Adaptation of the Nelson-Somogyi Reducing-Sugar Assay to a Microassay Using Microtiter Plates

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The Nelson-Somogyi assay for reducing sugars was adapted to microtiter plates. The primary advantages of this modified assay are (i) smaller sample and reagent volumes, (ii) elimination of boiling and filtration steps, (iii) automated measurement with a dual-wavelength scanning TLC densitometer, (iv) increased range and reproducibility, and (v) automated colorimetric readings by reflectance rather than absorbance.

Many colorimetric assays have been modified for simple testing in microtiter plates (1,2) and automated measurement in a microplate reader. These microassay systems facilitate rapid screening of a large number of column chromatography fractions and substantially conserve time and reagents. In addition, fractions can be screened for different enzymes simultaneously on the same microplate.

Most commercial microplate readers differentiate colorimetric reactions by transmitted light (absorbance) at a specific wavelength. Either insoluble substrates, used in many assays (3,4), or the precipitates formed during an assay prevent penetration of light and render measurement by transmitted light inaccurate.

We have adapted a thin-layer chromatography (TLC) double-beam densitometer to accept 96-well microtiter plates for determination of reducing sugars by a modified Nelson-Somogyi method. Each well is read by reflectance, thus avoiding the time-consuming filtration step necessary in the standard Nelson-Somogyi assay in cases when insoluble precipitate hinders reading by absorbance (transmission).

The principal result of this study is that for the first time, a sensitive, rapid microassay can be effectively substituted for the macroassay, thus conserving time and reagents.

MATERIALS AND METHODS

Macroassay for reducing sugars. The standard reducing-sugar assay was that of Nelson and Somogyi (5,6). Briefly, 1-ml samples were combined either with 10 mg substrate and 1 ml citrate buffer (0.1 M, pH 5.0) or with 1 ml substrate solubilized in 0.1 M citrate buffer (pH 5.0) at 1% (w/v) in 50-ml Folin tubes. A control for each sample was prepared with substrate and buffer. Tubes were incubated at 40°C for 24 h. After incubation, 2 ml of copper reagent, consisting of 4 parts KNa tartarate: Na₂CO₃:Na₂SO₄:NaHCO₃ (1:2:12:1.3) and 1 part CuSO₄·5H₂O:Na₂SO₄ (1:9), was added to each tube. Both copper reagents must be prepared by boiling to completely dissolve the components; they can then be stored at room temperature. They were mixed together just prior to use. After 1 ml of sample was added to the appropriate control tubes, all tubes were boiled for 10 min in a water bath. The tubes were then cooled completely, 2 ml of arsenomolybdate reagent (25 g ammonium molybdate in 450 ml H₂O + 21 ml H₂SO₄ + 3 g Na₃HA·SO₄·7H₂O dissolved in 25 ml H₂O) was added to each tube, and the tubes were shaken thoroughly before adjusting the final volume to 25 ml with water. Individual samples were filtered through filter paper, and colorimetric measurements were determined by transmitted light at 500 nm in a spectrophotometer.

Microassay for reducing sugars. The microassay for reducing sugars was a modification of the Nelson-Somogyi assay (5,6). In a 96-well microplate, 25 µl of sample and 25 µl of appropriate substrate solubilized in 0.1 M citrate buffer, pH 5.0, were placed in each well. The plate was covered with an acetate adhesive sheet and incubated at 40°C for 24 h. After incubation 75 µl of Somogyi copper reagent was added, and the wells were resealed.

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FIG. 1. Typical standard curve for the micromodification of the Nelson-Somogyi reducing-sugar assay from 100 to 2000 µg glucose/ml at 500 nm. The mean of five replicates is represented.

Substrates were prepared by dissolving the desired substrate in 0.1 M citrate buffer, pH 5.0 (1% w/v), and removing insoluble material by centrifuging at 5000g for 10 min (8). A unit of enzyme activity was defined as the amount needed to liberate reducing power equivalent to 1 µg of glucose per 24 h at 40°C. The background, consisting of simple sugars in the preincubated sample, was subtracted.

Microassay of protein. A Bradford microassay was used for simultaneous protein determinations of fractions (9) using bovine serum albumin (Sigma) for standard calibration.

RESULTS

A standard curve for glucose was determined with appropriate dilutions (Fig. 1). The linear range of our standard curve is 100 to 1500 µg glucose/ml. Representative results from three column chromatography separations are shown in Fig. 2.

The reducing-sugar microassay shows distinct enzyme peaks for xylanase, laminarinase, and CMCase.

