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ENZYMATIC QUANTITATIVE DETERMINATION OF HEXOSES, SINGLY AND IN MIXTURES WITH THEIR OLIGOSACCHARIDES

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Summary

Quantitative determinations of a number of naturally ocurring D-hexoses and their oligosaccharides can be accomplished by combinations of enzymatic methods without prior separation of the sugars. These spectrophotometric methods employ readily-available enzymes and commercial reagents. The procedures are particularly useful for the quantitative analysis of fructofuranosides, such as raffinose or stachyose, in the presence of sucrose, glucose and/or fructose; maltose or lactose mixed with galactose and/or glucose; melibiose in mixtures with raffinose, and other combinations.

Introduction

Many chemical and enzymatic methods have been described for qualitative and quantitative determination of naturally-occurring sugars (Aminoff *et al.*, 1970; Holligan, 1971; Holligan and Drew, 1971; Lewis and Smith, 1967). The latest employ gas-liquid chromatography (GLC). Since GLC is not yet widely available to botanists, this paper presents modified enzymatic methods for the rapid quantitative determination of mixtures of oligosaccharides and their component monosaccharides. These methods, which employ readily-available enzymes and commerical reagents, have been used in studies of carbohydrate physiology of orchid seedlings (Ernst, 1967; Ernst, Arditti and Healey, 1972).

Materials and methods

Sugars. Melibiose and stachyose were obtained from Calbiochem, Los Angeles, California and other sugars from Pfanstiehl Laboratories, Inc., Waukegan, Illinois. Only D-sugars were employed. They were dissolved either in distilled water and kept frozen until used or in methanol–water (7 : 3, v/v) and stored at room temperature.

Enzymes. Fructofuranosidase (invertase) melibiase free, 116 U/mg, derived from bakers' yeast, Grade IV was obtained from Sigma Chemical Company, St Louis, Mo. An alternative grade, purchased from Calbiochem Wallerstein invertase activity 873 (Meister, 1965), gave comparable results.

Yeast α-glucosidase (maltase) suspension, 2 mg protein/ml (20 U/ml) and galactose dehydrogenase (Galactose UV-test, TC-GA 15921 TGAN) were obtained from Boehringer Mannheim Corporation (BMC), San Francisco, California.

β-Galactosidase (lactase), derived from *Escherichia coli*, 5.5 mg protein/ml (398 U/mg)

in saturated ammonium sulphate and a prepared galactose oxidase reagent, Galactostat, were purchased from Worthington Biochemical Corporation, Freehold, New Jersey.

Phosphoglucose isomerase (PGI from yeast) in 2.4 M ammonium sulphate, 4 mg protein/ml, 1820 U/ml at 30° C was obtained from Calbiochem.

Thin layer chromatography (*TLC*). Method A. Borated Silica Gel G plates, developed with methyl ethyl ketone : acetic acid : methanol (6 : 2 : 2, v/v/v), were used for separation and identification (Jeffrey, Arditti and Ernst, 1969). Maltose and the products of its hydrolysis were detected with aniline-diphenyl amine (Bailey and Bourne, 1960) followed by naphthoresorcinol-H_2SO_4 (Lato *et al.*, 1968). This system detected sugars which could not be detected with aniline-diphenyl amine alone. Method B employs identical plates and visualizing techniques, but uses chloroform : acetic acid : water (5 : 4.3 : 0.7, v/v/v) as the developer.

Spectrophotometry. Spectrophotometric measurements were conducted in a Beckman DB-G instrument at 340 nm and 1 cm-light path. Mixing was by several gentle inversions. The cuvette chamber was maintained at 25° C to prevent bubble formation. Calculations were based on the micromolar extinction coefficient of 6.22 cm^2 for $NADH_2$ and $NADPH_2$ (Horecker and Kornberg, 1948).

Replication. All hydrolyses and determinations were replicated four times. The results reported are averages of these.

