Cellulose Derivatives with Low Degree of Substitution. 3. The Biodegradability of Cellulose Esters Using a Simple Enzyme Assay

Wolfgang G. Glasser,* Bruno K. McCartney, and Gamini Samaranayake

Department of Wood Science and Forest Products, and Biobased Materials Center, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0323

The biodegradability of cellulose ester derivatives using a degradation assay based on commercially available cellulolytic enzyme preparations was found to depend on two factors: degree of substitution (DS) and substituent size. The esters comprised a series of novel and unusual cellulose derivatives, different in both type of substitution and DS, and were prepared using homogeneous phase reaction conditions. The cellulose esters had acyl substituents ranging in size between propionyl and myristyl and DS values between 0.1 and nearly 3. Biodegradation was assayed by incubation with cellulolytic enzymes from two commercial sources followed by the determination of the concentration of reducing sugars. The rate of cellulolytic enzyme biodegradability (CEB) was found to differ with morphology. Two of three parent cellulose preparations and all cellulose products regenerated from homogeneous phase solution reached 75–80% of their maximum degradability within ca. 7 days of incubation with the enzyme system. The maximum degree of acylation (a) which the enzyme could tolerate before the polymer became undegradable and (b) which resulted in degradation in excess of 10% by weight ranged from 0.5 to at least 1.0 of a possible maximum of 3, depending on ester type. CEB per unit of DS was inversely related to the number of carbons present in the acyl substituent.

Introduction

The development of biodegradable materials for packaging, construction, transportation, etc., has become a topic of intense research. The general approach taken in creating biodegradable polymers (*1*) involves both fermentation-produced polymers and polymers derived from biological substrates by extraction, modification (optional), and reshaping. Poly(hydroxyalkanoates) are thermoplastic polyesters produced via fermentation from a variety of microorganisms having the enzymatic machinery for polymerizing hydroxyalkanoic acids. The second approach, reshaping of existing biological polymers, typically involves polysaccharides and lignin. While this approach is favored by the ready availability and abundance of these biobased materials, it is, however, hindered significantly by their resistance to being reshaped. Processing characteristics are typically improved by chemical modification, such as in the case of cellulose esters. These chemically modified derivatives lose, to some extent, one of their most important attributes, biodegradability. It is the objective of this study to gain an understanding of the relationship between cellulose modification (by esterification) and enzyme biodegradability.

Cellulose is a partially crystalline linear polymer with a melting point above its thermal decomposition temperature. Esterification is recognized as a method for reducing melting point (*2*). Commercial cellulose esters are produced heterogeneously, making it necessary to reach very high levels of substitution for uniformity. Cellulose esters of high degrees of substitution are no longer degraded by cellulolytic enzymes, and they are biodegradable only after significant structural modification has taken place, presumably by deacylation or depolymerization (*3*). However, with the advent of homogeneous phase cellulose derivatization, uniform cellulose ester derivatives with acyl substituents ranging in size from C-3 to C-16 have been prepared. This has been the subject of two prior articles in this series (*4, 5*). While the relationship between thermal characteristics and the structure of cellulose ester derivatives has been the subject of several other publications (*6, 7*), the relationship between cellulose ester structure and biodegradability has only recently begun to be examined (*3, 8, 9*).

We needed to define a simple assay in order to study the relationship between the chemical structure and biodegradability of cellulose and its derivatives. Biodegradation assays can be run using whole organisms (fungal or bacterial, aerobic or anaerobic) or with isolated enzymes. The enzymatic system offers the advantages of expediency, adaptability to micro or semi-micro scales, and low cost; the disadvantages include limited availability and specificity of isolated enzymes. The focus on an enzyme-based assay is most readily accomplished by using commercially available enzyme preparations. The preferred assay system should be rapid, relatively simple, insensitive to unwanted, nonenzymatic catalyzed reactions, quantitative, usable on a small (micro or semi-micro) scale, and of low cost. Current assays (*8–11*) in use within the research community include whole organisms and enzymatic assays utilizing the measurement of weight loss, microbial growth, carbon dioxide production, oxygen consumption, and changes in physical properties (tensile strength, viscosity, elasticity, etc.). The degradation of the physical structure of the substrate can also be monitored visually or by microscopy. Chemical methods include enzyme incubation followed by reducing-end (total as well as glucose-specific) analysis, which has been a well-established assay technique for some time (*12, 13*).

