Analyses of Reducing Sugars on a Thin-layer Chromatographic Plate with Modified Somogyi and Nelson Reagents, and with Copper Bicinchoninate

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Received September 25, 1995

Two novel methods to detect reducing sugar on a thin-layer chromatographic plate, using aqueous coloring reagents and a commercial microwave oven, were developed. After spraying the modified Somogyi reagent on the plate, irritating the reducing sugars, and then heating in a commercial microwave oven for a few minutes, the modified Nelson reagent was sprayed on the plates. Reducing sugars were only apparent as blue spots. On the other hand, after spraying the bicinchoninate reagent on the plates and then heating in the microwave oven, the sugar spots became reddish-violet. These two new methods enabled 0.1 µg of glucose per spot to be detected on a TLC plate. Non-reducing sugars (sucrose, trehalose, methyl 2-O-methylglucoside, and transfer products containing non-reducing ends) were not detectable by these methods.

Key words: thin-layer chromatography (TLC); modified Somogyi-Nelson reagent; copper bicinchoninate; commercial microwave oven; reducing sugars

The procedure for thin-layer chromatography (TLC) is very convenient and its sensitivity for detecting sugars (carbohydrates) is relatively high, so that TLC is commonly utilized for the analyses of low-molecular-weight sugars and their derivatives. Aniline diphenylamine-phosphoric acid,1,2 aniline phthalate3 and p-anisidine phosphate4 have been employed to detect reducing sugars on TLC plates.5,6 These spray reagents were prepared by using organic solvents such as ethanol, 1-butanol and acetone, thus limiting the spread of the sugar spots on the plate.7

The Somogyi8 and Nelson9 reagents system is one of the most common detection methods for quantitatively detecting reducing sugars in an aqueous solution. The copper bicinchoninate reagent is also used to detect reducing sugars with a micro-sample plate reader.10 The ranges of amount of reducing sugars that can be detected by the Somogyi-Nelson and bicinchoninate reagents are 5-600 µg of glucose per ml and 1-20 µg per ml, respectively. These reagents have never been used to detect reducing sugars on a TLC plate, because when these aqueous reagents are sprayed on the TLC plate, and the plate heated, it has been assumed that this would damage the surface of the plate.

This paper describes two new methods for analyzing reducing sugars by using modified Somogyi and Nelson reagents, and the copper bicinchoninate reagent.

Materials and Methods

Materials. Maltodextrins (G2, G4, G12, G12) were presented by Hayashibara Biochemical Laboratory (Okayama, Japan), and Kieselgel 60F254 plates (0.25 mm thickness) were purchased from Merck. Glucose, sucrose, sodium 2,2'-bicinchoninate, L-serine, sodium sulfate and ammonium molybdate were all purchased from commercial sources.

Preparation of the reagents. The Somogyi and Nelson reagents used in this experiment were prepared either by the original methods11,12 or by modified methods. As shown in the Table, the modified Somogyi reagent was prepared by halving normal amount of each component, except for CuSO4·5H2O, and the modified Nelson reagent was prepared by adding two volumes of conc. H2SO4 (2 ml) to the reference reagent. The copper bicinchoninate and p-anisidine phosphate reagents were prepared as described by Fox and Robyt,13 and by the method of Mukherjee and Srivastava,14 respectively, for use as spraying reagents. 5% H2SO4 in methanol was used to detect the total sugar content.

Thin-layer chromatography. Thin-layer chromatography (TLC) was performed by using the double-ascending method with a solvent system of 1-butanol ethanol water (5:5:2.5:3). The irrigated sugars were detected with 5% H2SO4 in methanol (100°C for 5 min), the modified Somogyi and Nelson reagents, or the copper bicinchoninate reagent.

Detection of the reducing sugars. To detect the reducing sugars, a TLC plate was sprayed completely with the modified Somogyi reagent, the excess water on the plate being quickly removed with an air dryer. The dried plate was then put into a Petri dish as shown in Fig. 1, the plate together with the Petri dish then being heated in a microwave oven (ER-435F, Toshiba, Japan) for 3 min (recommended time of 4 min in the range mode and cooled immediately to 4°C. After spraying the modified Nelson reagent onto the cooled plate, reducing sugar was detectable only as a blue spot. When the copper bicinchoninate reagent was also sprayed and the plates were heated in the microwave oven for only one minute, reducing sugar was detectable as a reddish-violet spot.

After the p-anisidine phosphate reagent had been sprayed onto the TLC plate, it was heated at 100°C for 5 min.

