Cellulose

Geoffrey Halliwell

All forms of cellulose can be described chemically as β-1→4-polyglucosides. The less complex forms are completely hydrolysed by β-1→4-polyglucosidases. An example of this type of enzyme is carboxymethylcellulase which hydrolyses carboxymethylcellulose to its constituent sugars. However, the term cellulase should only be used for a β-1→4-polyglucosidase when the particular enzyme does not hydrolyse undegraded cellulose to the extent that several micro-organisms can do (see below).

The more complex forms of cellulose, prepared with the minimum of degradative treatment, may have a degree of polymerization of up to 10000. Strong alkalis, particularly in the presence of oxygen, strong acids, and even mechanical treatment (*e.g.* grinding in a ball-mill) produce changes in the physical structure of the cellulose molecule. In extreme cases, such treatment breaks and shortens the glucose chains. The degradation products still resemble native cellulose chemically, since they consist of chains of glucose units and are insoluble in many solvents, but their degree of polymerization is much lower. β-Cellulose is a fraction of wood cellulose or degraded cotton cellulose that is soluble in 17.5% NaOH, but insoluble on neutralization. Its molecules have degrees of polymerization between 10 and 200.

Native cotton fibres contain very little non-cellulosic material and after minimal purification are the most suitable substrate for studies with cellulases. Cellulose powders and filter papers prepared from cotton are often contaminated with small amounts of non-cellulosic material. For example, xylose has been isolated after the degradation of cellulose powder (Alphacel) by rumen organisms[1], and short chain polysaccharides of mixed composition have been extracted with water from filter paper, cotton linters and also from purified wood α-cellulose[2]. Wood cellulose contains even more impurities which tend to increase in amount during attempts at purification. Such forms of cellulose are therefore more likely to be attacked by micro-organisms or mixed enzyme preparations than is the native, undegraded cellulose of cotton fibres.

Truly cellulolytic micro-organisms hydrolyse all forms of cellulose, including native undegraded forms, to give soluble products. One of the best sources of cellulase are the bacteria from the rumen of sheep, which can hydrolyse 70 to 95% (as measured by loss in weight) of 50 mg. of each of the following substrates in 66 hours[3]: cotton fibres, cellulose powder (Whatman), phosphoric acid swollen cellulose powder and hydrocellulose (formed by the action of concentrated HCl on cotton wool).

Much of the early work on cellulases is of limited value due to the use of extracts which only hydrolysed undegraded cellulose to a slight extent. Other reasons are: prolonged incubation with the risk of contamination, the use of enzyme preparations which were not always cell-free and the lack of adequate controls. Whilst sheep rumen micro-organisms can bring about a significant hydrolysis of undegraded cellulose, for example, cotton fibres, similar hydrolysis does not occur with cell-free extracts. If extensive hydrolysis is eventually obtained with cell-free extracts the process may well be found to occur in stages, perhaps involving earlier steps that do not render cellulose soluble. If the enzymatic hydrolysis is small (less than 10% hydrolysis or loss of a substrate, which has been prepared by degradative treatment) it can generally be assumed that cellulase is not concerned, but that the enzymatic effect is due to hydrolysis of the shorter cellulose chains or the non-glucose polymers present as impurities (see[1,2]) by a glucosidase or β-1→4-polyglucosidase.

As a result of the stability of undegraded cellulose, for example, native cotton fibres, towards enzymes there is no standard method for the enzymatic determination of this type of substrate. However,

---

[1] *W. D. Kitts* and *L. A. Underkofler*, J. agric. Food Chem. *2*, 639 [1954].
[2] *G. W. Huffman, P. A. Rabers, D. R. Spriesterbach* and *F. Smith*, Nature [London] *175*, 990 [1955].
[3] *G. Halliwell*, J. gen. Microbiol. *17*, 166 [1957].

degraded forms of cellulose are rapidly and completely hydrolysed by enzymes. If the cellulose is treated with phosphoric acid, it swells and is transformed into a more reactive form without any great change in the degree of polymerization[4]. Swollen cellulose or swollen cotton fibres are rendered 97% soluble in 22 hours by the action of cell-free culture filtrates from *Myrothecium verrucaria*[5,6]. The extent of the enzymatic hydrolysis is best determined by the loss of weight of the insoluble substrate. Methods which depend upon determination of the reducing sugar involve assumptions about the mode of action of cellulase and the nature of the product. Loss of weight of cellulose after enzymatic hydrolysis can be determined colorimetrically by either of two methods:

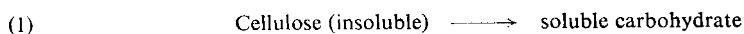
1. Directly[5]: the insoluble cellulose is filtered off (sintered glass filter), washed and determined quantitatively after oxidation with potassium dichromate.
2. Indirectly[7]: from the amount of soluble carbohydrate formed in the aqueous phase.