An assay system which operates within relatively mild conditions using limited capabilities for biodegradation

* Author to whom correspondence should be addressed.

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can only be used as an indicator of biodegradability. The likelihood of overall and complete biodegradation of a material can be predicted only under controlled laboratory conditions that simulate the complex microbial systems that exist in the environment. Nevertheless, a great deal of fundamental information may be gained, using milligram quantities of test material, by studying relatively simpler defined enzyme–substrate systems.

It was the objective of the present study to test the relationship between cellulose ester structure in terms of type and degree of substitution and the ability of a cellulolytic enzyme to recognize and degrade the polymer.

Experimental Section

I. Materials. *1. Assay Materials.* The cellulose standards included two microcrystalline cellulose preparations (sources: Aldrich Chemical Company, Milwaukee, WI, cat. no. 31,069-7; Whatman CF-11, cat. no. 4021-050), and a medium intrinsic viscosity (ca. 8.6 dL g^{-1}) commercial dissolving pulp. The *Trichoderma longibrachiatum* enzyme preparations were provided by Genencor, Inc. (Cytolase 123 and Multifect XL) and ALKO, Ltd. (Econase EP 1262). Stanbio OT-V glucose reagent (6% (w/v) *o*-toluidine in glacial acetic acid) was obtained from Fisher (Norcross, GA). Acetate buffer (sodium form, 20 mM, pH 4.9) and glucose standard were obtained from Sigma (St. Louis, MO). These materials were used as received.

2. Cellulose Ester Derivatives. The cellulose ester derivatives were those described in the two prior articles in this series (*4*, *5*). In brief, they were prepared using novel, homogeneous phase reaction conditions involving cellulose solutions in dimethylacetamide (DMAc) and lithium chloride (LiCl). Solutions of cellulose (2% (w/v)) in DMAc/LiCl (9%) were treated either with an appropriate acid chloride or acid anhydride and triethylamine (equimolar amount) as the base catalyst or with acid anhydride or (free) acid and dicyclohexylcarbodiimide and pyrrolidinopyridine. The cellulose ester products were recovered by precipitation in water, and they were purified by extraction with methanol, ether, and other appropriate solvents in accordance with procedures described elsewhere (*4*, *5*).

II. Methods. *1. CEB Assay Procedure.* Cellulose/cellulose derivative samples were powdered and sieved using a 40-mesh screen. Samples in other physical forms were also used as substrates. Approximately 250 mg of substrate was allowed to soak in exactly 25 mL of acetate buffer in sealed flasks for 24 h prior to the addition of enzyme. Aliquots of enzyme preparation (100 μL) were added to the substrate suspension at the onset, and the assay was run once each day. The time of degradation began upon the addition of the first enzyme aliquot. The assay suspensions were kept sealed in Erlenmeyer flasks at 50 °C, and they were agitated at 30 rpm in a covered shaker bath for the duration of the assays. The production of reducing sugars (glucose) was determined using an *o*-toluidine-based assay procedure (*14*, *15*) (Stanbio Direct Glucose Procedure No. 0270, Stanbio Laboratory, Inc., San Antonio, TX), with colorimetric absorbance readings taken at 636 nm or scanned between 700 and 500 nm using a Perkin-Elmer Lambda 6 UV/vis spectrophotometer with PECS 5 software. The concentration of reducing sugars (glucose) produced by enzymatic hydrolysis was determined by comparison with a control (buffer and enzyme preparation only) and standard glucose solutions in the Stanbio procedure. HPLC analysis of the hydrolysis broth was performed using a Waters system equipped with a Waters 410 differential refractometer and Biogel size exclusion chromatography columns (Bio-Rad, Richmond, CA) having pore sizes of 120 (SEC 20 XL, two columns) and 250 Å (SEC 30 XL, one column). HPLC-grade water served as the mobile phase, which was eluted at a rate of 0.6 mL/min.

Table 1. Enzyme Assay Conditions in Accordance with Suppliers' Recommendations

	enzyme preparation	
	Cytolase 123	Econase EP 1262
supplier	Genencor Inc.	ALKO, Ltd.
organism source	*Trichoderma longibrachiatum*[a]	*Trichoderma longibrachiatum*[a]
optimal temperature (°C)	40–60	40–50
optimal pH	4.0–5.5	4.0–5.5
enzyme/substrate dosage used	100 μL/250 mg	100 μL/250 mg
buffer used (pH)	20 mM sodium acetate (pH 4.9)	20 mM sodium acetate (pH 4.9)

[a] Formerly *T. reesei*.