Assay of individual sugars and mixtures

Glucose

Glucose was determined by the hexokinase/glucose-6-phosphate dehydrogenase method (Cori and Larner, 1951; Keller, 1965; Peterson and Young, 1968; Slein, 1965) with a commercial reagent, Glucose Stat-Pack (Calbiochem), referred to as 'glucose reagent'. The reaction sequence is as follows:

$$\text{glucose} + \text{ATP} \xrightarrow[\text{(hexokinase)}]{} \text{glucose-6-phosphate (G-6-P)} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP} \xrightarrow[\text{(G-6-P dehydrogenase)}]{} \text{NADPH}_2 + \text{6-phosphogluconate.}$$

Fructose

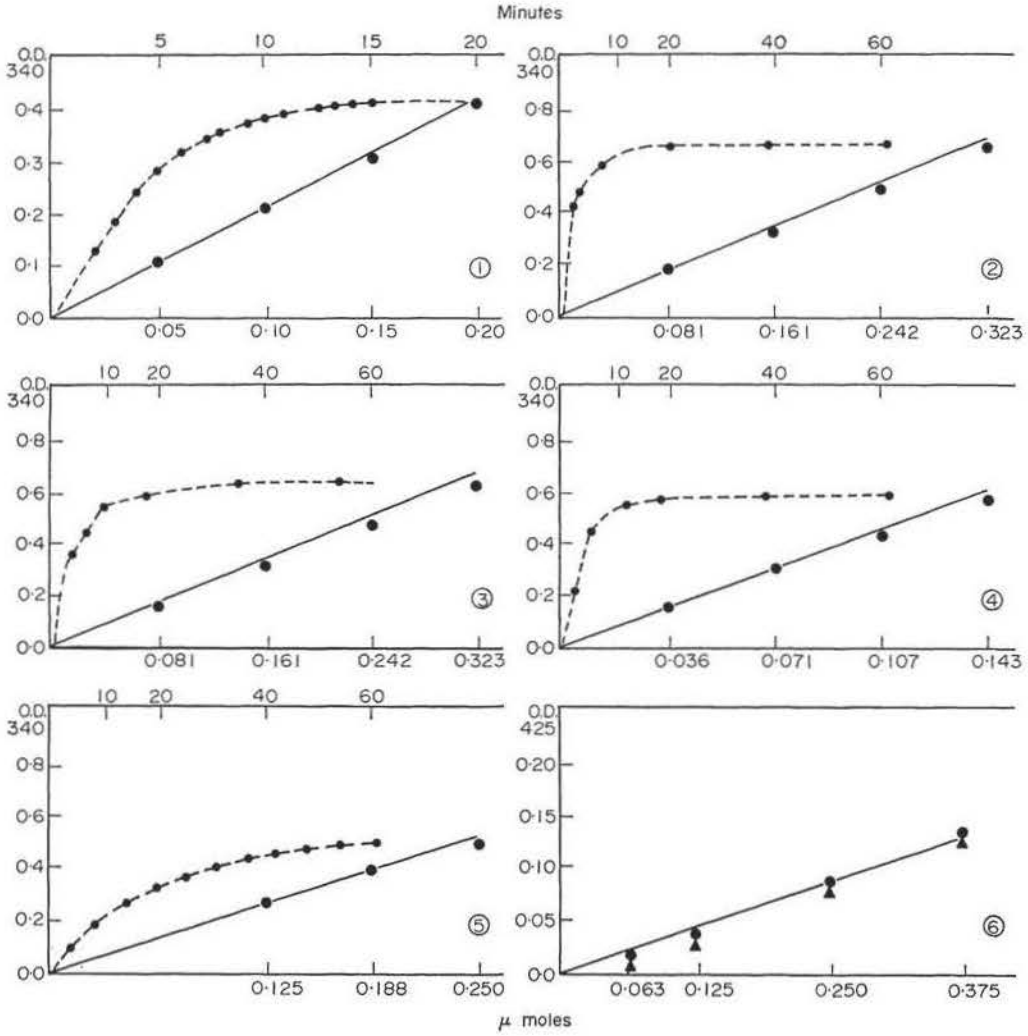
Fructose was determined by converting it into glucose-6-phosphate with PGI (Schmidt, 1961) added to the glucose reagent. This enzyme mixture is referred to as the 'fructose reagent'. Thus, following glucose determination, 5 μl PGI (about 9 U) were added to each 3 ml of the glucose assay reagent containing the sugar sample. Stable absorbance values and quantitative recoveries of known amounts of sample were obtained after 15 minutes at 25° C (Fig. 1) when fructose was alone or in mixture with glucose, sucrose or raffinose.

Galactose

Measurements of galactose were by the galactose dehydrogenase method (Finch *et al.*, 1969; Rommel *et al.*, 1968; Wallenfels and Kurz, 1962) using Galactose UV-test.

