# Preparation and Utilization of Cellulose Substrates Regenerated after Treatment with Hydrochloric Acid†

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A versatile method for the preparation of amorphous, hydrochloric acid regenerated, cellulose substrates is described. The procedure involves dispersion of microcrystalline cellulose in reagent grade HCl at room temperature, cellulose dissolution via temperature reduction to −30 °C, and substrate regeneration in cold water or acetone. The substrates' degree of polymerization may be varied by altering a hydrolysis period following cellulose dissolution. The relative crystallinity of the substrates may be varied by modifying the drying techniques used following substrate regeneration. The hydrochloric acid regenerated substrates are highly susceptible to cellulase-catalyzed saccharification when compared to commercial cellulose powders. The substrates are free of the inorganic esters common to other acid-swollen/regenerated cellulose preparations. The presented dispersion–dissolution procedure is also readily applicable to the preparation of soluble cellodextrins, allowing their preparation without the utilization of fuming hydrochloric acid.

## INTRODUCTION

Cellulase enzyme complexes and their component enzymes are of interest due to their role in the ecology of natural biosystems and their potential use in the industrial processing of renewable biomass to fuels and chemical feedstocks. Cellulase preparations are often characterized by noting their hydrolytic activity on several structurally unique cellulose substrates (Beldman et al., 1985). Cellulases from different microbial sources (Mackenzie et al., 1987), as well as enzyme preparations from the same microbe cultured under different conditions (Labudova and Farkas, 1983), may markedly differ in their relative activities toward different substrates. The relative activity of a particular enzyme preparation with respect to different substrates provides an indication of the enzyme's potential as a catalyst for the saccharification of different cellulosic substrates. Structurally distinct substrates are also an essential component of studies analyzing the physical properties of cellulose which dictate cellulase/cellulose interactions and the kinetics of saccharification (Sinitsyn et al., 1989). The substrates commonly included in such studies include a relatively crystalline, inert, water-soluble cellulose, a relatively amorphous, more reactive, water-insoluble cellulose, and a chemically modified water-soluble cellulose (Wood and Bhat, 1988).

The relatively inert, insoluble substrates, such as microcrystalline or powdered cellulose, are generally of wood origin and available commercially. The soluble, chemically modified substrates, such as (carboxymethyl)cellulose, are also available commercially. In contrast, the more amorphous, relatively reactive, insoluble cellulose substrates are most often prepared in the investigator's laboratory by mechanical or chemical treatment of wood or cotton-derived cellulose powders (Wood, 1988). Chemical methods designed to increase the enzymatic susceptibility of cellulose are based on disruption of the fiber's intermolecular hydrogen bonding. In these procedures, the cellulose is first swollen or dissolved in an appropriate solvent and subsequently regenerated by precipitation. The chemical and physical properties of the regenerated celluloses are dependent on the methods used for their preparation (Turbak et al., 1980).

This study describes a simple method for the preparation of regenerated cellulose substrates from hydrochloric acid. The simple dissolution process presented may be used for the preparation of either relatively reactive, insoluble cellulose substrates or soluble cellodextrins. The insoluble hydrochloric acid regenerated (HCR) substrates provide an acid-regenerated cellulose free of the inorganic esters present in currently used substrates regenerated from phosphoric (Wood, 1988) or sulfuric (Sasaki et al., 1979) acid.

## MATERIALS AND METHODS

**Materials.** The microcrystalline cellulose starting material, Avicel PH101, was a gift from FMC Corp., Princeton, NJ. Hydrochloric acid, reagent grade, and standardized NaOH solution were from Fisher Scientific, Pittsburgh, PA. Glucose oxidase was from Sigma Chemical Co., St. Louis, MO, *Trichoderma viride* cellulase (Cellulysin) from Calbiochem Corp., La Jolla, CA, and cupriethylenediamine from Olin, Pisgah Forest, NC.