2. CEB Degradability Calculation. Cellulolytic enzyme biodegradability (CEB) was calculated using the following formula:

$$\mathrm{CEB} = (G/180)[162 + f(w - 1)]$$

where G is the glucose equivalent yield (wt %), f is the degree of substitution, and w is the molecular weight of the acyl moiety.

Results and Discussion

I. Cellulolytic Enzyme Biodegradability (CEB) Assay. There is a wide variety of purified or partially purified enzyme preparations commercially available for the hydrolysis of cellulose. These preparations with a broad range of activities (*16*) (including "hemicellulases", etc.) allow the assay to be used as a screening method for many polysaccharides and their derivatives. Two enzyme systems were selected: Cytolase 123 (Genencor Corporation) and Econase EP 1262 (ALKO, Ltd.). Both enzyme preparations are derived from the same organism, *Trichoderma longibrachiatum* (formerly *T. reesei*), and both enzymes have similar optimal ranges of conditions (Table 1). Enzymes were used at 20-fold the suppliers' recommended dosage. At this "saturation dose", both sources of enzymes produced comparable results. The heterogeneous incubation mixture was kept agitated by shaking in a shaker bath. No contamination of the mixtures was noticed within the first ca. 10 days. At longer incubation time periods, the occasional occurrence of contamination by organisms was indicated by (a) cloudiness of the otherwise clear supernatant of the suspension mixture and/or (b) the development of pressure in the tightly closed reaction vials. In general, contamination was not found to present a problem until after a 2-week incubation period.

The wavelengths of the absorption maxima for colored complexes produced by the degradation products of cellulose derivatives were found not to vary significantly from that of glucose. The concentration of reducing substances was determined as if it were glucose. This measured quantity was defined as the glucose equivalent concentration. This was subsequently converted to the weight percent glucose equivalent yield on the basis of the weight of the sample tested. The calculation is presented in the Experimental Section.

The procedure does not rule out the possibility that mono- as well as oligosaccharides produce positive assay results. This imparts no error to the experimental data

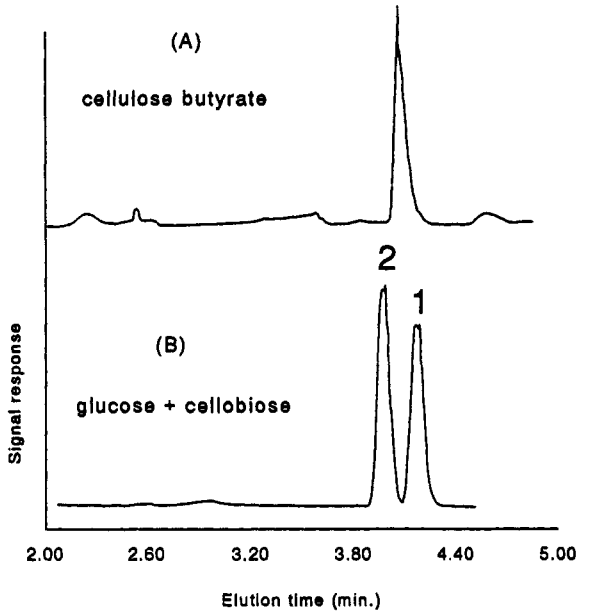

Figure 1. Size exclusion chromatograms of degradation products of cellulose butyrate (sample DS = 0.2) (A) and of a model mixture of glucose (1) and cellobiose (2) (B); CEB procedure.

since each reducing end determined is a bond broken, and the biodegradability is defined as a percentage of bonds broken (see the equation given in the Methods section). It was therefore of interest to examine the degradation product mixtures for the possible presence of oligosaccharides. The size exclusion chromatogram of a typical degradation product mixture (Figure 1) provides unambiguous evidence for the absence of oligosaccharides in the incubation broth. The sample chromatogram reveals a slightly noisy base line at the location of polysaccharides, a zero-response at the position where disaccharides appear, and a strongly positive response for monosaccharides. A weak base-line deflection at a higher elution time than monosaccharides could possibly indicate the presence of low molecular weight substances in the incubation mixture, such as acetic or butyric acid. There were, however, no other signs of ester hydrolysis. The shape of the monosaccharide peak, with several subtle shoulders on the downside slope, may indicate the presence of several types of monosaccharides, such as glucose in addition to 6-glucose (and others) monobutyrate. [This is the subject of continuing studies.] These (preliminary) results allow the conclusion that the CEB assay determines the monosaccharide yield by cellulose derivative degradation.