Raffinose

Hydrolysis of raffinose was carried out under conditions recommended for inversion of sucrose (Bergmeyer and Klotzsch, 1965). Aqueous solutions of the trisaccharide,



EXPLANATION OF FIGURES

Fig. 1–5. Solid lines show theoretical values (lower abscissa); dots represent μ moles sugar recovered. Broken line is reaction rate in minutes (upper abscissa).

Fig. 1. Fructose. Points representing recovery were obtained after 15-minutes incubation. Reaction rates were determined with 0.01 M solutions.

Fig. 2. Raffinose. Points representing recovery were obtained following 1-hour incubation with invertase. Reaction rates were determined with 0.1 M solutions.

Fig. 3. Stachyose. Points representing recovery were obtained following 1-hour incubation with invertase. Reaction rates were determined with 0.1 M solutions.

Fig. 4. Maltose. Points representing recovery were obtained following 1-hour incubation with α-glucosidase. Reaction rates were determined with 0.01 M solutions.

Fig. 5. Lactose. Points representing recovery were obtained following 75-minutes incubation with β-galactosidase in glucose reagent. Reaction rates were determined with 10 μl of 0.025 M solutions in 3 ml glucose reagent.

Fig. 6. Melibiose (▲) and raffinose (●). Points representing recovery were obtained following 1-hour incubation with Galactostat reagent.

ranging in molarity from 0.025 to 0.1, were incubated with 5.2 times their volumes of 0.1 M sodium acetate buffer (pH 4.6) containing 0.77 mg/ml invertase, at 37° C, for 1 hour. Although melibiose samples (0.1 M) were included to check for possible melibiase activity, the hydrolysed samples yielded only melibiose and fructose by TLC and glucose was also shown to be absent by enzymatic analysis. Hydrolysis was complete in 20 minutes and recovery corresponded to 96.4–98.7% (Fig. 2).

The quantity of raffinose (measured as fructose) contained in 20 μl of hydrolysed sample added to the fructose reagent is derived as follows, where O.D. Fru is observed change in optical density, total volume of reagents in cuvette = 3 ml and micromolar extinction coefficient for $NADPH_2$ = 6.22:

$$\text{micromoles } NADPH_2 \text{ formed} = \frac{3 \times \text{O.D. Fru}}{6.22} = \frac{\text{O.D. Fru}}{2.073};$$

$$\therefore \text{ micromoles } NADPH_2 \text{ per ml} = \frac{\text{O.D. Fru}}{2.073} \times \frac{1}{0.02}.$$

By correcting for the dilution factor, 6.2, occurring during the hydrolysis step and allowing for the relationship between the molecular weights of raffinose and fructose, raffinose content (%) in the sample prior to hydrolysis can be calculated. Based on a 20-μl sample in 3 ml, raffinose content (%) $= \dfrac{0.4345 \times 6.2 \times \text{O.D.} \times 504.43}{180.16}$

where 0.4345 is a factor converting O.D. into g/100 ml fructose as follows:

$$\frac{180.16}{2.073} \times \frac{1}{0.02} = 4345 \ \mu\text{g/ml} = 0.4345 \text{ g/100 ml (\%)}.$$

Sucrose/raffinose mixtures

Hydrolysis of sucrose and raffinose mixtures under the conditions outlined above will result in a hydrolysate containing glucose (from sucrose) and fructose (from sucrose and raffinose). Fructose values in excess of double the optical density obtained for glucose represent hydrolysed raffinose. Therefore with O.D. Glc = observed change in optical density with glucose reagent, and other factors as above, the following calculations apply:

$$\text{micromoles sucrose} = \frac{\text{O.D. Glc}}{2.073};$$

$$\therefore \text{ per cent sucrose in sample prior to hydrolysis} = \frac{0.4345 \times 6.2 \times \text{O.D. Glc} \times 342.3}{180.16},$$

and, since subtraction of O.D. Glc from O.D. Fru gives fructose derived from raffinose only, as the glucose and fructose content of sucrose are equivalent,

$$\text{micromoles raffinose} = \frac{\text{O.D. Fru} - \text{O.D. Glc}}{2.073};$$

$\therefore$ per cent raffinose in sample prior to hydrolysis

$$= \frac{0.4345 \times 6.2 \times (\text{O.D. Fru} - \text{O.D. Glc}) \times 504.43}{180.16}.$$

Aqueous and methanol–water solutions of 0.025–0.075 M sucrose and 0.075–0.025 M raffinose, as well as 0.1 M sucrose only and 0.1 M raffinose only, were hydrolysed as for raffinose. TLC of samples taken after 1 hour showed absence of sucrose and raffinose and presence of melibiose, glucose and fructose. The recovery of sucrose was 97.2–101.7% and that for raffinose 95.7–98.7% when analysed with the glucose and fructose reagents. Presence of methanol had no adverse effect on this method (but see maltase assay below).

