

R60-34

ANALYTICAL BIOCHEMISTRY 2, 127-132 (1960)

Measurement of Carboxymethylcellulase Activity

GAIL LORENZ MILLER, ROBERT BLUM, WILLIAM E. GLENNON,
AND ANNE L. BURTON

*From the Pioneering Research Division, Quartermaster Research and
Engineering Center, Natick, Massachusetts*

Received April 18, 1960

INTRODUCTION

The method outlined by Reese¹ (10) for measuring cellulase activity, involving action of the enzyme on carboxymethylcellulose followed by determination of the reducing power of the resulting sugars with the aid of the dinitrosalicylic acid reagent of Sumner and Sisler (11), appears to be one of the most useful of many reported. Carboxymethylcellulose is favored over native cellulose as a substrate because of the great advantage of its solubility in water, while dinitrosalicylic acid reagent is favored over other reagents for determination of reducing sugar because of its great convenience. To avoid ambiguity, however, with regard to the presumptive relationship of enzymes acting on carboxymethylcellulose to those acting on native cellulose, the former will be designated carboxymethylcellulase, as suggested by Festenstein (1).

Through experience with the method, technical details have been worked out to produce a simplified procedure which will be described in the present paper. The use of iodine-potassium iodide (4, 7) for determination of the reducing sugar produced in the test is also described. Comparisons are made of results obtained with the two different sugar reagents and with carboxymethylcellulases from different sources.

SOURCES OF CARBOXYMETHYLCELLULOSE

Samples of carboxymethylcellulase studied were a lyophilized preparation from *Myrothecium verrucaria* QM 460 (6), cellulolytic culture filtrates from *Trichoderma viride* QM 6a, *Penicillium pusillum* QM 137g, and *Pestalotiopsis westerdijkii* QM 381 and an acetone precipitate of a filtrate from Basidiomycete QM 806. The filtrates and the acetone precipitate were prepared and placed at our disposal by Dr. E. T. Reese and Dr. Mary Mandels of this laboratory.

¹ Reese, E. T., private communication.

TECHNICAL LIBRARY
QUARTERMASTER RESEARCH &
ENGINEERING CENTER
NATICK, MASS.

REAGENTS

Buffered Carboxymethylcellulose Substrate

Forty-four and eight-tenths grams of Hercules 50T carboxymethylcellulose (CMC) are dissolved in 2800 ml water at 90°C. This is best accomplished with the aid of a 1-gal Waring blender which effectively disperses gelatinous lumps of the CMC. The mixture is cooled, 800 ml of 0.5 *M* citrate buffer (35 gm citric acid monohydrate and 98 gm sodium citrate dihydrate per liter), 0.4 gm Merthiolate, and 0.4 gm glucose are added and the solution is diluted to 4 l. The reagent is stored at 4°C. The final solution thus prepared contains 1.00% CMC based on a 12% moisture content found for the original CMC powder. The citrate concentration is 0.1 *M*; the pH, 5.0; the glucose concentration, 0.1 mg/ml. This level of glucose (0.1 mg/ml) provides a safe excess over the level required to compensate for the destruction of reducing sugar in the color test (5), yet does not contribute to too high a blank reading. The Merthiolate serves as a preservative.

Dinitrosalicylic Acid Reagent

Modified dinitrosalicylic acid reagent containing Rochelle salts (5) is prepared by dissolving a mixture of 40 gm dinitrosalicylic acid, 8 gm phenol, 2 gm sodium sulfite, and 800 gm Rochelle salts in 2 l of 2% sodium hydroxide and diluting to 4 l.

Iodine-Potassium Iodide Reagents

Two-hundredths normal iodine-10% potassium iodide, 0.75 *M* sodium carbonate, and 1.2 *N* phosphoric acid reagents are prepared as described elsewhere (7).

PROCEDURES

Procedure Using Dinitrosalicylic Acid as Sugar Reagent

(a) One-milliliter aliquots of cold (4°C) CMC substrate are added to 1-ml aliquots of enzyme samples and of glucose standards in 14-mm o.d. colorimeter tubes. The samples are mixed by inverting 10 times. (b) The mixtures are incubated at 50°C for a desired period of time, usually 20 min, and are then cooled in running tap water. (c) Three-milliliter aliquots of dinitrosalicylic acid reagent are added to each digest and the reactants are mixed by inverting five times. (d) The mixtures are then heated in a boiling water bath for 15 min, followed by cooling in running tap water adjusted to ambient temperature. (e) Readings of absorbance are finally made in a Beckman model DU spectrophotometer

at a wavelength of 640 $m\mu$ and a slit width of 0.2 mm. Shorter wavelengths will be found suitable where readings are made in colorimeters using color filters.

Procedure Using Iodine-Potassium Iodide as Sugar Reagent

Steps (a) and (b) are those described in the preceding section. (c) Following the procedure for aldoses (7), 1 ml of 0.75 *M* sodium carbonate and 2 ml of 0.02 *N* iodine-potassium iodide are next added to each tube, and the contents of the tubes are mixed by twirling. (d) The tubes are allowed to stand at 25°C for 1 hr. (e) Each mixture is acidified with 5 ml of 1.2 *N* phosphoric acid and is carefully stirred with a glass rod. (f) Absorbances are measured in a Beckman model DU spectrophotometer at a wavelength of 480 $m\mu$ and a slit width of 0.2 mm.