The results obtained with the two methods should agree. The second method is more rapid if interfering compounds are absent.

Method 1

Principle

Cellulase catalyses the reaction:



The enzyme is allowed to act on the substrate, the reaction mixture is filtered, the residue is oxidized with dichromate-H_2SO_4 reagent and the colour is measured. The difference between the experimental and control tubes represents the amount of cellulose hydrolysed by the enzyme[5]. The range of the method is from 0.2 to 1.2 mg. The lower limit is controlled by the accuracy with which weighings can be made on a microbalance. Swollen cellulose powder or swollen cotton fibres are hydrolysed to about 97% in 22 hours with the method described below.

Reagents

1. Potassium dichromate, $K_2Cr_2O_7$, A. R.
2. Sulphuric acid, 98% (w/v), M. A. R.
3. Sodium sulphite, $Na_2SO_3 \cdot 7\,H_2O$, A. R.
4. Glucose, A. R.
5. Acetic acid, A. R.
6. Sodium acetate $\cdot$ 3 H_2O, A. R.
7. Cellulase
   cell-free culture filtrate of *Myrothecium verrucaria*, for preparation, see Appendix, p. 71.

Purity of the enzyme preparation

Cellulase preparations usually contain enzyme contaminants, which hydrolyse other carbohydrates, such as soluble degradation products of cellulose (cellulose dextrins), soluble derivatives of cellulose (*e.g.* carboxymethylcellulose), cellobiose and glucosides. However, these contaminants do not interfere if the insoluble, residual material is determined.

[4] *C. S. Walseth*, Techn. Papers Pulp Paper Ind. *35*, 228 [1952].
[5] *G. Halliwell*, Biochem. J. *68*, 605 [1958].
[6] *G. Halliwell*, Biochem. J. *79*, 185 [1961].
[7] *G. Halliwell*, Biochem. J. *74*, 457 [1960].

Apparatus

1. Sintered glass filter sticks (1 cm. diameter, porosity 3 for cellulose powder, porosity 2 for cotton fibres).
2. Filtration apparatus. For the equipment and technique, see[8], but make glass to glass connections with polyethylene tubing.

Preparation of Solutions (for 500 determinations)

I. Potassium dichromate (*ca.* 0.5% w/v):

Dissolve 5 g. $K_2Cr_2O_7$ in 20 ml. hot distilled water, allow to cool and dilute to 1000 ml. with 98% H_2SO_4. Store in a glass-stoppered flask protected from dust.

II. Sodium sulphite (*ca.* 20% w/v):

Dissolve 2 g. $Na_2SO_3 \cdot 7\,H_2O$ in 10 ml. distilled water. Store in a closed vessel with a high ratio of volume to surface area. Store at 2°C and, if necessary, prepare freshly every two weeks. Check the suitability of the solution as follows: add 0.2 ml. of this solution to 2 ml. solution I and 10.9 ml. distilled water. The mixture must have the same optical density at 430 mμ as mixtures of the same composition prepared with fresh solutions.

III. Glucose standard solution:

a) Stock solution (3.5% w/v):
Dissolve 3.5 g. glucose in distilled water and make up to 100 ml. Store the solution at 2°C.

b) Dilute solution (0.175% w/v):
Dilute solution a) 20-fold with distilled water.

IV. Acetate buffer (0.2 M; pH 5.5):

Mix 87.5 ml. 0.2 M sodium acetate solution (2.72 g. Na acetate·3 H_2O/100 ml.) with 12.5 ml. 0.2 M acetic acid (dilute 1.2 g. = 1.14 ml. acetic acid with distilled water to 100 ml.) and check the pH.

V. Cellulase

Use the cell-free culture filtrate prepared according to p. 71 undiluted.

Stability of the solutions

The potassium dichromate solution keeps for about three months at room temperature (20° C). Store the enzyme preparation at 2° C. In the absence of substrate the enzyme preparation loses 11% of its activity against swollen cellulose powder in 1 hour at 20° C[6].

