 for the other activities, at temperature 37°C. The maltase activity at 0.139 M substrate concentration has been arbitrarily set at 100.

Substrate	Enzymes acting upon each substrate	Relative activity at 0.139 M substrate concentration	Relative activity at 0.028 M substrate concentration
Maltose	{Invertase (= Maltase I) Maltase II Maltase III}	100	150
Sucrose	Invertase (= Maltase I)	50	30
Trehalose	Trehalase	25 **	23 **
Isomaltose	{"Specific" isomaltase? † Maltase II Maltase III}	8—10	8—10
Turanose	{Invertase (= Maltase I) Maltase II Maltase III}	7—8	7—8
Phenyl-α-D-glucopyranoside	{"Specific" phenyl-α-D-glucopyranosidase? † Maltase II Maltase III}	5—6	6—7
Melezitose	{Maltase II Maltase III}	0.20***	0.05***
Lactose	{Lactase?}	10	9
Cellobiose		5	3
Gentiobiose		0.20	0.15
Phenyl-β-D-glucopyranoside		0.50	0.45

* Prepared as described earlier. [2]

** Provided ethanol precipitation (which inactivates trehalase) has been omitted, and that the preparation has been obtained from the upper part of the jejunum. [2,12]

*** Calculated as complete hydrolysis of the trisaccharide. [13]

† "Specific" isomaltase and "specific" phenyl-α-D-glucopyranosidase may be identical.[14]

0.8 and the *lactase/invertase activity quotient* 0.3. Maltase I accounted for 15 % only of the total maltase activity, the remaining 85 % of the total maltase activity was exerted by maltase II and maltase III.

METHODS FOR DETERMINATION OF INTESTINAL GLYCOSIDASE ACTIVITIES

Substrates

Maltose (4-(α-D-glucopyranosyl)-D-glucose) monohydrate was obtained from Merck A.G. (Germany), *sucrose* (2-(α-D-glucopyranosyl)-β-D-fructofuranoside) and *lactose* (4-(β-D-galactopyranosyl)-D-glucose) monohydrate from Baker Co. (U.S.A.), *trehalose* (1-(α-D-glucopyranosyl)-α-D-glucopyranoside) dihydrate, *melezitose* (α-D-glucopyranosyl-

(1→3)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside) dihydrate. and *cellobiose* (4-(β-glucopyranosyl)-D-glucose) from Pfanstiehl Chemical Co (U.S.A.), *turanose* (3-(α-D-glucopyranosyl)-D-fructose), *gentiobiose* (6-(β-D-glucopyranosyl)-D-glucose) and *phenyl-β-D-glucopyranoside* dihydrate from Nutritional Biochemicals Co. (U.S.A.). *Isomaltose* (6-(α-D-glucopyranosyl)-D-glucose) and *phenyl-α-D-glucopyranoside* were prepared as described earlier [14,12].

Incubation conditions and units

The *standard conditions* for determination of intestinal glycosidase activities are as follows. The enzyme acts in a 0.028 M solution of substrate in the presence of 0.05 M maleate buffer [16] at optimum pH (*i.e.* pH 6.5 for all α-glucosidase activities except trehalase, pH 6.0 for trehalase, β-galactosidase and β-glucosidase activities) at 37°C. Toluene is used as a preservative. The degree of hydrolysis is not allowed to exceed 15 % (within this limit the reaction follows zero order kinetics and the (initial) velocity of hydrolysis is proportional to the amount of enzyme present).

One unit of glycosidase activity is the activity which causes 5 % of hydrolysis in 2.0 ml of reaction mixture in 60 min under these conditions. 5 % of hydrolysis in 2.0 ml of reaction mixture at 0.028 M substrate concentration is equal to the formation of *1 mg of glucose* with maltose, isomaltose, trehalose, cellobiose or gentiobiose as substrate, *1 mg of invert sugar* (= 0.5 mg of glucose) with sucrose or turanose as substrate, and *0.5 mg of glucose* with phenyl-α-D-glucopyranoside, phenyl-β-D-glucopyranoside or lactose as substrate.

Preparation of the substrate solutions

The reaction mixture is generally obtained by mixing equal volumes of substrate solution and a suitably diluted enzyme solution. Since the reaction mixture shall contain 0.028 M substrate and 0.05 M maleate buffer, the substrate solution shall contain 0.056 M substrate and 0.10 M buffer. The substrate solutions are prepared (according to Table 5) by dissolving the substrates in 0.10 M maleate buffer [16] of suitable pH.

Table 5. Composition of substrate solutions. The solutions also contain 1 ml of toluene per 100 ml and should be stored in a refrigerator.

