Jansen, E. F., Jang, R., Bonner, J., *Food Res.* **25,** 64 (1960).
Kew, T. J., Veldhuis, M. K., *Proc. Fla. State Hort. Soc.* **73,** 293 (1960).
Kew, T. J., Veldhuis, M. K., to U.S. Gov't., U. S. Patent 2,995,448 (Aug 8, 1961).
MacDonnell, L. R., Jansen, E. F., Lineweaver, H., *Arch. Biochem.* **6,** 389 (1945).
Mizrahi, S., Berk, Z., *J. Sci. Food Agr.* **21,** 250 (1970).
Pilnik, W., Voragen, A. G. J., "The Biochemistry of Fruits and Their Products," Academic Press, New York, N. Y., 1970, pp 53–87.
Rouse, A. H., Atkins, C. D., *Fla. Agr. Exp. Sta. Bull.* 570 (1955).
Scott, W. C., Kew, T. J., Veldhuis, M. K., *J. Food Sci.* **30,** 833 (1965).
Senn, V. J., Murray, M., O'Connor, R. T., U. S. Dept. of Agriculture, ARS-72-8 (1955).
Vas, K., Nedbalek, M., Scheffer, H., Kovacs-Proszt, G., *Fruchtsaft-Ind.* **12,** 164 (1967).

Received for review May 10, 1972. Accepted August 8, 1972. Mention of brand names is for identification only and does not imply recommendation by the U. S. Department of Agriculture.

Hydrolysis of Wood and Cellulose with Cellulytic Enzymes

Wayne E. Moore,* Marilyn J. Effland, and Merrill A. Millett

A procedure is described for determining the digestibility of cellulose, wood, modified cellulose, and modified wood with cellulytic enzymes. It is useful as a screening test to evaluate the effects of various physical and chemical treatments to improve the accessibility of cellulose carbohydrates to ruminants. Although not economical at the present time, fine grinding was the most effective treatment for increasing the digestibility of wood and cellulose. Treatment of aspen (*Populus tremuloides* Michx.) from pulpwood logs that contained mostly sapwood with dilute alkali increased the digestibility from 10% for the untreated wood to 50% for the treated wood. Treatment with liquid ammonia increased the digestibility to 36%. Reasons for the differences in digestibility found are discussed.

The carbohydrate content of wood varies between 70 and 80%. Considering the large quantities of wood available from low-grade forests, residues from logging operations, sawdust, shavings, and mill wastes, it is very tempting to develop means for treating wood so as to make the carbohydrate accessible to ruminants (Scott *et al.*, 1969). For such work, a screening test was needed to evaluate the effects of various treatments on the digestibility of wood. Such a procedure is described and is applied to substrates that have been treated by various physical and chemical means. It is based on measuring the hydrolysis of the polysaccharides by a cellulolytic enzyme.

MATERIALS AND METHODS

Preliminary work with several enzymes showed "Onazuka" SS (Kanematsu New York, Inc., New York, N. Y. 10004; 1500 μ/g of activity based on manufacturer's values for decomposition activity on filter paper) to be the most active of the commercially available cellulases tried. Therefore, it was selected for further work. This enzyme is derived from *Trichoderma viride* and contains appreciable quantities of hemicellulases. The powdered enzyme contains lactose, galactose, glucose, and arabinose amounting to 50% reducing sugar calculated as glucose. The principal sugar is lactose.

The enzyme solution was buffered at pH 4.6 with acetate buffer. It was prepared as follows.

Acetate Buffer. Dissolve 68 g of $NaC_2H_3O_2$–$3H_2O$ in about 500 ml of H_2O; add 29 ml of glacial acetic acid and dilute to 1 l.

Enzyme. Stir 25 g of powdered enzyme into a mixture of 900 ml of H_2O and 100 ml of acetate buffer for 1 hr. Filter off insoluble material.

The enzyme solution was stored in a refrigerator at 40°F. The solution was reasonably stable; a solution stored for 5 weeks had the same activity as a freshly prepared solution.

Substrates. The following substrates were used: a high-purity cotton linters pulp from Buckeye Cellulose Corp., equivalent to ICCA Pulp No. 1 of the International Committee for Cellulose Analysis; aspen (*Populus tremuloides*); a high α rayon grade softwood sulfite pulp designated ICCA Pulp No. 4; alfalfa (a reference feeding sample for ruminants, obtained from University of Wisconsin); cotton linters and aspen ground in a vibratory ball mill for 30 min (National Bureau of Standards, 1950; Pew and Weyna, 1962); cotton linters and aspen treated with liquid ammonia for 1 hr at 30° at approximately 150 psi (the samples were then air dried); cotton linters and aspen treated with dilute alkali for 1 hr at 30°. Five-gram samples were ground to pass a 40-mesh sieve and treated with 100 ml of 1% NaOH. The treated wood was then thoroughly washed and air dried.