II. Application of CEB Assay to Cellulose Standards. The CEB assay was tested using several unmodified parent cellulose preparations (Table 2). The cellulose control tests produced excellent agreement between four cellulose samples and a striking difference with a fifth preparation (Figure 2). Whereas for four of the control celluloses, assay results ranged from 55 to 80% degradation of cellulose to glucose within the first 2 days (they all reached 80% degradation on the 5th day), cellulose preparation A (Whatman CF-11) showed a much slower initial rate of degradation than the other control samples, with no significant change in degradation rate for the 2-week period. Since the same cellulose preparation, after regeneration from homogeneous solution (DMAc/LiCl) in either powder (sample D) or bead (sample E) form with virtually unchanged molecular weights, produces results in complete agreement with the other cellulose controls, the difference in degradation behavior can safely be attributed to supermolecular, morphological differences.

Table 2. Characteristics of Cellulose Preparations

sample	source	treatment	$\bar{M}_n \times 10^{-3}$	$\bar{M}_w \times 10^{-3}$	MHS[e] constant
A	Whatman CF-11	none	89.3	155.6	0.85
B	Aldrich[a]	none	58.2	151.9	0.78
C	Eastman Kodak[b]	none	634.8	1552	0.71
D	Whatman CF-11	regeneration[c]	107	184	0.80
E	Whatman CF-11	regeneration[d]	107	184	0.80

[a] Fibrous α-cellulose. [b] Dissolving pulp with intrinsic viscosity of 8.6 dL g^{-1}. [c] Involving dissolution in DMAc/LiCl and regeneration by precipitation into warm (60 °C) methanol in water (50%). [d] Involving regenerating dissolved cellulose (see footnote c) in the form of spherical beads instead of in powder form. This regeneration procedure has been found to produce amorphous (noncrystalline) cellulosic gel particles (*17*). [e] Mark–Houwink–Sakurada constant.

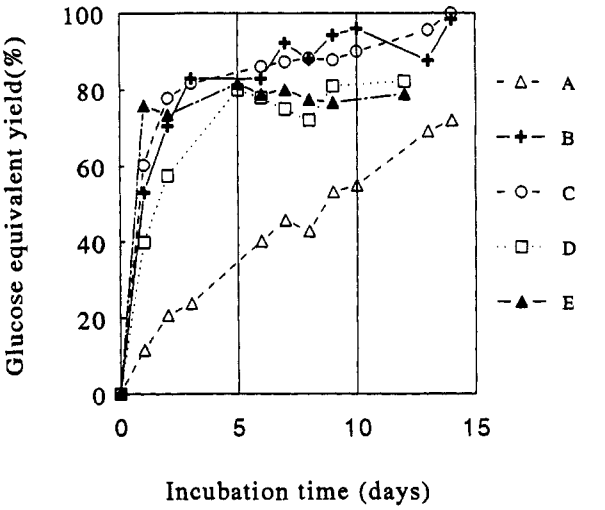

Figure 2. Cellulolytic enzyme biodegradability of various cellulose preparations. Sample identification is in accordance with Table 2.

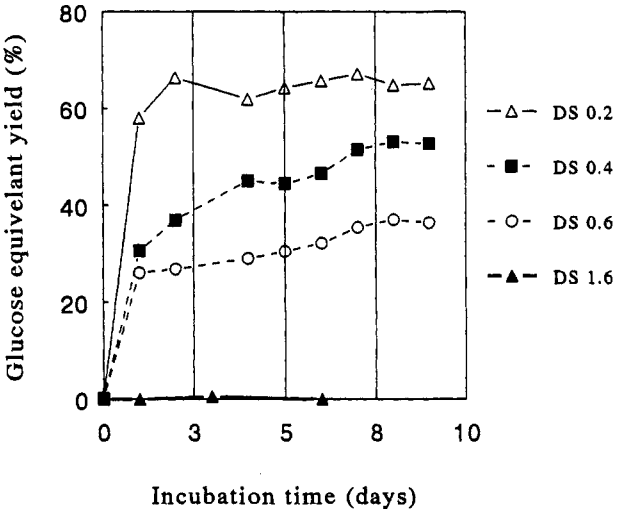

Figure 3. Cellulolytic enzyme biodegradability of cellulose butyrates with rising DS.

