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Determination of Reducing Sugars with 3-Methyl-2-benzothiazolinonehydrazone

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Many polysaccharide-degrading enzymes are commonly assayed by quantifying the amount of reducing sugars released during the assay. Typically the reducing sugars are quantified by one of a number of colorimetric procedures (1–4). Colorimetric assays based on the reduction of copper have been around for decades and are still commonly used (1, 2). The most sensitive of these uses the chromogen bicinchoninic acid (BCA)[1] to quantify the amount of copper reduced. Improved versions of this method (2) are useful in the range of 1 to 20 nmol sugar and have been applied to the assay of endoglucanase activity (5). Unfortunately this type of "reductometric" assay is not specific for sugars and other compounds capable of reducing copper, most notably protein, also react. This assay is in fact one of the most commonly used methods to quantify protein concentrations.

Other methods for determining sugars exist, but all are significantly less sensitive than the BCA method. A method based on condensation of sugars with 2-cyanoacetamide (4) is simple and is commonly used to assay polygalacturonase activity (6). It is, however, only about one-fifth as sensitive as the BCA procedure. Furthermore, it relies on the measurement of a product with an absorbance maximum at 276 nm, a wavelength where many other biological compounds absorb. Our goal was to develop a visible spectrophotometric method for quantifying reducing sugars with high sensitivity, like the BCA method, but free from interference by protein.

[1] Abbreviatons used: BCA, bicinchoninic acid; MBTH, 3-methyl-2-benzothiazolinone hydrozone; DTT, dithiothreitol.

The colorimetric determination of aldehydes by 3-methyl-2-benzothiazolinone hydrozone (MBTH) is well known (7). In this reaction an aldehyde combines with two molecules of MBTH in a two-step process. In the first step, which occurs at neutral pH, the aldehyde condenses with a single MBTH to form an adduct. In the second step, which occurs under acid and oxidizing conditions, this adduct reacts with a second MBTH to form a highly colored final product (λmax at 620 nm). This method can be used to determine a number of aldehydes and is specific for aldehydes versus ketones.

Honda *et al.* (8) showed that sugars, if incubated with MBTH under alkaline conditions and elevated temperatures, can also undergo a condensation reaction with a single MBTH molecule, analogous to the first step in the aldehyde procedure. This simple condensation reaction yielded a colored product (λmax at 390 nm) which could be used to quantify reducing sugars. No value was given for molecular absorptivity but they reported that the sensitivity of this assay was in the range of 50 to 350 nmol, considerably less sensitive than the BCA procedure. These authors also refer to two older reports (9, 10) which indicate that the MBTH adduct of sugars, like the adducts of aldehydes, can undergo the oxidative addition of a second molecule of MBTH to yield a more highly colored final product. This would appear to be a highly sensitive method for quantifying reducing sugars although we know of no modern reports of its use. We have confirmed these older reports and have developed a practical and sensitive method for determining reducing sugars useful in the assay of enzymes like polygalacturonase.

Our standard procedure for determining sugars was as follows. In a 12 × 75-mm test tube, a sample containing 0 to 20 nmol of sugar in 100 μl water was mixed with 100 μl of 0.5 N NaOH followed by 100 μl of MBTH reagent. This MBTH reagent was prepared by mixing equal volumes of 3 mg/ml MBTH and 1 mg/ml DTT immediately before use. The samples were then heated for 15 min at 80°C in an aluminum heat block. As the samples were removed from the heat 200 μl of a solution containing 0.5% $(FeNH_4(SO_4)_2) \cdot 12\ H_2O$, 0.5% sulfamic acid, and 0.25 N HCl was added and the samples allowed to cool to room temperature. Finally 0.5 ml (or larger volume depending on the absorbance of the sample) of H_2O was added and the absorbance at 620 nm was determined. In cases where proteins or other acid-insoluble materials are present the samples were centrifuged before reading. The MBTH and DTT stock solutions could be stored in the refrigerator for at least 1 week. The acidic Fe solution was stable at room temperature.

Honda *et al.* (8) reported that the first step of the reaction, the formation of the MBTH sugar adduct,

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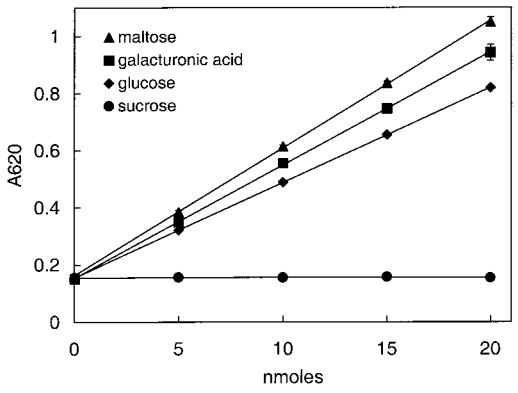