Substrate	Gram of substrate per 100 ml of substrate solution	pH of maleate buffer
Maltose monohydrate	2.00	6.5
Sucrose	1.90	6.5
Trehalose dihydrate	2.10	6.0
Isomaltose	1.90	6.5
Turanose	1.90	6.5
Phenyl-α-D-glucopyranoside monohydrate	1.52	6.5
Phenyl-α-D-glucopyranoside anhydric	1.42	6.5
Phenyl-β-D-glucopyranoside dihydrate	1.62	6.0
Melezitose dihydrate	3.00	6.5
Lactose monohydrate	2.00	6.0
Cellobiose	1.90	6.0
Gentiobiose	1.90	6.0

Determination of the degree of hydrolysis

The method which is most suitable for the determination of the degree of hydrolysis of the substrate, will vary with the properties of the substrates.

A) *3,5-Dinitrosalicylic acid.* The 3,5-dinitrosalicylate reagent, introduced by Sumner [17] for the determination of reducing sugars, is very suitable for the determination of the degree of hydrolysis of nonreducing substrates as sucrose, trehalose or melezitose. (It is, however, not suited for phenyl-glycosidase activity determinations [2]). The method has the advantage that the presence of proteins does not interfere, and therefore protein precipitation is not necessary.

For glycosidase activity determinations 1.0 ml of substrate solution is mixed with 1.0 ml of enzyme solution (diluted to contain 0.5—3.0 units of glycosidase activity per ml) in a test tube immersed in a water bath at 37°C. After 60 min the reaction is interrupted by the addition of 2.0 ml of 3,5-dinitrosalicylate reagent (most readily prepared as described by Hostettler *et al.*[18]) A blank with the same composition is prepared, in which the 3,5-dinitrosalicylate reagent is added *before* the mixing of the substrate and enzyme solutions. The tubes are then heated in a boiling water bath for 10 min, chilled for a few min in running tap water, and diluted with 20.0 ml of distilled water. The red colour produced is measured in a Beckman B spectrophotometer at 530 mμ using 1 cm cuvettes. The colour is stable for at least 24 h.

The amount of monohexoses produced in each sample is calculated from a standard curve made with known amounts of glucose (0.5—3.0 mg). Glucose and fructose have the same extinction coefficient with the 3,5-dinitrosalicylate reagent.

If the enzyme solution contains 1 unit/ml of *invertase* or *trehalase*, 1 mg of monohexoses will be formed during the procedure described above. If more than 3 mg are formed, the determination has to be repeated with a more dilute enzyme solution.

The amount of reducing sugars produced from a trisaccharide as *melezitose* will depend on whether one or both of the glycosidic links are hydrolyzed.

The 3,5-dinitrosalicylic acid method is not sensitive enough when only a small amount of the substrate or the enzyme is available, or when it is desired to use very dilute substrate solutions. In these cases the amount of monohexoses produced from a non-reducing substrate may be determined with Somogyi-Nelson's copper reduction method [19,20], after the precipitation of the proteins with Somogyi's Zn-Ba-reagents [21].

B) *Glucose oxidase.* When the substrate is a *reducing* disaccharide, the 3,5-dinitrosalicylic acid method is not applicable. The amount of glucose liberated in those cases may be determined specifically with a reagent containing glucose oxidase. This reagent does not react with fructose or galactose. It does, however, react with maltose and isomaltose,[2] and cannot, therefore, be used for the determination of the degree of hydrolysis of these substrates.

The glucose oxidase reagent is used for the determination of turanase, lactase, cellobiase and gentiobiase activity. It cannot be used for phenyl-glucosidase activity determinations, since free phenol interferes with the production of the colour.

The determination of glycosidase activity is performed in the following way. The enzyme solution is diluted with water to contain 0.2—1.0 units/ml of cellobiase or gentiobiase activity or 0.5—2.0 units/ml of any of the other activities. In a 7 × 120 mm test tube 0.1 ml of the diluted enzyme solution is mixed with 0.1 ml of the substrate solution, and the tube is immersed in a water bath at 37°C for 60 min. Then 0.8 ml of water is added, and the enzymatic reaction is interrupted by immersing the tube in a boiling water bath for 2 min. A blank is prepared with the same composition, which immediately after the mixing of enzyme and substrate is immersed in a boiling water bath for 2 min.

When turanose is used as substrate, the water added after incubation is replaced by 0.8 ml of 0.1 M acetate buffer pH 5.0. (Turanose will undergo partial alkaline hydrolysis when boiled at a pH higher than 6.0.) This is not necessary with the other substrates. The addition of acetate buffer does not interfere with the subsequent enzymatic determination of the amount of glucose liberated.

After chilling the tubes, 0.5 ml of the contents of each tube is transferred to a 17 × 180 mm tube, added 3.0 ml of the glucose oxidase reagent (prepared as described by Huggett and Nixon [22]), mixed and placed in a water bath at 37°C for 1 h for development of the colour. After that time the colour produced is measured in a Beckman B spectrophotometer at 420 mμ using 1 cm cuvettes.