Assay of polysaccharide-degrading enzymes. Polysaccharide-degrading enzymes from Postia placenta (Fr.) M. Lars. et Lomb. [ = Poria placenta (Fr.) Cke.] were partially purified by gel chromatography as previously described (7). Fractions from multiple Sepharose 6B (Pharmacia, Piscataway, NJ) column runs that contained desired enzyme activity were pooled and subsequently passed down Fractogel TSK HW-55 (F) (EM Science, Gibbstown, NJ) columns in phosphate-buffered saline (pH 7.4) to yield partially separated hemicellulase components of the enzyme complex. Enzyme fractions (3 ml) were assayed simultaneously for xylanase, laminarinase, carboxymethylcellulase (CMCase), and protein in 96-well microplates (Dynatech, Chantilly, VA). Polysaccharide-degrading enzyme activities from the brown-rot fungus P. placenta were determined using the following substrates: xylan (P-L Biochemical Co., Milwaukee, WI) for endo-β-1,4-xylanase, carboxymethylcellulose (Hercules) for endo-β-1,4-glucanase, and laminarin (Sigma, St. Louis, MO) for endo-β-1,3-glucanase.

FIG. 2. Reducing-sugar microassay on Fractogel HW-55 fractions to determine xylanase, CMCase, and laminarinase enzyme activity. Protein content, as determined by the Bradford microassay, is shown for the same fractions.
TABLE 1
Results of Nelson-Somogyi Standard Reducing-Sugar Assay and Microplate Adaptation

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme units (µg glucose ± SD) a,b</th>
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<tbody>
<tr>
<td></td>
<td>Standard assay</td>
</tr>
<tr>
<td>Laminarin</td>
<td>506 ± 246</td>
</tr>
<tr>
<td>Xylan</td>
<td>233 ± 90</td>
</tr>
<tr>
<td>Carboxymethylcellulose</td>
<td>98 ± 43</td>
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a Number of replicates: 3 for standard assay, 5 for microassay.
b Statistically, no significant difference between standard and microassay results (one-way analysis of variance; p > 0.05).

DISCUSSION

The standard Nelson-Somogyi reducing-sugar assay has been used routinely for estimating polysaccharide-degrading enzyme activities (5,6,10). The standard Nelson-Somogyi assay is quite manageable when only a small number of assays are needed, but it becomes unwieldy when numerous chromatography fractions require assay for multiple enzyme activities and protein. A modification of the Nelson-Somogyi assay was adapted to 96-well microtiter plates by utilizing reflectance measurement in a TLC scanning densitometer. We believe this is the first report of a microplate adaptation of an assay that requires boiling.

The barriers to microadaptation of the standard Nelson-Somogyi assay were the boiling and filtration steps in which insoluble substrates or precipitates are removed prior to measuring absorbance (500 nm) in a spectrophotometer. We replaced the boiling step with a 30-min heating step at 80°C, which minimizes damage to the polystyrene plates. The filtration step was eliminated by measuring the subsequent color change by reflectance rather than absorbance. If solubilized substrate is used, optical density can be measured by transmitted light in a commercial microplate reader (unpublished result). Sample volumes were conserved by reducing the test volumes from 1.0 to 0.025 ml, and reagent proportions were adjusted to fit the 0.30-ml well capacity. The sensitivity of the macroassay is greater (5 compared with 100 µg glucose/ml) (unpublished results), but it requires 40 times more sample volume.

The two assays performed on the same sample (Table 1) gave equivalent values for laminarinase, xylanase, and carboxymethylcellulase. Precision, as measured by standard deviation from the mean, was greater in the microassay (Table 1).

The microassay has proven invaluable for rapid surveys designed to locate polysaccharide-degrading enzyme from chromatography fractions, as illustrated in Fig. 2. The substantially smaller test volume reduces loss of precious enzyme. The ability to run multiple samples as quickly as single samples improves efficiency, and test reagents can be added with multichannel pipets. The linear range of the Nelson-Somogyi assay has been increased on the upper end from 400 to 1000 µg glucose/ml.

The standard Nelson-Somogyi reducing-sugar assay has been successfully adapted to microtiter plates with automated reading by reflectance in a TLC scanning densitometer. Examples of other applications for the reducing-sugar microassay include a clinical application for blood sugar determinations (11,12) and monitoring of reducing sugars in the wine industry (13). Other complex assays that include either a heating or a filtration step may also be automated by microassay.

REFERENCES