All samples except those ground in the vibratory mill were ground in a Wiley mill to pass a No. 40 U.S. standard sieve. Since it was reported that high concentrations of sugars inhibit cellulolytic enzymes (Katz and Reese, 1968), 0.2-g samples were used in 6 ml of enzyme solution for the digestion.

An incubator was constructed from a forced draft oven whose temperature could be controlled within ±0.2° from 30 to 50°. Rotating racks were provided to hold vials of 8-ml capacity (17 mm diameter by 60 mm long) equipped with Teflon-lined screw caps. To provide agitation and to prevent packing or sticking of the ground sample during the digestion, the sample tubes were rotated end-over-end at 7 rpm.

The effectiveness of the enzyme system was evaluated by determining the loss in weight after digestion and by measuring the total sugars, as glucose, produced in the liquor.

The FPL method (American Society for Testing and Materials, 1970; Saeman *et al.*, 1954) for chromatographic analysis was used where the papers are irrigated with a butanol-

Forest Products Laboratory, Forest Service, U. S. Department of Agriculture, Madison, Wisconsin 53705.

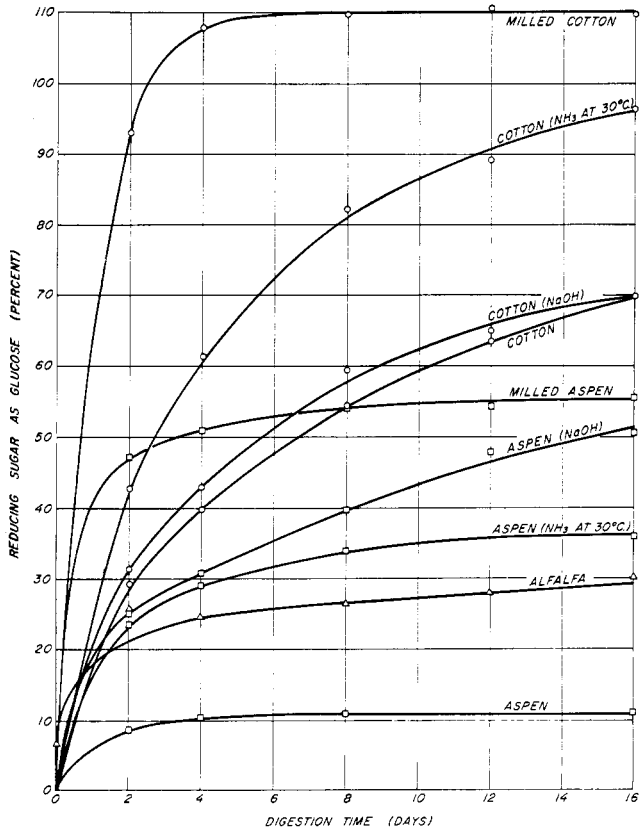

Figure 1. Enzyme digestion of wood and cellulose treated in various ways. Reaction rates with "Onazuka" SS cellulase, 1500 μ/g activity at 40°. Yields of total sugars with increasing digestion times

pyridine–water mixture, 10:3:3 by volume. The sugar spots are located by spraying with aniline phthalate.

Procedure for Enzyme Digestion. Determine moisture in sample.

Analyses are run in triplicate. Weigh into vials about 0.2 g of air-dry samples. Warm enzyme solution to 40°. Add 6 ml of enzyme solution to sample vials and to vials which are to be controls. Place vials in incubator at 40° and rotate for the time intervals selected. At the various time intervals, remove vials from the incubator and place in an ice–water bath to slow or stop the reaction.

Centrifuge by placing vials in protector cups which contain $^1/_2$-in.-diameter wood dowels to support the vials. (In this work, an International centrifuge, head No. 823, cup No. 302, was used at a speed of 1750 rpm for 15 min.)

Dilute a 1-ml aliquot of the supernatant liquor for reducing sugar determinations (Shaffer and Somogyi, 1933; Somogyi, 1952). After applying corrections for sugars in the controls (digestion with enzyme and no carbohydrate), calculate percent of sugar produced, based on oven-dry weight of sample.