Table Comparison of the Components of the Reference and Modified Somogyi Nelson Reagents

<table>
<thead>
<tr>
<th>Component</th>
<th>Reference reagent (g)</th>
<th>Modified reagent (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Na2CO3 (anhydrous)</td>
<td>12g</td>
<td>24g</td>
</tr>
<tr>
<td>Rochelle salt</td>
<td>6g</td>
<td>12g</td>
</tr>
<tr>
<td>CuSO4·5H2O</td>
<td>4g</td>
<td>4g</td>
</tr>
<tr>
<td>NaHCO3</td>
<td>8g</td>
<td>16g</td>
</tr>
<tr>
<td>Na2SO4·(anhydrous)</td>
<td>9g</td>
<td>18g</td>
</tr>
<tr>
<td>in H2O</td>
<td>1000ml</td>
<td>1000ml</td>
</tr>
<tr>
<td>B (NH4)2MoO4·4H2O</td>
<td>25g</td>
<td>25g</td>
</tr>
<tr>
<td>H2SO4</td>
<td>42ml</td>
<td>21ml</td>
</tr>
<tr>
<td>Na2HAsO4·7H2O</td>
<td>3g</td>
<td>3g</td>
</tr>
<tr>
<td>in H2O</td>
<td>500ml</td>
<td>500ml</td>
</tr>
</tbody>
</table>

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Enzyme and reaction conditions. Isoamylotranse (EC 3.2.1.94) from Arthrobacter globiformis T6 was prepared by the method of Sawai et al.11) Reaction mixtures for the production of transfer products contained 0.4 units of isomaltodextranase, 5% dextran (M, 3000), and 7% sucrose or 7% trehalose in 1 ml of a 0.1 M sodium acetate buffer (pH 5.3). After reacting for 20-30 h at 30 C, 2μl of the mixture was spotted onto a TLC plate and developed by the double-ascending method. Sugars on the plate were detected by each spray reagent just mentioned.

Results and Discussion

Application of aqueous reagents sprayed on to TLC plates

Spray reagents for detecting sugars on a TLC plate have been reported by several researchers, e.g., aniline diphenylamine-phosphoric acid,12) aniline phthalate,13) p-anisidine phosphate,41) and 4-aminophippuric acid.12) However, these reagents have been prepared in organic solvents to prevent migration of the sugar spots. Thus, any reagent that is insoluble in an organic solvent could hardly be used as a coloring reagent. We considered that if the heating process after spraying an aqueous reagent such as the Somogyi–Nelson and copper bichinoninate reagents was shortened to a few minutes, the reagent would then be able to be used for the detection of reducing sugars on the plate. It was hypothesised that, if a microwave oven was used to heat the plates for a few minutes, no damage to the chromatograms would be caused.

Selection of aqueous reagents and modified reagents

We selected one of the general reagents and one of the most sensitive reagents that are used for determining reducing sugars in enzyme reaction mixtures and in carbohydrate samples. Somogyi and Nelson reagents in their reaction with reducing sugars have the characteristic of controlling the pH value of the sample solution; the addition of one volume of the Somogyi reagent equivalent to the sample solution increases the pH to 9–10 and, after the addition of two volumes of the Nelson reagent, the pH drops to 1–2 to develop molybdenum blue. Therefore, the compositions of the Somogyi and Nelson reagents for spraying on to the TLC plates were modified as shown in the Table so that similar volumes of the reagents could be