**Substrate Preparation.** A schematic of the preparation procedure is presented in Figure 1. Eight grams of cellulose is initially dispersed with constant stirring in 100 mL of 37.5% HCl at 23 °C. The cellulose suspension resulting from the above dispersion is then cooled to between −25 and −35 °C. During cellulose dissolution the temperature of the cellulose–HCl suspension was lowered relatively slowly by placing the suspension in a −37 °C freezer, which approximates a first-order cooling rate constant of −0.047 $min^{-1}$. The dissolved cellulose is regenerated directly, or it is transferred to a 25 °C water bath for a prescribed hydrolysis period prior to regeneration. Regeneration of substrate is accomplished by dilution of the cellulose solution with ice-cold (1 °C) water or 4 °C acetone while vigorous agitation is maintained. A common blender was used for agitation of diluent during regeneration and particle size breakdown. Washing of regenerated samples was accomplished by repeated suspension and filtration. Neutralization was done by the addition of 5 M NaOH. Solvent drying was accomplished by refluxing the regenerated substrate for 30 min in the appropriate solvent followed by filtration. The procedure was repeated with solvents as indicated in the text. Oven-dried samples were dried in a vacuum oven at

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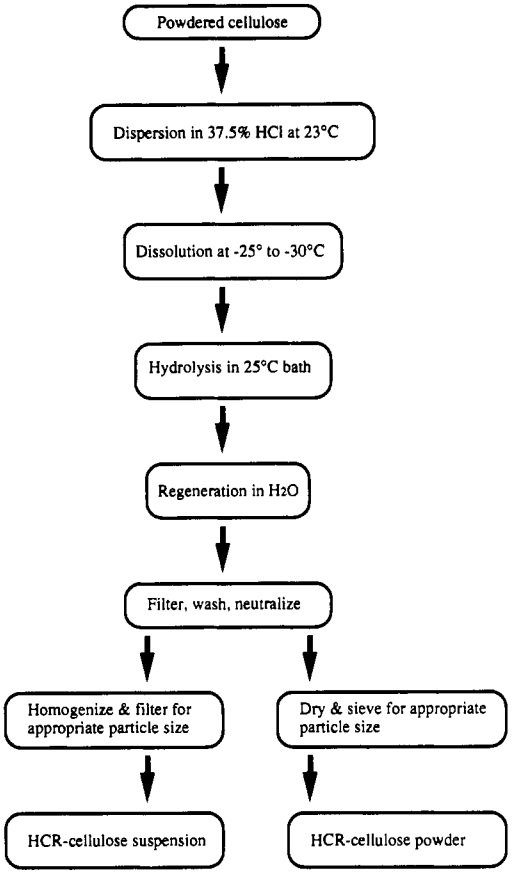


**Figure 1.** Schematic for the preparation of HCl-regenerated (HCR) celluloses.

65 °C for 14 h. The dried substrates were sieved to obtain fractions of uniform particle size. The particle size range from 124 to 355 μm was used for all analyses in this study. The concentration of the HCl solvents used for substrate preparations were determined by titration to the chlorophenol red endpoint (Christian, 1977). Phosphoric acid swollen cellulose was prepared as described by Hsu and Penner (1989) utilizing 8 g of cellulose/100 mL of 78% phosphoric acid.

**Substrate Characterization.** The amount of glucose released upon hydrolysis of a given mass of regenerated substrate by sequential hydrolysis with $H_2SO_4$ (Englyst et al., 1982) was determined by the enzymatic quantification of released glucose (Glucose Oxidase Assay Kit 510-A, Sigma). The extent of hydrolysis of cellulose chains was estimated by determining the viscosity average degree of polymerization of the substrate solubilized in 0.5 M cupriethylenediamine. Measurements were made with a Cannon-Fenske viscometer according to ASTM standard D 1795 (ASTM, 1986). The crystallinity index of substrates was calculated from diffractograms obtained with an automated Philips X-ray diffractometer using nickel-filtered Cu Kα radiation. The diffraction intensity was measured between Bragg angles (2θ) of 10 and 30. Crystallinity index (CrI) values were determined as described previously (Hsu and Penner, 1989). The enzyme susceptibility of substrates was determined with *T. viride* cellulase. Reaction conditions were 32 μg of enzyme/mL, 0.2% (w/v) substrate, 50 mM sodium acetate buffer, pH 5.0, 37 °C, in a gyratory bath oscillating at 140 rpm. The reaction was terminated at 24 h, and solubilized reducing sugar equivalents were measured colorimetrically (Liaw and Penner, 1990). Inorganic phosphorus analyses were done with a Jarrell-Ash Model 9000 inductively coupled argon plasma spectrometer. Samples were dry-ashed at 500 °C for 6 h, allowed to cool to room temperature, and dissolved in 10% HCl for analysis. Solubilized cellodextrins present after water precipitation of the HCl hydrolysate were quantified as reducing sugar equivalents (Nelson, 1944; Somogyi, 1952) and total sugar equivalents (Roe, 1955) by utilizing glucose as a calibration standard.

