High-Performance Liquid Chromatography of Carbohydrates in Enzymic Hydrolysate of Waste Paper

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Key Words
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Summary
Waste paper was pretreated with 5M sulfuric acid at 28 °C to give a gel-like solution which was then hydrolyzed with cellulase, purified from *Trichoderma viride*, to form carbohydrates. The determination of the carbohydrates was accomplished using an aminopropyl-bonded silica HPLC column and an RI detector. The detection limits were 20 ppm; xylose, glucose, and cellobiose were the major components. Salts from the buffer solution may affect the activity of cellulase. The analysis of carbohydrates can be performed under acid conditions. HPLC analysis of carbohydrates from enzymatic hydrolysis is reliable and successful.

Introduction
Enzymatic hydrolysis of various biomass resources becomes more and more important in biotechnology, food science, and the fuel industry [1-3]. Generally, hydrolysis of cellulose wastes and lignocellulosic materials by cellulase would produce glucose and cellobiose [4-5]. The determination of total reducing sugar in solution is usually based on colorimetric measurements [6-8]. Recently, enzymatic methods developed for the determination of glucose have become popular [8-9]. Nevertheless, chromatographic methods including gas-liquid chromatography (GLC) and high-performance liquid chromatography (HPLC) are useful for simultaneous determination of carbohydrates and for final confirmation [10]. Liquid chromatography is most widely utilized in carbohydrate analysis because of low volatilities and derivatization is unnecessary. Therefore, an aminobonded silica column for the analysis of saccharides was first used by Palmer [11]. Subsequently, chemically bonded phases containing aminooalkyl functional groups on silica surfaces have been widely employed [12-13]. These columns are characterized as normal phase chromatography.

Previously, the carbohydrates produced from cellulose by hydrolysis of waste paper pretreated with *Trichoderma reesei* were analyzed by HPLC with UV detection [14]. In this process we can get useful products from wastes and partly help environmental protection. However, crude instrumentation made difficulties both for the fermentation of pretreated waste paper and the carbohydrate analysis. This paper is an extension of this work and reports on the carbohydrates analysis. Cellulose in waste paper is now hydrolyzed using purified cellulase from *Trichoderma viride* and a commercially available aminopropyl-bonded silica HPLC column utilized for the analysis of carbohydrates with refractive index (RI) detection.

Experimental

Materials
D(+)-glucose, D(+)-xylose, cellobiose, CaCl₂ · 2H₂O, K₂HPO₄, (NH₄)₂SO₄, H₂SO₄ and chromatography grade CH₃CN were from Merck (Darmstadt, Germany). MgSO₄ was from Wako Pure Chemical industries’ Ltd. (Osaka, Japan). Guaranteed reagent NaOH was from Shimakyu’s Pure Chemicals (Osaka, Japan). Cellulase (EC3.2.1.4) produced by *Trichoderma viride* was from Sigma (St. Louis, MO, USA). The HPLC column (Lichrophor 100 NH₂, 250 × 4 mm I.D.) was packed with a chemically-bonded aminopropyl group on silica gel (5 μm) (Merck, Darmstadt, Germany). A guard column was mounted with a manu-CART system. All water was filtered and deionized before use. Waste paper (A4-type paper used for computer printout) was collected in our laboratory.

Instrumentation
The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-9A dual-piston, solvent-delivery module...
with a high-sensitivity filter unit, a Rheodyne (Cotati, CA, USA) Model 7125 injection valve with a 20 µl sampling loop, a Shimadzu Chromatopac CR-6A data processor, a Shimadzu Model SPD-6A variable-wavelength UV detector, and a Shimadzu Model RID-6A refractive index detector. The analytical column was kept in a Shimadzu CTO-6A column oven to maintain constant temperature.

Standard Solution Preparation

Carbohydrate standard solutions were prepared separately by measuring suitable amounts of analytical-reagent grade carbohydrates and dissolving them directly in water. Successive dilutions of these stock solutions were used for lower concentrations. Reference carbohydrate standards were prepared by the same procedures.

Waste Paper Pretreatment

About 6.7 g waste paper were torn to slips and put in a 250 ml Erlenmeyer flask, 150 ml 5M H₂SO₄ were added to digest the paper. The flask was continuously shaken for 24 hours at 200 rpm. The digestion temperature was 28 °C. Whatman No. 5 filter paper was used to filter quickly the digested waste paper solution. The waste paper left on the filter paper was washed with 50 ml 1M NaOH followed by 100 ml deionized water and then transferred into a 300 ml beaker. About 150 ml of the deionized water was also added. This solution was further subjected to ultrasonic treatment (Ultrasonics, Inc., Sonicator W-385, New York, USA) for 50 minutes with output control: 7; pulser cycle time: 2 sec; and 50 % duty cycle. A gel-like solution that was suitable for enzymatic hydrolysis was produced.

Enzymatic Hydrolysis

Crude cellulase from Trichoderma viride was commercially available for hydrolyzing the cellulose in pretreated waste paper. Therefore, two aliquots of 50 ml of solution were diluted with 450 ml deionized water and buffer solution separately. The buffer solution [7] was prepared by dissolving 1.25 mg CaCl₂ · 2H₂O, 125 mg MgSO₄, 1.1 g K₂HPO₄, and 150 mg (NH₄)₂SO₄ in 500 ml deionized water. The pH of this buffer solution was 8.56. The pH of the aliquot waste paper solution with the above buffer added was 8.13. The pH of the aliquot waste paper solution mixed with deionized water was 3.55. Finally, the pH of the two aliquots was adjusted to 4.8, then 100 ml of the aliquot was taken for enzyme hydrolysis. The amount of cellulase added was 2 ml of a 15 mg ml⁻¹ solution. Hydrolysis was performed at 40 °C with constant shaking at 200 rpm for one week.

HPLC Determination of Carbohydrates

When hydrolysis was complete the solutions were filtered through Whatman No. 41 paper followed by a 0.45 µm microporous membrane (Gelman Science, Ann Arbor, MI, USA). The pH values of the hydrolysates containing either buffer solution or deionized water after filtration were 3.31 and 3.36 respectively. The filtered hydrolysates were analyzed by HPLC with RI detection. The column temperature was 29 °C. Since the mobile phase (CH₃CN/H₂O; 75 : 25 v/v) was kept at a constant isocratic elution rate of 1.0 ml min⁻¹, the column pressure was about 80 kg cm⁻² during analysis.

Results and Discussion

The suitability of the RI detector for the analysis of carbohydrates was tested in terms of sensitivity and accuracy [15]. The calibration data of carbohydrates show good linearity between 1000 ppm and 25 ppm. The detection limits of xylose, glucose, and cellobiose were all 20 ppm.

Figure 1 indicates that xylose is another major component in the hydrolysate besides glucose and cellobiose. The source of xylose should be from hemicellulose in the pretreated paper solution. Hemicellulose which is composed of D-xylose, D-mannose, and D-galactose is one of the structural polysaccharides in plants. Recently, low relative molecular mass xylanase [16] was purified from Trichoderma viride and used to cleave the specific bonds as β (1 → 4) or β (1 → 3) glycosidic

![Figure 1](image_url)