RESULTS

Definition of Unit of Carboxymethylcellulase Activity

For convenience the unit of carboxymethylcellulase activity may be defined as that activity shown by an amount of enzyme in 1 ml of solution which will produce an amount of reducing sugar equivalent to 1.0 mg glucose when incubated for 20 min at 50°C with 1 ml of 1% carboxymethylcellulose dissolved in 0.1 *M* citrate buffer at pH 5, as determined with the dinitrosalicylic acid reagent, or 0.5 mg glucose, as determined with the iodine-potassium iodide reagent. This unit closely approximates the Cx unit of Reese and co-workers (2, 10). The reason for the different amounts of glucose corresponding to 1 unit of activity when the different sugar reagents are used will become clear from results given in the next section.

*Dilution Curves for Carboxymethylcellulase from
Myrothecium verrucaria*

Data obtained for amounts of reducing sugar formed when various dilutions of a solution of carboxymethylcellulase from *Myrothecium verrucaria* are incubated with carboxymethylcellulose for widely varying periods of time (20 min and 24 hr) are shown graphically in Fig. 1. The amounts of reducing sugar indicated by the dinitrosalicylic acid reagent (upper curves) are about twice those indicated by the iodine-potassium iodide reagent (lower curves). This can be explained, at least to a large degree, by the fact that with the dinitrosalicylic acid reagent oligosaccharides from cellulose yield on an equimolecular basis much more color than does glucose,² while with the iodine-potassium iodide

² Miller, G. L., and Slater, R. W., unpublished work.

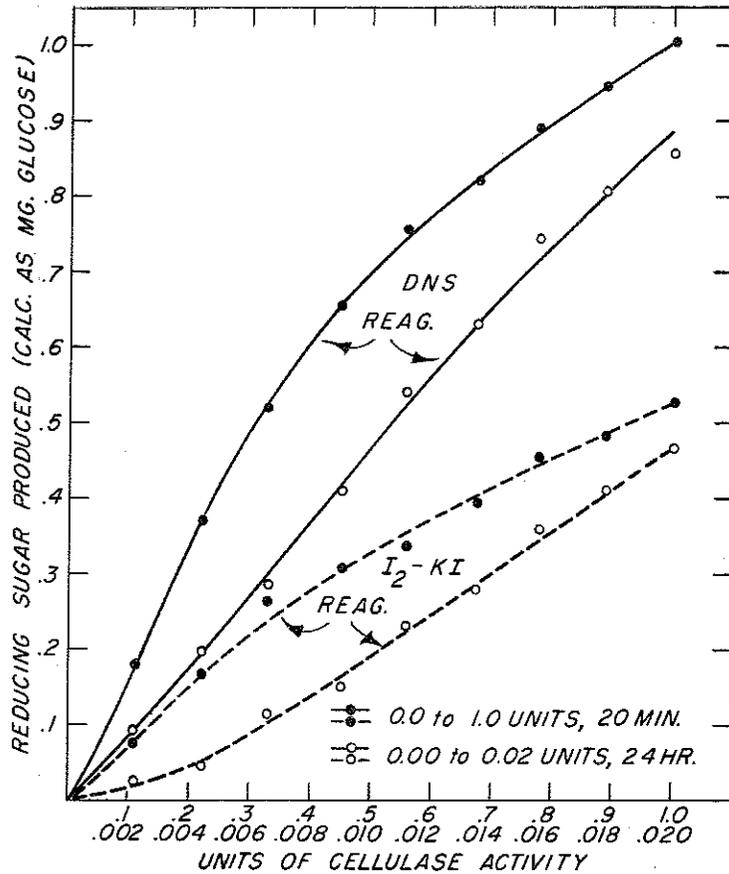


FIG. 1. Dilution curves for carboxymethylcellulase from *Myrothecium verrucaria*, obtained with different ranges of enzyme activity and different periods of incubation and with different sugar reagents.

reagent the degrees of reaction of oligosaccharides and glucose are the same (7).

Comparison of Dilution Curves for Carboxymethylcellulases from Different Fungi

Dilution curves of carboxymethylcellulases from *Myrothecium verrucaria* and *Trichoderma viride*, measured under conditions of 20 min incubation, but covering 10 times the range of activity given in Fig. 1, are shown in Fig. 2. It is clear that the dilution curves for the different cellulases are different from one another and cannot be superimposed. Curves obtained for carboxymethylcellulases from other fungi, not shown