**Table I. Influence of Hydrolysis Period on Properties of Hydrochloric Acid Regenerated Substrates[a]**

| substrate | hydrolysis time,[b] min | temp,[c] °C | DP[d] | solubilized glucose equiv,[e] μmol/mL | CrI,[f] % |
|---|---|---|---|---|---|
| microcrystalline cellulose | | | 240 ± 4 | 1.11 ± 0.06 | 86.7 |
| HCR[g] cellulose | 0 | −27.0 | 214 ± 3 | 4.10 ± 0.12 | 26.5 |
| | 15 | 18.0 | 160 ± 4 | 3.68 ± 0.30 | 31.6 |
| | 30 | 22.0 | 120 ± 3 | 2.88 ± 0.05 | 34.4 |
| | 60 | 23.2 | 69 ± 6 | 1.49 ± 0.04 | 47.6 |

[a] Mean ■ SEM. [b] Hydrolysis period in 25 °C water bath following cellulose dissolution. [c] Temperature of cellulose solution just prior to water precipitation. [d] Viscosity average degree of polymerization. [e] Reaction conditions were 60 mL of a 0.2% (w/v) cellulose suspension containing 2 mg of *T. viride* cellulase in a 50 mM sodium acetate buffer, pH 5.0, at 37 °C for 24 h. [f] From X-ray diffractograms (Hsu and Penner, 1989). [g] HCR, hydrochloric acid regenerated.

## RESULTS AND DISCUSSION

**Dissolution of Cellulose in HCl.** The initial step in the procedure is the dispersion, or wetting, of the cellulose powder. The powder is to be equally dispersed, resulting in a uniform suspension without aggregation. Attempts to disperse cellulose in reagent greater than 37.5% HCl result in aggregates of swollen cellulose which are not readily dissolved during subsequent low-temperature treatments. Experience indicates that fresh HCl reagents, labeled as 36–38% HCl, generally approach the 38% HCl level and therefore must be diluted prior to use in this procedure.

Immediately after cellulose dispersion, the temperature of the suspension is lowered to approximately −30 °C to solubilize the cellulose. Cooling may be accomplished by transferring the suspension to an appropriate freezer or by immersion in a solid $CO_2$–ethanol bath. Cellulose was observed to go into solution at approximately −25 °C. The rate of cooling of the suspension and, consequently, the time required for cellulose dissolution were influenced by the extent of mixing. Cellulose dissolution was also dependent on the concentration of the HCl solvent used. Solubilization attempts using reagent of less than 36.5% HCl were not successful. This lower limit of 36.5% HCl for cellulose dissolution and the upper limit of 37.5% HCl for cellulose dispersion define the concentration range appropriate for this procedure.

**Regeneration of Insoluble Cellulose.** Cellulose dissolved in 37.5% HCl is readily hydrolyzed as temperatures approach 25 °C. This property may be utilized to prepare substrates with the desired degree of polymerization (DP). The cold cellulose solution resulting from the dissolution process, ∼−30 °C, may be transferred to a 25 °C bath for a prescribed hydrolysis period. Under these conditions, the cellulose remains in solution and cellulose hydrolysis occurs as the solution approaches the bath temperature (Table I). The extent of hydrolysis for a given period of time is somewhat empirical, being dependent on the experimental conditions, particularly the rate of heat transfer. Representative data characterizing this hydrolysis period are presented in Table I. As expected, the greater the hydrolysis time, the lower the DP of the resulting substrate. The inverse correlation between the dried substrate's DP and its CrI is in agreement with the general consensus from studies evaluating the recrystallization of cellulose fibers during acid hydrolysis (Fan et al., 1987). The direct correlation between the substrate's DP and its susceptibility to enzymatic hydrolysis is expected on the basis of the inverse correlation between CrI and DP and the widely reported