FIG. 1. Standard curves for various sugars. Reactions were performed as described in the text in a final volume of 1 ml. Each point is the mean of triplicate determinations. Standard errors are indicated by error bars; in most cases they are smaller than the symbol size. Correlation coefficients for the regression lines were 0.9998 or greater.

required high pH and elevated temperatures. We also found that this reaction required the addition of NaOH and heating of the samples. Maximum final color development was observed if the samples were heated for 4 min at 100°C, 15 min at 80°C, or 60 min at 60°C. Heating the samples at these temperatures for longer times led to increased blanks and reduced color yield. For our standard protocol we adopted 80°C for 15 min. The inclusion of DTT in the reagents was found to give more consistent blanks and reduce the tendency of the absorbance in the blank to increase during heating. Its inclusion was not required for color formation and had no effect on the color yield.

For the second step, the oxidizing reagent was added to the samples as they were removed from the heating block and color formation occurred as the samples cooled to room temperature. Under these conditions color formation was rapid, going to completion in about 5 min. Once formed, the blue colored product was stable for several days at room temperature while the yellow background color in the blank gradually decayed. The absorbance spectrum of the product formed by the reaction of glucose with MBTH had a peak at 620 nm and had the same shape as that reported for the reaction of formaldehyde with MBTH (7).

Standard curves for several sugars are shown in Fig. 1. The response was linear up to 20 nmol for various reducing sugars. The useful range of the assay could be extended up to 100 nmol by diluting the samples with additional water prior to reading the absorbance at 620 nm. As expected, the nonreducing sugar sucrose did not form a colored product. The relative responses of a number of sugars are given in Table 1. Most common monosaccharides gave very similar responses. Interestingly, the ketose fructose gave the identical response as the aldose glucose. Since ketones generally do not react with MBTH, the most likely explanation for this would be the isomerization of the fructose to glucose under the alkaline conditions of the reaction (11). The disaccharides maltose, lactose, and cellobiose all gave similar responses, about 25% greater than the corresponding monosaccharides. The tetramer cellotetrose gave an even greater color yield.

In general there is no correspondence between the relative color yield at 620 nm in this two-step reaction and the color yield at 390 nm from the direct condensation of sugars with MBTH reported by Honda *et al.* (8). In fact in several cases the relative reactivities are exactly reversed. For example, *N*-acetylglucosamine gave 2.7 times more color than glucosamine at 620 nm (Table 1), but only 0.33 as much at 390 nm in the direct condensation reaction (8). Similarly, galacturonic acid produced more color at 620 nm than glucuronic acid while the reverse was true at 390 nm. We have no explanation for this difference.

A significant deficiency in the BCA method for sugar determination is interference from proteins and reductants. For example, the addition of 50 μg of BSA to samples in the BCA procedure (2) caused an increase in the blank of 1.5 OD. With our MBTH method this same amount of protein increased the blank by only 0.12 OD. This concentration of protein in the sample (0.5 mg/mL) also had no effect on either the linearity or the slope of a standard curve. On the other hand, any compound that is incompatible with Fe will interfere

TABLE 1

Millimolar Extinction Coefficients at 620 nm for Various Sugars Reacted with MBTH

Sugar	ϵ	Sugar	ϵ	Sugar	ϵ
D-Glucose	31.1[a]	L-Arabinose	31.9	D-Xylose	33.7
D-Fructose	31.1	Maltose	41.3	D-Galacturonic acid	36.7
D-Mannose	31.7	Lactose	39.0	D-Glucuronic acid	23.5
D-Galactose	29.7	Cellobiose	39.6	D-Glucosamine	17.4
D-Ribose	33.3	Cellotetrose	55.0	*N*-Acetylglucosamine	48.1

[a] $M^{-1}\ cm^{-1} \times 10^{-3}$.

with our assay. Thus EDTA or other chelators, if present in significant amounts, as well as citrate and phosphate buffers, cannot be used. At a concentration of 0.1 M, acetate and succinate buffers did not interfere nor did the "good" buffers Mes (2-(*N*-morpholino)ethanesulfonic acid) or Mops (2-(*N*-morpholino)propane sulfonic acid). Tricine (*N*-tris[hydroxymethyl]methylglycine) caused small increases in the blank.

The procedure outlined here offers a useful alternative to existing methods for the determination of reducing sugars. The method offers high sensitivity and, unlike methods based on copper reduction such as the BCA procedure, neither proteins nor reducing agents such as DTT interfere. This makes the method well suited for use in enzyme assays, particularly where specific activity levels are low and large amounts of protein are present. It also has advantages over the cyanoacetamide method in that it is severalfold more sensitive and yields a product which absorbs in the visible rather than the ultraviolet region. We are currently using this assay to measure polygalacturonase activity in crude preparations from sources such as peaches and carrots.

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