Procedure

Experimental material

Native cotton fibres consist of about 94% cellulose. Other cellulose preparations contain more impurities, including other polysaccharides (hemicelluloses and pectins), lignin, lipids, proteins, pigments and minerals. These impurities must be removed before the enzymatic determination of cellulose as follows:

---

[8] *G. Halliwell*, Analytic. Chem. *22*, 1184 [1950]; in *K. Paech* and *M. V. Tracey:* Modern Methods of Plant Analysis. Springer-Verlag, Berlin 1955, Vol. 2, p. 497.

Dewax cotton fibres with ethanol and ether and finally boil with 1% NaOH under nitrogen for 6 hours[9–11]. Remove lipids from other plant material with ethanol and ether[12], pectins with hot water or 0.5% ammonium oxalate solution[13] and most of the lignin with 1% $NaClO_2$ solution-0.05 N acetic acid[12]. Treat the product, holocellulose, with 5%, then with 24% KOH to extract the hemicellulose[12]. Stir the purified cellulose preparation in a 20-fold volume of 90% phosphoric acid for 2 hours at 1° C (to cause swelling to occur). Filter the mixture, wash the residue with water, with 1% Na_2CO_3 solution and then again with water until the material is alkali and acid-free[14]. Analyse the wet residue. Store in water until ready for assay. Determine the dry weight on a sample which has been dried for 4 hours or overnight at 105° C.

Enzymatic reaction

Prepare 25 ml. test tubes (1.9 cm. diameter, 12.5 cm. long) as follows:

*Experimental**)	*Control*
0.2—1.2 mg. swollen cellulose	0.2—1.2 mg. swollen cellulose
1.3 ml. buffer (solution IV)	1.3 ml. buffer (solution IV)
1.7 ml. distilled water	2.7 ml. distilled water
1.0 ml. cellulase solution (V)**)	

Incubate the tubes in a 37° C water bath for 22 hours and shake occasionally. Then filter the contents of the tubes through filter sticks. Wash the residues with a total volume of 20 ml. distilled water, suck the water gently through the filter sticks. Suck the residue and the walls of the test tubes dry by using maximum vacuum.

Colour reaction

Wavelength: 430 mμ.

Add to the *experimental* and *control* tubes and to two empty test tubes (*blank* tubes):

0.9 ml. distilled water

2.0 ml. dichromate solution (I).

Rinse the sintered glass discs of the filter sticks (*experimental* and *control* tubes) several times with the mixture (suck through with slight vacuum and expel with compressed air) and leave the filter sticks in the tubes. Stopper the tubes with glass marbles (do not rest on the filter stick), heat for $1^1/_2$ hours in a boiling water bath and then cool for 5 min.

Add to the *experimental* and *control* tubes

3.0 ml. distilled water

---

*) Instead of adding all the enzyme at one time, it can be added in portions over the incubation period. In this way maximum hydrolysis of the substrate is reached sooner.

**) With less active preparations use less distilled water and more enzyme solution.

[9] Comittee of the Division of Cellulose Chemistry of the American Chemical Society, Ind. Engng. Chem., analytic. Edit. *15*, 748 [1923].

[10] *A. B. Corey* and *H. Le B. Gray*, Ind. Engng. Chem., analyt. Edit. *16*, 853, 1130 [1924].

[11] *R. K. Worner* and *R. T. Mease*, J. Res. nat. Bur. Stand. *21*, 609 [1938].

[12] *E. Wise, M. Murphy* and *A. A. Addieco*, Paper Trade J. *122*, No. 2, 35 [1946].

[13] *R. L. Whistler A. R. Martin* and *M. Harris*, J. Res. nat. Bur. Stand. *24*, 555 [1940].

[14] *C. S. Walseth*, Tech. Papers Pulp Paper Ind. *35*, 228 [1952].

and mix. Allow the solutions to cool and rapidly suck them through the filter sticks into clean, ground-glass stoppered test tubes (for apparatus, see[8]). Suck two

1.0 ml. portions of distilled water

through each filter stick. Continue to wash the old test tube and the filter stick with a further

5.0 ml. distilled water.

Stopper the tubes and mix the contents (filtrate + washings).

To each of the *blank* tubes add

10.0 ml. distilled water,

mix, and to one of the *blank* tubes add

0.2 ml. Na_2SO_3 solution (II)

and remix.

Measure the optical densities of all the tubes at 430 mμ (Ilford violet filter No. 601) against the *blank* tube containing Na_2SO_3.

Standard curve

Pipette into ground-glass stoppered test tubes:

0.057 to 0.8 ml. glucose standard solution (IIIb) (corresponding to 0.1 mg. to 1.4 mg. glucose)

distilled water to 0.9 ml.

In addition prepare two test tubes (blank tubes) with

0.9 ml. distilled water.