**Table II. Effects of Drying Methods on Crystallinity and Enzymatic Susceptibility of Hydrochloric Acid Regenerated Substrates[a]**

| substrate | drying method | CrI,[b] % | solubilized glucose equiv,[c] μmol/mL |
|---|---|---|---|
| microcrystalline cellulose | | 88.1 | 1.16 ± 0.01 |
| HCR[d] cellulose (water ppt) | oven-dried | 39.9 | 2.47 ± 0.17 |
| | solvent-dried | | |
| | M/M/A[e] | 30.0 | 3.95 ± 0.27 |
| | M/M/A/A | 26.8 | 4.26 ± 0.26 |
| | freeze-dried | 7.5 | 4.57 ± 0.23 |
| | never dried | | 7.66 ± 0.45 |
| HCR cellulose (acetone ppt) | acetone-dried | 22.9 | 2.39 ± 0.15 |

[a] Hydrolysis condition was 15 min in 25 °C water bath following cellulose dissolution. [b] From X-ray diffractograms (Hsu and Penner, 1989). [c] Mean ± SEM. Reaction conditions were 60 mL of a 0.2% (w/v) cellulose suspension containing 2 mg of *T. viride* cellulase in a 50 mM sodium acetate buffer, pH 5.0, at 37 °C for 24 h. [d] HCR, hydrochloric acid regenerated. [e] M, methanol; A, acetone.

inverse relationship between a substrate's CrI and its susceptibility to enzymatic hydrolysis (Sasaki et al., 1979; Sinitsyn et al., 1989).

The dissolved cellulose is regenerated/precipitated from solution by dilution with either 1 °C water or 4 °C acetone. In the present study, 100 mL of cellulose solution was diluted into 600 mL of vigorously agitated diluent. Substrates regenerated in either acetone or water, followed by solvent drying, were found to have similar crystallinity indexes but different susceptibilities to enzymatic hydrolysis (Table II). Following regeneration, the cellulose is washed exhaustively to neutralize and remove chloride ions. A maximum particle size for the washed, neutralized substrate may be defined by filtration. Suspensions with maximum particle sizes of less than 6 μm are readily obtained. HCR substrate suspensions may be used as is or dried for ease of handling and storage.

Drying the HCR celluloses significantly influences their CrI. Diffractograms of the never dried HCR celluloses indicated no crystallinity, while those of the dried substrates indicated significant crystalline character. The degree of crystallinity of the dried substrates was dependent on the drying method (Table II). The freeze-dried HCR cellulloses had the lowest CrI and greatest enzymatic susceptibility of the dried substrates. The enzymatic susceptibilities of the solvent-dried substrates, particularly the treatment utilizing two acetone washes, were similar to that of the freeze-dried substrates. Oven-drying resulted in substrates with the highest CrI and the most resistance to enzymatic saccharification. Similar drying effects have been observed for other cellulose preparations (Merchant, 1957; Wood, 1988).

**Properties of HCl-Regenerated Celluloses.** Complete hydrolysis of the regenerated cellulose, followed by enzymatic quantification of the liberated glucose, indicates essentially no chemical modification of the regenerated substrate other than the expected hydrolysis of cellulose chains. The degree of polymerization of the substrate is dependent on the amount of time the cellulose solution is in the 25 °C bath prior to precipitation, as discussed previously. However, some hydrolysis of the polymer occurs during the dispersion and dissolution process, and this sets an upper limit for the DP of approximately 214 when the microcrystalline cellulose starting material is used. Diffractograms of the dried substrates have a maximum at $2\theta = 20$, indicative of the cellulose II structure observed for other swollen and regenerated celluloses