That the degradation rate changes as a function of material morphology has been well-recognized in previously published literature (*18*).

The degradation behavior of two samples (A and C, Table 2) was examined by statistical means. The results indicated (a) that CEB determinations have a mean standard deviation of 2–4%, and (b) that glucose yields were ca. 40 and 80% after 5 days of incubation, depending on substrate source (and morphology).

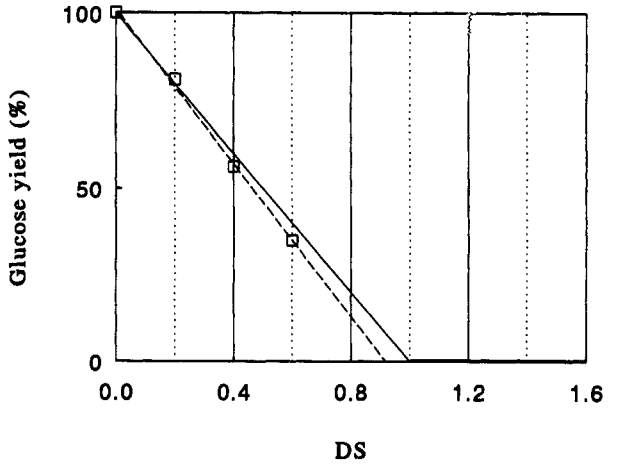

Figure 4. Relationship between experimental and theoretical glucose yield. Experimental glucose yield represents data from Figure 3 normalized to the 5-day value of control cellulose from Figure 2. The theoretical glucose yield is calculated assuming uniform distribution of monosubstituted anhydroglucose units.

It can thus be concluded that the CEB assay produces meaningful results for most cellulose controls and for regenerated cellulose within a 2-day incubation period, that the results are highly reproducible, and that differences in degradation rate are related to morphological differences.

III. Application of CEB Assay to Cellulose Derivatives. ***1. Effect of Degree of Substitution (at Constant Substituent Type).*** Extensive studies on the biodegradability of cellulose ethers have established that the degree and the uniformity of substitution are decisive factors for their biodegradability (*12, 13*). Major conclusions from those studies include the following: (1) If each of the anhydroglucose (AHG) units possesses a substituent, the derivative is not biodegradable (*12*). (2) Derivatives undergo biodegradation, initially with a faster rate, along contiguous unsubstituted AHG units, and then with a slower rate at isolated unsubstituted AHG units (*13*).

Parallel behavior is observed for cellulose ester derivatives prepared by novel, homogeneous phase reaction chemistry. This new methodology is presumed to give cellulose derivatives having their acyl substituents (a) uniformly distributed along the polymer backbone and (b) located predominantly (but not exclusively) in position 6 (*4*). This implies that a cellulose ester with a DS of 1.0 is a polymer, not of anhydroglucose, but of anhydroglucose monoester (position 6). Likewise, a derivative with DS 0.5 has (theoretically) zero cellobiose repeat units but, instead, has cellobiose monoester building blocks. While it is well-recognized that enzymes are highly substrate specific, it is as yet unknown how fast the ability of cellulases to recognize cellulose as a target molecule is lost when the backbone of the macromolecule is modified with alien substituent groups. Studies on the biodegradation of cellulose ethers using microorganisms (as well as isolated enzymes) have shown that a DS < 1 is required for the scission of (unsubstituted) polymer repeat units to occur (*19–21*). In contrast, cellulose acetates with DS values between 1.7 and 2.5 were all found to biodegrade using mixed culture systems, at least partly following deacetylation (*3*).

The degradation behavior of several cellulose butyrates with different DS values (range 0.2–2.4) (Figure 3) reveals that degradability is significantly related to DS and that the degradation after 2 days is between 60 and 80% of the maximum achievable. This suggests that a 2-day assay produces meaningful results.