Pipette into all the tubes

2.0 ml. dichromate solution (I),

mix carefully, stopper the tubes and heat in a boiling water bath for 30 min. Cool in water, add

10.0 ml. distilled water

to each tube, stopper and mix.

To one of the blank tubes add

0.2 ml. Na_2SO_3 solution (II),

and mix. Read the optical densities of all the solutions at 430 mμ against the blank tube containing sulphite. Plot the optical densities (ordinate) against the mg. glucose/tube (abscissa) (standard curve).

Calculations

Calculate the difference in optical density between the experimental and control tubes. Obtain the amount of glucose corresponding to this value from the standard curve and multiply by 0.9 to obtain the mg. cellulose/tube.

Sources of Error

After the enzymatic reaction the washing procedure removes soluble carbohydrates and other compounds that could interfere in the colour reaction. Interfering compounds include the enzyme and, if present, certain buffer solutions such as those containing citrate, which are oxidized by the dichromate reagent. If a culture filtrate of *M. verrucaria* is used as the source of cellulase, the amount of enzyme

protein required for the complete hydrolysis of the sample is negligible in comparison to the amount of cellulose taken. Acetate and phosphate buffers do not interfere in the colour reaction.

Specificity

Cell-free culture filtrates with cellulase activity have not yet been separated into individual enzymes with different substrate specificity. Such filtrates frequently hydrolyse many substrates, for example, degraded cellulose, carboxymethylcellulose, cellulose dextrins, glycosides, cellibiose and hemicelluloses. In the method described here, in which only insoluble cellulose is determined, the presence of enzymes other than cellulase does not appear to be detrimental. On the contrary, these enzymes may promote the subsequent breakdown of the initial products of cellulolysis.

Method 2

Principle

The principle of this method is the same as that for Method 1 except that the soluble carbohydrates formed on enzymatic hydrolysis are determined instead of the insoluble residue[7]. The procedure is more rapid and is applicable when it is possible to use acetate, phosphate or a similar buffer (but not citrate buffer*)), and when compounds which interfere in the dichromate oxidation, such as proteins, are absent or are only present in small amounts (*e.g.* the enzyme protein). The method allows the determination of 0.4 to 3 mg. swollen cellulose (powder or fibres) corresponding to 0.1 to 0.7 mg. soluble carbohydrate/0.9 ml. filtrate.

Reagents

See Method 1, p. 65.

Preparation of Solutions (for 500 determinations)

See Method 1, p. 66, but:

I. Potassium dichromate (*ca.* 0.25% w/v):
Dissolve 2.5 g. $K_2Cr_2O_7$ in 20 ml. hot water, cool and dilute to 1000 ml. with 98% H_2SO_4. Store in a stoppered flask protected from dust. The solution keeps for at least 3 months at room temperature (20° C).

II. Glucose standard solution:
c) Dilute solution (0.0875% w/v):
Dilute stock solution (IIIa from p. 66) 40-fold with distilled water.

Procedure

Experimental material

See Method 1, p. 66.

Enzymatic reaction

Prepare 15 ml. centrifuge tubes (1.6 cm. diameter, 11 cm. long; round-bottom) as follows:

	Experimental	*Control*	
		without enzyme	*without substrate*
swollen cellulose	0.4 to 3 mg.	0.4 to 0.3 mg.	—
buffer (solution IV)	1.3 ml.	1.3 ml.	1.3 ml.
distilled water	1.7 ml.	2.7 ml.	1.7 ml.
enzyme solution (V)	1.0 ml.	—	1.0 ml.

*) Citrate interferes in the dichromate oxidation.

Incubate all the tubes in a 37°C water bath for 22 hours and shake occasionally. Centrifuge for 3 min. at 1 800 g and room temperature. If neccessary, filter the supernatant through a small sintered glass filter (porosity 3).

Colour reaction

Wavelength: 430 mμ

Pipette into ground-glass stoppered test tubes

0.9 ml. supernatant or filtrate (corresponding to 0.1 – 0.7 mg. soluble carbohydrate)

from the *experimental* and *control* tubes. In addition pipette into two test tubes (*blank* tubes)

0.9 ml. distilled water.

Add to all the tubes

2.0 ml. dichromate solution (I from p. 66),

stopper the tubes and heat in a boiling water bath for 20 min. Cool in water, add to each tube

6.0 ml. distilled water,

mix and reduce one of the *blank* tubes with

0.1 ml. sulphite solution (II).

Read the optical densities of all the tubes at 430 mμ (Ilford violet filter No. 601) against the *blank* containing sulphite.