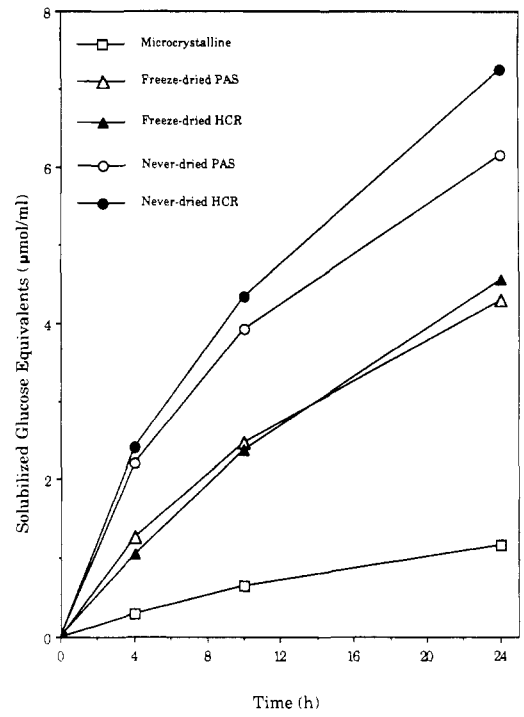


**Figure 2.** Comparison of the 24-h time course for the enzymatic saccharification of microcrystalline, hydrochloric acid regenerated (HCR) and phosphoric acid swollen (PAS) celluloses. Reaction conditions were 60 mL of a 0.2% (w/v) cellulose suspension containing 2 mg of *T. viride* cellulase in a 50 mM sodium acetate buffer, pH 5.0, at 37 °C.

(Atalla, 1983). The cellulose II crystallinity indices of the freeze-dried and solvent-dried (2× acetone) substrates were 71.4 and 78.8, respectively. Crystallinity index values reported in Tables I and II are calculated as cellulose I (see Materials and Methods), due to the correlation between this parameter and the susceptibility of the substrate to enzymatic hydrolysis (Sasaki et al., 1979; Sinitsyn et al., 1989). The enzymatic susceptibility of the HCR substrates may best be considered relative to that of other substrates used for the characterization of cellulase enzyme systems. Figure 2 illustrates the time course for the saccharification of the freeze-dried and never dried HCR substrates along with that for microcrystalline cellulose and phosphoric acid swollen (PAS) cellulose. Microcrystalline cellulose is commonly used as a crystalline, inert, insoluble substrate. PAS cellulose is routinely used as a relatively amorphous, relatively reactive, insoluble substrate (Wood, 1988). Figure 2 illustrates that the HCR substrates are significantly more susceptible to enzymatic saccharification than microcrystalline cellulose and similar to the corresponding PAS substrates.

**Utilization of HCR Cellulose.** Native cellulose occurs as a complex, semicrystalline, fibrous aggregate possessing several polymorphs (Atalla, 1983). The complex structures of cellulose make it difficult to interpret mechanistic studies analyzing the interaction between cellulases and their native substrates. One experimental approach to the study of cellulose/cellulase interactions is to utilize a series of structurally unique substrates. HCR cellulose is an appropriate substrate for this type of study. The HCR substrate is readily susceptibile to enzymatic digestion, is relatively amorphous, and retains a degree of polymerization similar to that of the more crystalline starting material. The properties of HCR cellulose are similar to those of the PAS substrates, which are well accepted in both descriptive and mechanistic studies of cellulase enzymes (Wood, 1988). Consequently, results derived from

studies utilizing PAS and HCR substrates should be complementary. One advantage of the HCR substrates, relative to PAS substrates, is their absence of phosphate esters. Results obtained from studies utilizing PAS substrates may be influenced by the chemistry, such as metal chelation (Penner et al., 1987), of the phosphate esters formed during the dissolution process required to prepare PAS cellulose. In this regard, the PAS substrates prepared for this study contained an average of 310 ppm of inorganic phosphorus. The complementary nature of the HCR and PAS substrates illustrates the general necessity of utilizing several cellulose substrates to avoid artifacts associated with any particular one.

With minimal modification this procedure may be adapted for the preparation of soluble cellodextrins. Analysis of the ratio of total sugar equivalents to reducing sugar equivalents present after precipitation of a 1-h hydrolysate indicated an average degree of polymerization of approximately 3 and a total yield of approximately 835 μmol of glucose/cellodextrin product. The currently accepted method for preparing these oligosaccharides includes dissolution of cellulose in 40% (fuming) HCl and, consequently, requires the use of HCl gas (Pereira et al., 1988). The dissolution process described in this paper represents a significant simplification of that procedure in that it avoids the use of 40% HCl. Cellulose dissolution directly in concentrated HCl at −30 °C for the preparation of cellodextrins has been reported (Halliwell and Vincent, 1981). This method has not proved successful due to the aggregation of the cellulose starting material when added directly to cold HCl. This aggregation is avoided if the cellulose is dispersed, as described previously, prior to the lowering of the temperature of the suspension.

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