Determine the loss in weight of the sample by washing the residue into a No. 2 Gooch crucible fitted with an asbestos mat. The sample is washed with 200 ml of water in small portions. The air-dried crucibles are dried in a vacuum oven at 60° and 2 cm pressure for 4 hr. Percent loss in weight is calculated from the original oven-dry weight of sample and weight after digestion. Corrections for solubility of the samples in the buffered solution containing no enzyme were found to be negligible for the samples in Figure 1. For samples that were degraded during pretreatment, this correction should be applied.

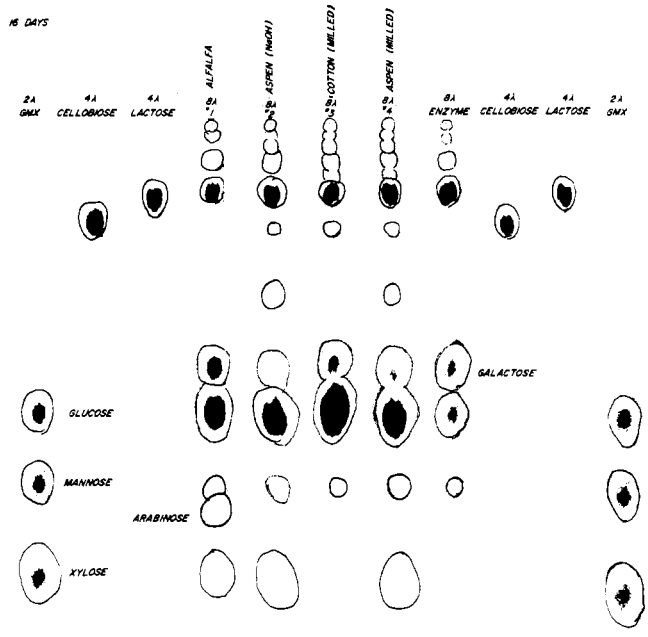

Figure 2. Paper chromatogram for enzyme digestion of samples used in Figure 1. Liquors from samples and from enzyme controls were spotted at loadings of 4 and 8 λ. GMX is a known mixture of glucose, mannose, and xylose. Cellobiose and lactose are known sugar solutions

Table I. Comparison of Total Sugars Produced and Loss in Weight during Digestion of Aspen and Cotton with "Onazuka" SS Cellulase for 96 Hr at 40°

Loss in weight, %	Total sugars calculated as anhydroglucose, %
Cotton linters	
37.04	34.60
36.70	35.81
36.92	35.98
Avg 36.89	35.46
Aspen	
9.90	8.44
9.85	8.77
9.92	8.81
Avg 9.89	8.67

RESULTS

The reducing sugar content of the controls increased from a value of 48% at the start of the incubation to 60% after 16 days at 40°. These increases are due to the hydrolysis of lactose which the enzyme preparation contains. An examination of chromatograms such as that in Figure 2 revealed a decrease in lactose and an increase in galactose and glucose in the enzyme controls for the 16-day digestion.

Losses in weight during digestion agree with results for reducing sugars produced. In cellulose, the glucose units exist as anhydro units. During hydrolysis, each unit reacts with a molecule of water to produce glucose. In Table I, the sugar results are calculated as anhydroglucose by multiplying by the factor 162/180. Replicability is good. The amount of hydrolysis by the "loss of weight" procedure is about one percentage unit higher than by the "total sugars" procedure. This comparison holds for a number of samples. The latter procedure, because of its simplicity, was adopted for further work.

The enzyme hydrolysis of a number of untreated and treated substrates is given in Figure 1. The ordinate is expressed as

percent sugar (calculated as glucose). Since 100 g of cellulose (cotton) reacts with 11 g of H_2O to form glucose, the maximum theoretical yield of 111% is approached in the digestion of the milled cotton. Alfalfa was included as a representative forage sample.

Paper chromatograms of the sugar products from the enzyme digestions were prepared at each time interval. From the supernatant liquor, 4 and 8 λ loadings were spotted on paper, along with a mixture of glucose, mannose, and xylose (GMX), and the single sugars cellobiose and lactose. A representative chromatogram is shown in Figure 2. The high content of lactose in the enzyme preparation is immediately apparent. The relative amounts of glucose formed enzymatically from each substrate are also noted.

DISCUSSION

Vibratory ball milling is a very effective way to improve the digestibility of a cellulosic material. This was first demonstrated by Pew and Weyna (1962).

X-Ray studies at our laboratory (Caulfield and Steffes, 1969) have shown that cotton, ball milled for 30 min, is less than 7% crystalline. On wetting with water, however, 50% of the initial crystallinity of the cellulose is recovered. A reduced crystallinity and increased specific surface area make the cellulose increasingly accessible to the enzyme. At the present time, however, vibratory milling is not an economical process.