Standard curve

Pipette into ground-glass stoppered test tubes:

0.12 to 0.8 ml. glucose standard solution (IIIc) (corresponding to 0.1 to 0.7 mg. glucose)

distilled water to 0.9 ml.

In addition prepare two test tubes (blank tubes) with

0.9 ml. distilled water.

Pipette into all the tubes

2.0 ml. dichromate solution (I from p. 66),

mix, stopper the tubes and heat for 20 min. in a boiling water bath. Cool in water, add

6.0 ml. distilled water

to each tube, stopper the tubes and mix carefully. Reduce one of the blanks by the addition of

0.1 ml. sulphite solution (II).

Read the optical densities of all the solutions at 430 mμ against the reduced blank. Plot the optical densities (ordinate) against the mg. glucose/tube (abscissa) (standard curve).

Calculations

Read off from the standard curve the glucose values ($G_{sam.}$, G_{C1} and G_{C2}) corresponding to the optical densities of the experimental and the two control tubes. Add together the glucose content of the control tubes and subtract the sum from the glucose content of the experimental tube. Multiply the difference by 0.9 to obtain the mg. cellulose/tube (colour reaction):

$[G_{sam.} - (G_{C1} + G_{C2})] \times 0.9$ = mg. cellulose/colour reaction mixture.

To obtain the mg. cellulose in the enzymatic reaction mixture multiply by 4 instead of 0.9:

$[G_{sam.} - (G_{C1} + G_{C2})] \times 0.9 \times \frac{4}{0.9} = [G_{sam.} - (G_{C1} + G_{C2})] \times 4$ = mg. cellulose/enzymatic reaction mixture.

Sources of Error

Compounds which are oxidized by dichromate interfere with the determination. If the cellulose preparation to be analysed contains such compounds and they are soluble, method 1 must be used. An advantage of method a, in contrast to methods dependent on the determination of reducing sugar, is that it enables a correction to be made for the fraction of the cellulose preparation which consists of small molecules (reducing or non-reducing) and which is soluble in the assay mixture in the absence of the enzyme.

Appendix

Isolation of the cellulase preparation

Grow *Myrothecium verrucaria* (strain I. M. I. 45541, Commonwealth Mycological Institute, Kew, Surrey, England) in 50 ml. Erlenmeyer flasks[6] containing 50 mg. cellulose powder (Whatman) in 10 ml. salt solution *) or 50 mg. dewaxed cotton fibres in 4.5 ml. salt solution *). Inoculate the solutions with 1 ml. of a thick suspension of spores which have been obtained from cultures of *M. verrucaria* on filter paper strips on agar slopes. Shake the flasks horizontally **) (frequency of 120 cycles and an amplitude of 5 cm. for cellulose powder, 60 cycles and an amplitude of 3.8 cm. for cotton fibres). Allow the cultures to grow for 5 to 10 days at 28 to 30° C. At the end of the incubation, filter the culture through a coarse sintered-glass filter and centrifuge the filtrate for 30 min. at 54000 g and 2° C. Check the activity of a portion of the culture filtrate by one of the methods described above and, if necessary, dilute with distilled water so that 1 ml. of the solution contains sufficient enzyme to completely hydrolyse the highest amount of cellulose stated in the method within the given time. If the culture filtrate is not sufficiently active, see the footnote **) below.

---

*) The salt solution[15] (pH 6.6) contains in 1000 ml.: 0.2 g. KH_2PO_4; 0.15 g. K_2HPO_4; 2.0 g. $NaH_2PO_4 \cdot H_2O$; 1.5 g. Na_2HPO_4; 0.6 g. NH_4NO_3; 3.8 g. $NaNO_3$; 0.3 g. $MgSO_4 \cdot 7\,H_2O$; 0.05 mg. $ZnSO_4 \cdot 7\,H_2O$; 0.054 mg. $Fe_2(SO_4)_3 \cdot 6\,H_2O$; 0.0025 mg. $CuSO_4 \cdot 5\,H_2O$; 0.0055 mg. $MnSO_4$; 0.057 mg. H_3BO_3; 0.024 mg. ammonium phosphomolybdate.

**) If the cells cannot be shaken, grow them in 250 ml. Erlenmeyer flasks containing 0.5 g. cotton fibres and 25 ml. salt solution. Cultures which have been shaken (*i.e.* aerated) hydrolyse cellulose faster than ones which have not.

15) *P. R. Saunders*, *R. G. H. Siu* and *R. N. Genest*, J. biol. Chemistry *174*, 697 [1948].