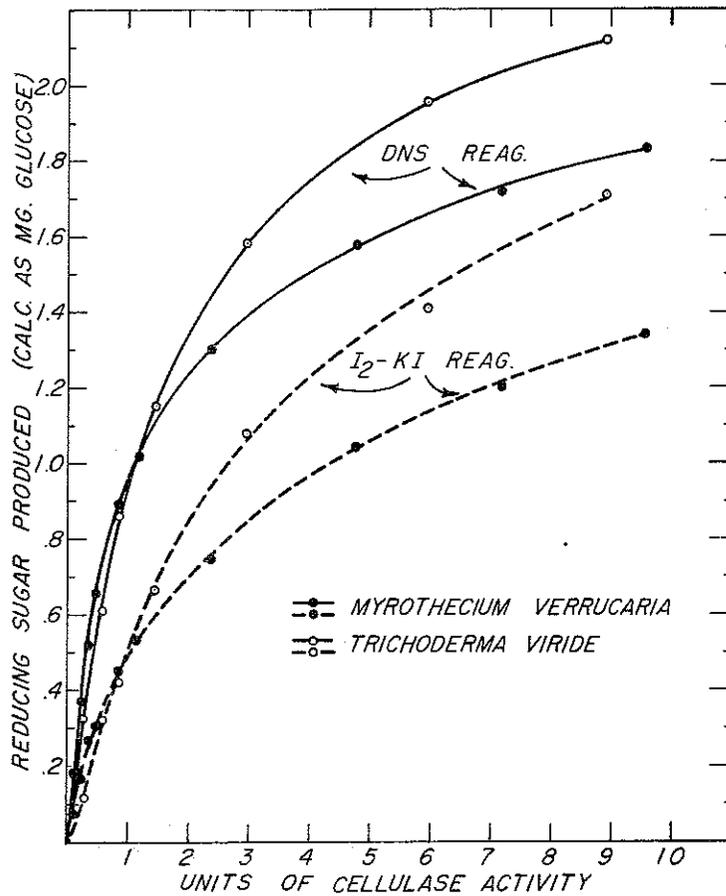


FIG. 2. Comparison of dilution curves for carboxymethylcellulases from different fungi, obtained over a wide range of enzyme activity and with different sugar reagents.

on the figure, also were found to be distinctive. Further, the differences in the dilution curves for different carboxymethylcellulases were found to be reproducible.

DISCUSSION

Within certain limitations the procedures described in this paper will be found to be highly useful. For many purposes dilution curves of the types shown in Figs. 1 and 2 can be used as standards of reference for estimations of activities of unknowns, although where this is done the enzyme used for the dilution curve and that contained in the unknown should correspond to one another. The wide ranges of activities which

can be measured is clearly apparent from the data. When relative values of activity suffice, as in measurements of zone-electrophoretic fractions, values of absorbance may be used as first approximations of enzyme activity, with excellent reproducibility (8). The dinitrosalicylic acid reagent is more convenient for measuring reducing sugar than the iodine-potassium iodide reagent. However, the latter should prove to be the more reliable in kinetic studies because of its greater specificity for aldehyde groupings.

It can be shown for the present data that a constant product of enzyme activity and time fails to yield a constant amount of reducing sugar, as required by first-order reaction kinetics (9). Further, the dilution curves of the different enzymes reveal, to varying degrees, anomalous *S* shapes. These deviations may be due to the operation of complicating factors such as inhomogeneity of substrate (degree of substitution, particle size), multiplicity of enzyme components (2, 3, 6, 8), and presence or absence of activators or inhibitors. It may be presumed that other cellulase methods described in the past are subject to similar deviations. Methods for controlling such factors are not available at present. Caution is particularly advisable in making comparisons of activities of enzymes from different sources. Empirical applications of the described procedures should, however, aid in resolving the apparent complexities of cellulase systems.

SUMMARY

Methods are described for measurement of carboxymethylcellulase activity involving action of the enzyme on carboxymethylcellulose followed by determination of reducing power of resulting sugars with the aid of dinitrosalicylic acid or iodine-potassium iodide. Results obtained with the different reagents and with carboxymethylcellulases from different sources are compared. The applicability of the methods is discussed.

REFERENCES

1. FESTENSTEIN, G. N., *Biochem. J.* **65**, part 2, 23, (1957).
2. GILLIGAN, W., AND REESE, E. T., *Can. J. Microbiol.* **1**, 90 (1954).
3. HASH, J. H., AND KING, K. W., *J. Biol. Chem.* **232**, 381 (1958).
4. JERMYN, M. A., *Australian J. Sci. Research, Ser. B*, **5**, 409 (1952).
5. MILLER, G. L., *Anal. Chem.* **31**, 426 (1959).
6. MILLER, G. L., AND BLUM, R., *J. Biol. Chem.* **218**, 131 (1956).
7. MILLER, G. L., AND BURTON, A. L., *Anal. Chem.* **31**, 1790 (1959).
8. MILLER, G. L., BLUM, R., AND HAMILTON, N. F., *J. Chromatog.* **3**, 576 (1960).
9. MOELWYN-HUGHES, E. A., in "The Enzymes" (Sumner, J. B., and Myrbäck, K., eds.), Vol. 1, Part 1, p. 28. Academic Press, New York, 1950.
10. REESE, E. T., SIU, R. G. H., AND LEVINSON, H. S., *J. Bacteriol.* **59**, 485 (1950).
11. SUMNER, J. B., AND SISLER, E. B., *Arch. Biochem.* **4**, 333 (1944).