Stachyose

Hydrolysis of stachyose was carried out as for raffinose. Hydrolysis was slower than for raffinose and the optical density obtained with the fructose reagent levelled off after about 40 minutes (Fig. 3). Recoveries ranged from 93.5–96.7% (Fig. 3).

Sucrose/stachyose mixtures

Hydrolysis of mixed aqueous sucrose and stachyose solutions was carried out as for sucrose/raffinose mixtures. TLC of samples taken after hydrolysis showed absence of sucrose and stachyose, but the presence of manninotriose, glucose and fructose. In assays of mixtures in various proportions as for sucrose and raffinose, recovery ranged from 98.7 to 104.6% for sucrose and 94.2 to 95.7% for stachyose Calculations for sucrose are those given for sucrose/raffinose mixtures, whereas stachyose recovery was calculated as follows:

$$\text{micromoloes stachyose} = \frac{\text{O.D. Fru} - \text{O.D. Glc}}{2.073},$$

and, per cent stachyose in sample prior to hydrolysis

$$= \frac{0.4345 \times 6.2 \times (\text{O.D. Fru} - \text{O.D. Glc}) \times 666.6}{180.16}.$$

Maltose

Maltose was measured as glucose following hydrolysis. Aqueous maltose solutions (0.025–0.1 M) and maltose dissolved in methanol–water were hydrolysed with maltase as follows: 50 μl maltose solution, 400 μl, 0.1 M sodium acetate buffer (pH 6.0), and 250 μl maltase suspension (20 U) were incubated at 37° C for 1 hour. Samples of glucose only (0.1 M) dissolved in water or methanol–water were included as controls. Unreacted maltose samples showed the presence of 0.09% glucose (by enzymatic procedure) as impurity, and minor amounts of a higher oligosaccharide (probably maltotriose) by TLC (Table 1). On completion of hydrolysis, TLC of aqueous solutions showed the presence of glucose only. With methanol–water solutions, a second spot appeared with a higher R_F value than glucose. Since α-methyl-D-glucoside (meGlc) can be synthesized by action of maltase from yeast where methanol is present (Bourquelot, Hérisse and Bridel, 1913) we tested for its presence chromatographically. The data show that meGlc was present in the hydrolysate (Table 1). Hydrolysis of aqueous maltose solutions resulted in stable optical density values after 40 minutes (Fig. 4). Methanol–water solutions gave a recovery of only 67.5% with maltose and of 69.2% with glucose.

Lactose

Hydrolysis and estimation of lactose was by an adaptation of a previous method

(Reithel and Venkataraman, 1956). To each 3 ml of glucose reagent were added 10 μl of 0.0125–0.25 M aqueous lactose solutions, free of glucose. Following the addition of 44 units of β-galactosidase (lactase) in 20 μl, lactose was hydrolysed and measured as glucose over a period of 75 minutes. Recoveries ranged between 96.5 and 100.3% (Fig. 5). Lactose content (%) in the sample prior to hydrolysis

$$= \frac{0.869 \times \text{O.D. Glc} \times 342.3}{180.16}.$$

In this as well as all previous examples involving oligosaccharides, the presence of glucose and/or fructose (free or as phosphate) must be determined prior to hydrolysis. Calculations for oligosaccharides should be corrected accordingly.

Table 1. *Thin layer chromatography of maltose, glucose and derivatives*

Carbohydrate	Chromatography Method A R_F	Colour	Method B R_F	Colour
Glucose	0.57	blue	0.25	blue
Maltose	0.43	blue	0.14	blue
Maltotriose (?)	0.19	blue	0.07	blue
α-Methyl-D-glucoside	0.68	reddish-blue	0.41	reddish-blue
Unknown	0.68	reddish-blue	0.41	reddish-blue

Melibiose

Galactose oxidase oxidizes terminal galactosyl units in oligosaccharides (Avigad *et al.*, 1962). Employing Galactostat, we obtained standard curves with melibiose, lactose, raffinose and stachyose. These were of different slopes at equivalent concentrations. Hence melibiose can be measured directly with the Galactostat reagent if other galactosyl containing sugars or galactose are absent. The same is true for raffinose stachyose and lactose.