Although dilute alkali has little effect on the digestibility of cotton, it has a marked effect on the digestibility of aspen. Subsequent work has shown that the dilute alkali treatment causes a marked increase in swelling capacity in water for aspen, but has little effect on the swelling of cotton (Tarkow and Feist, 1969). For example, the fiber saturation point of aspen treated with 1.5% NaOH at room temperature increased from 52 to 100%, whereas cotton was essentially unchanged.

The treatment of aspen and cotton with liquid ammonia also raises the digestibility of the parent material. To a degree, this is due to a transformation of the cellulosic structure to cellulose III (Barry *et al.*, 1936; Legrand, 1951); however, the treatment also increases the subsequent swelling in water (Tarkow and Feist, 1969).

The very low disgestibility of untreated aspen is typical of unmodified wood.

In the chromatograms for the cotton and aspen, small amounts of cellobiose are evident. The amounts of cellobiose decrease with increased time of digestion. The amounts were estimated to be small enough to cause little error in the determination of digestibility by reducing sugar analysis.

As expected, the principal sugar product was glucose.

In the aspen samples, undetermined spots appeared near the starting line. These are believed to be incompletely hydrolyzed oligosaccharides. The spots near the origin stained pink when sprayed with aniline phthalate. This indicates that the unhydrolyzed polymers contained xylose. In the solvent system used, uronic acids remain at the origin. For the aspen samples, small unidentified spots occur between the cellobiose and galactose spots. The chromatogram of the 2-day aspen digestion showed no xylose spots, in spite of a high level of xylose in aspen. The 16-day digestions showed xylose spots. A comparison of the intensity of these xylose spots with spots from acid-hydrolyzed aspen indicates that the enzyme system hydrolyzes the glucan groups more readily than it does the xylan fraction.

It should be pointed out that some treatments for improving digestibility may degrade the cellulose to nonsugars. For example, in the case of samples irradiated with high-velocity electrons, differences between "loss in weight" and "reducing sugars" may be as high as 50%. The degraded material contains some solubles which are not analyzed as sugars. An estimate of the amount of degradation of carbohydrates that has occurred during irradiation may be obtained from a determination of sugars after acid hydrolysis (ASTM, 1970; Saeman *et al.*, 1954) on the original sample, on the residue after digestion, and on the liquors produced. Because there may be losses in degraded wood due to increased solubility of nonsugars during *in vitro* rumen digestion (Mellenberger *et al.*, 1970; Millett *et al.*, 1970), results by this method should be corrected for solubility in solutions containing no rumen fluid for comparison with the described enzyme hydrolysis.

The procedure described here clearly evaluates the effects of various physical and chemical treatments on the digestibility of cellulosic materials.

LITERATURE CITED

American Society for Testing and Materials, Standard D 1915-70, Chromatographic Analysis of Chemically Refined Cellulose (1970).
Barry, A. J., Peterson, F. C., King, A. J., *J. Amer. Chem. Soc.* **58,** 333 (1936).
Caulfield, D. F., Steffes, R. A., *Tappi* **52,** 1361 (1969).
Katz, M., Reese, E. T., *Appl. Microbiol.* **16,** 419 (1968).
Legrand, C., *J. Poly. Sci.* **7,** 333 (1951).
Mellenberger, R. W., Satter, L. D., Millett, M. A., Baker, A. J., *J. Animal Sci.* **30**(6), 1005 (1970).
Millett, M. A., Baker, A. J., Feist, W. C., Mellenberger, R. W., Satter, L. D., *J. Anim. Sci.* **31**(4), 781 (1970).
National Bureau of Standards, Research Paper 2116, *J. Res. Nat. Bur. Stand.* **45,** 109 (1950).
Pew, J. C., Weyna, P., *Tappi* **45,** 247 (1962).
Saeman, J. F., Moore, W. E., Mitchell, R. L., Millett, M. A., *Tappi* **37,** 336 (1954).
Scott, R. W., Millett, M. A., Hajny, G. J., *Forest Prod. J.* **19,** 16 (1969).
Shaffer, P. A., Somogyi, M. J., *J. Biol. Chem.* **100,** 695 (1933).
Somogyi, M. J., *J. Biol. Chem.* **195,** 19 (1952).
Tarkow, H., Feist, W. C., *Advan. Chem. Ser.* **95,** 197 (1969).

Received for review March 13, 1972. Accepted August 10, 1972.