Fig. 2. Sensitivity for Reducing Sugars by Various Spray Reagents (I), and Color Comparisons of Reducing and Non-reducing Sugars (II) on a TLC Plate.
(I) Two μl of serially diluted standard glucose was quantitatively spotted on to a TLC plate. After spraying each reagent, the resulting plates were heated as described in the Materials and Methods section. A, 5% sulfuric acid in methanol; B, modified Somogyi–Nelson reagent; C, copper bichinoninate reagent; D, p-anisidine phosphate reagent. 1, 50 μg ml; 2, 25 μg ml; 3, 10 μg ml; 4, 5 μg ml; 5, 1 μg ml; 6, 500 μg ml; 7, 100 μg ml; 8, 50 μg ml; 9, 10 μg ml; 10, 5 μg ml.
(II) Two μl of serially diluted standard glucose was quantitatively spotted on to a TLC plate. After spraying each reagent, the resulting plates were heated as described in the Materials and Methods section. A, 5% sulfuric acid in methanol; B, modified Somogyi–Nelson reagent; C, copper bichinoninate reagent; a, glucose; b, trehalose; c, sucrose; d, methyl α-D-glucoside; e, mannose; see the legend under Fig. 2(I) for the other keys.
used. The pH value of the modified Somogyi reagent was 9.7, and that of a mixture of equal volumes of the modified Somogyi and Nelson reagents was 1.0, whereas that of the original reagent was 4.0. This suggested that the pH value on a TLC plate could be favorably controlled by spraying the modified reagents. A comparison of the original with the modified Somogyi–Nelson reagents, and with the copper bicinchoninate reagent indicated that the modified Somogyi–Nelson reagent and copper bicinchoninate reagent were well suited for detecting reducing sugars on a TLC plate (Fig. 2). The copper bicinchoninate reagent directly colored the spots of the sugars on plates heated in the microwave oven, but the color was only stable for 2–3 h. In contrast, the color by the modified Somogyi–Nelson reagent remained stable for more than 24 h.

Comparison of the coloration of reducing and non-reducing sugars

Serially diluted glucose solutions (from 100 μg/spot to 10 ng/spot) were spotted on TLC plates, sprayed with the modified Somogyi–Nelson reagent, copper bicinchoninate reagent and p-anisidine phosphate reagent, and then heated as described in the Materials and Methods section. In the case of the modified Somogyi–Nelson reagent, the reducing sugars were detectable as blue spots (Fig. 2(I)B), while when using the copper bicinchoninate reagent, the reducing sugars were detectable as red-violet spots (Fig. 2(I)C). Spot 6 containing 1 μg of glucose per spot was readily detectable by the modified Somogyi–Nelson reagent and copper bicinchoninate reagent. These reagents could detect 0.1 μg of glucose per spot, but 5% sulfuric acid in methanol and the p-anisidine phosphate reagent (Figs. 2(I)A and D) hardly detected the same amount of glucose.

We compared the coloration of reducing and non-reducing sugars on TLC plates by using glucose, mannose, methyl α-1-glucoside, trehalose, and sucrose (Fig. 2(II)). Reducing sugars, glucose and mannose, were colored by the modified Somogyi–Nelson reagent and copper bicinchoninate reagent, whereas non-reducing sugars, methyl α-1-glucoside, trehalose and sucrose, were not detectable by either reagent. If the time of heating in the microwave oven was longer than 6 minutes after spraying on the modified Somogyi reagent and longer than 2 minutes after spraying on the copper bicinchoninate reagent, a non-reducing sugar spot, especially for sucrose, would be revealed as a faint color. Both reducing and non-reducing sugars (more than 1 μg/spot) were readily detectable by the H₂SO₄–MeOH system.

The two coloring methods using the Somogyi–Nelson reagent and copper bicinchoninate reagent were tested on a mixture of maltodextrins which had been irrigated on the TLC plate (Fig. 3). One μg of maltodextrins per spot was clearly detectable by each method. One hundred μg of sucrose was not detectable by either the
modified Somogyi–Nelson reagent, or the copper bicincho-
ninate reagent. This indicates that these reagents did not 
react with non-reducing sugars under the above conditions.

Application for analyzing enzyme reactions

Isomaltodextran from A. globiformis T6\(^{13,15}\) releases
isomaltose units successively from the non-reducing ends 
of dextran, and has the transfer activity and condensation 
activity of isomaltose. The methods reported here were 
adapted for an analysis of the transfer action of
isomaltodextranase on the non-reducing sugar acceptors, 
sucrose and trehalose. As shown in Fig. 4, TP1 and TP2, 
which were produced by the transfer action of Arthrobacter 
globiformis T6 isomaltodextranase, were not detectable with 
the copper bicinchoninate reagent, whereas they were clearly 
detectable by 5% sulfuric acid in methanol. In addition, 
TP1 and TP2 were not detectable with the modified
Somogyi–Nelson reagent.\(^{17}\) This indicates that TP1 and 
TP2 were oligosaccharides containing a non-reducing end. 
By using the methods already mentioned, it is readily 
detectable whether transfer products are reducing or 
non-reducing sugars.

Acknowledgments. We thank Hayashibara Biochemical Laboratory
Inc. for generously presenting the multooligosaccharides (G2-G7). This 
reasearch was supported in part by a Grant-in-Aid for Pioneering Research
Project in Biotechnology from Ministry of Agriculture, Forestry, and 
Fishery of Japan.

References