Melibiose/raffinose mixtures

Melibiose present in mixtures with raffinose was determined as follows. Raffinose was hydrolysed with invertase, yielding equimolar amounts of fructose and melibiose. Total melibiose was then determined with the Galactostat reagent. Correction was made for melibiose derived from raffinose on the basis of fructose present in the hydrolysate (see raffinose determination). Thus, 0.1 M raffinose treated with invertase for 1 hour as above, was diluted with water to a concentration of 2.5 mM. Aliquots of 25–150 μl from this solution were each added to 1.5 ml Galactostat reagent and diluted to 3 ml with water. After incubation for 1 hour at 37° C, the reaction was stopped with 0.2 ml 0.5 M EDTA-Na_4 (Sempere, Gancedo and Asensio, 1965) and the optical density read at 425 nm. Melibiose treated in like manner was used as control (Fig. 6).

Discussion

Various techniques involving the enzymes glucose oxidase, invertase and melibiase in conjunction with determination of carbohydrate by reducing power, anthrone, manometry or optical rotation have been previously developed for assay of glucose, fructose, sucrose, melibiose and raffinose in various combinations (Eddy and Mapson, 1951; Potter and Williams, 1958; Böttger and Steinmetzer, 1959; Johnson *et al.*, 1964 and De Whalley, 1965). Problems include: (1) the necessity to remove interfering substances,

e.g. ascorbic acid with ascorbic acid oxidase; (2) colour development by the anthrone method, which requires the inclusion of standard solutions and depends on temperature and duration of reaction; (3) the determination of optical rotation which requires special apparatus, and measurements may be low where large amounts of optically active species are present (Bergmeyer and Klotzsch, 1965); and (4) melibiase is not commercially available.

The methods described here for these five sugars have important advantages over these techniques. They are simpler, accurate and, because of the enzymes involved, entirely specific. Except for melibiose, they do not require a calibration curve for each analysis and utilize equipment generally found in biological laboratories. Raffinose can also be measured with Galactostat but this reagent will also react with galactose and other galactosyl-glycosides. The fructose reagent can also be used to determine stachyose after treatment with invertase (Fig. 3).

The method of quantitative analysis of sucrose/raffinose or sucrose/stachyose mixtures should also be suitable for the determination of other galactosyl sucroses with a fructofuranosyl group detachable by invertase, such as verbascose (Bourquelot and Bridel, 1910) and ajugose. A difficulty may be the decreasing rate of hydrolysis with increasing molecular weight (Adams, Richtmyer and Hudson, 1943; Courtois, 1958). Although mixtures of glucose, fructose, sucrose and raffinose occur commonly in plant materials, stachyose or verbascose are less widespread (Bailey, 1965) but such mixtures could be measured quantitatively without separation by our methods.

Pan, Nicholson and Kolachov (1953) reported the determination of mixtures of glucose, maltose and other oligosaccharides fermentable by yeast, but substantial differences in the degree of fermentation, based upon type of yeast employed, have been shown by Kempf and Lindemann (1954) who could not confirm the quantitative fermentation of glucose in 90 minutes and that of maltose in 150 minutes with commercial baker's yeast. Quantitative analysis of glucose and maltose in mixture is readily achieved by our method. Although maltotriose would not be distinguished from maltose, this is not an important shortcoming because maltotriose does not occur naturally in plant tissues (Pazur, 1970).

Lactose has been detected in such plant tissues as the fruit of *Achras sapota* (Venkataraman and Reithel, 1958) and pollen of *Forsythia* (Kuhn and Löw, 1949). Chromatographically purified α-galactosidase was employed by us to avoid the interferences encountered with less pure preparations. The Galactostat reagent can be used for lactose determination only if galactose or other sugars with a terminal galactosyl group are absent. The Galactose UV-test reagent permits the measurement of this sugar in the presence of other galactosides. However, analogues of D-galactose such as L-arabinose and D-fucose will also react (Finch *et al.*, 1969; Hu and Grant, 1968).

In addition to the mixtures we have analysed, these methods may be employed for a variety of other combinations.

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