A standard curve is made with known solutions of glucose, containing 10, 30 and 50 μg of glucose/0.5 ml (0.5 ml of the standard solution is mixed with 3.0 ml of the glucose oxidase reagent and placed at 37°C for 1 h).

If the enzyme solution contains 1 unit/ml of glycosidase activity, 50 μg of glucose will be formed under these conditions with cellobiose or gentiobiose as substrates, and 25 μg with turanose or lactose as substrates.

C) *Acid copper reagent.* For the determination of the degree of hydrolysis of maltose and isomaltose is used an acid copper reagent, which is reduced to a much smaller extent by reducing disaccharides than by monosaccharides [2].

The enzyme solution is diluted with water to contain 1.0—3.0 units/ml of glycosidase activity. In a 7 × 120 mm test tube 0.1 ml of enzyme solution and 0.1 ml of substrate solution are mixed, and the tube is immersed in a water bath at 37°C for 60 min. Then 1.0 ml of a 0.3 N $Ba(OH)_2$-solution, 1.0 ml of a 0.15 M $ZnSO_4$-solution and 1.8 ml of water are added (the $Ba(OH)_2$- and $ZnSO_4$-solutions are previously titrated against each other and adjusted so as to give an ion-free supernatant [21]) which precipitate the proteins and interrupt the enzymatic reaction. A blank is prepared with the same composition, which is precipitated immediately after mixing the enzyme and the substrate. The content of each tube is transferred to a centrifuge tube and centrifuged for 5 min.

Then 2.0 ml of the clear supernatant is transferred to a Folin tube for sugar determination (with a narrow part to prevent oxidation of the cuprous oxide by air, and graduated at 12.5 and 25.0 ml) and 2.0 ml of acid copper reagent is added (prepared as described by Tauber and Kleiner [22]). The tubes are immersed in a boiling water bath for exactly 10 min, and chilled with tap water for 2 min. 2.0 ml of Benedict's molybdate reagent [23] is added and after a further 2 min water to the 25 ml mark. The blue colour is immediately measured in a Beckman B spectrophotometer at 660 mμ, using 1 cm cuvettes.

When the determination is performed in this way, the Folin tube will contain 0.1 ml of reaction mixture. Even when more dilute substrate solutions are used (*e.g.* for K_s determinations) it is impossible to use a greater amount of the reaction mixture for the sugar determination, since the maleate buffer then will interfere with the reaction [2]. For maltase activity determinations the reagents in such experiments may be replaced by the modified reagents described by Caputto *et al.*[24] which are less sensitive to the interference by the buffer. These reagents are not suitable for isomaltase activity determinations [2].

The degree of hydrolysis is calculated from a series of tubes containing mixtures of maltose (isomaltose) and glucose corresponding to 0, 5, 10 and 15 % hydrolysis of the substrate. One unit of maltase (isomaltase) activity per ml in the enzyme solution used will cause 5 % of hydrolysis of the substrate. If the degree of hydrolysis exceeds 15 %, the determination should be repeated with a more dilute enzyme solution.

In determinations with this reagent it is necessary to include a new standard series in each set of tubes that is boiled.

Maltase III (heat-stable maltase) in the presence of maltase I and maltase II is determined by the same method, but after heating the enzyme solution in 0.01 M phosphate buffer pH 6.0 at 60°C for 45 min as described earlier [25].

D) *Phenol determination reagent.* For the determination of the degree of hydrolysis of phenyl-α-D-glucopyranoside and phenyl-β-D-glucopyranoside the amount of phenol liberated is determined with the commercial Folin-Ciocalteau reagent [26] (obtained from G.T.Gurr Ltd., England).

The procedure is as follows. 0.5 ml of diluted enzyme solution (containing 0.5—2.0 units/ml of glycosidase activity) is mixed with 0.5 ml of substrate solution at 37°C. After 60 min the proteins are precipitated by the addition of 1.0 ml of 0.3 N $Ba(OH)_2$, 1.0 ml of 0.15 M $ZnSO_4$ and 1.0 ml of water (see above). A blank is prepared with the same composition, which is precipitated immediately after mixing the enzyme and the substrate.

After centrifugation for 5 min, 1.0 ml of the clear supernatant is transferred to another test tube. To this tube is added 2.0 ml of Folin-Ciocalteau reagent (the commercial reagent diluted with four volumes of distilled water) and 6.0 ml of 1 N Na_2CO_3. The tube is immersed in a water bath at 37°C for 20 min, and then the blue colour produced is measured in a Beckman B spectrophotometer at 675 mμ using 1 cm cuvettes.

A standard curve is made with solutions of phenol in water (32.5 and 65.0 μg of phenol/ml), treated in the same way as the supernatants of the precipitated reaction mixture.

If the enzyme solution contains 1 unit of glycosidase activity/ml, 32.5 μg of phenol will be formed during these conditions.

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