The decrease in monosaccharide (derivative) yield with rising DS (Figure 4) closely follows the theoretical yield calculated for the cellulose butyrate samples assuming that only unsubstituted units are released and that only they produce a positive assay response. This is consistent with the results obtained with whole microorganisms and cellulose ethers (*19, 20*), and it supports the view that the biodegradation of cellulose acetate (DS > 1.5) commences with deacetylation (*3*).

2. Effect of Substituent Type on Degradation Rate. The relationship between degradation rate and degree of substitution (Figure 5) reveals that cellulose derivatives have variable degradation rates that are not obviously related to the degree of substitution. (The results shown represent data of >10% degradation within 10 days of enzyme incubation.) Whereas cellulose propionates of low DS degrade faster than those of high DS (Figure 5A), cellulose butyrate shows the opposite behavior (Figure 5B). This variability may be a consequence of differences in morphology. These results suggest that, while it is desirable to terminate the assay after a short time period so as to avoid microbial contamination of the incubation mixture, the highly variable relationship between degra-

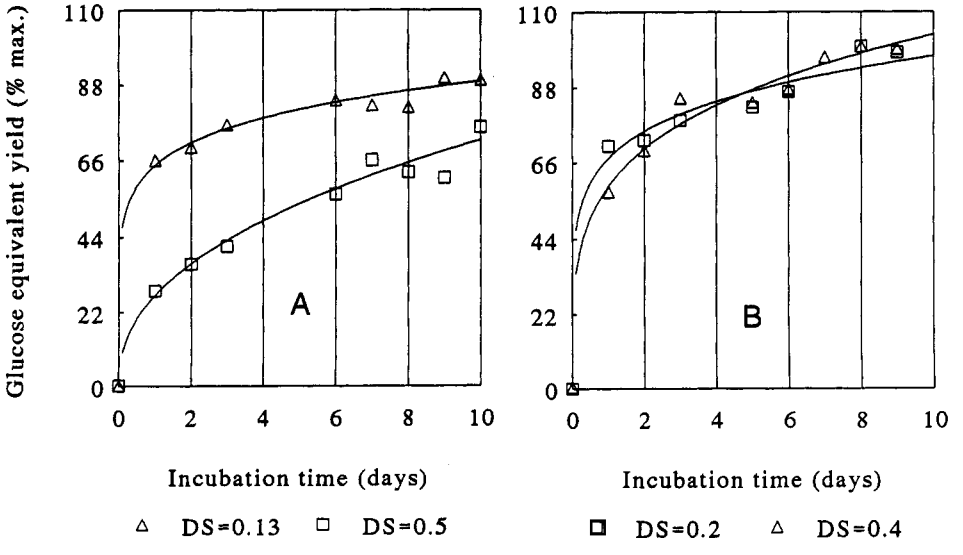

Figure 5. Relationship between glucose equivalent yield (in percent of total maximum) and incubation time for cellulose propionates (A) and cellulose butyrates (B) of different DS.

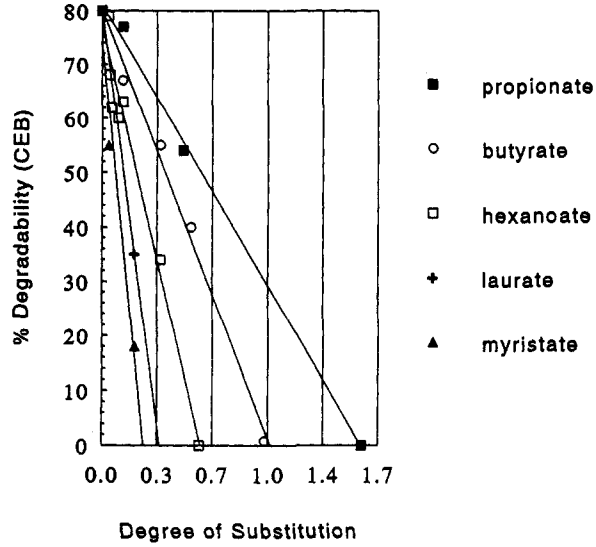

Figure 6. Cellulolytic enzyme biodegradability (CEB) vs DS for several cellulose derivatives.

Table 3. Cellulolytic Enzyme Biodegradability (CEB) of Cellulose Ester Derivatives

	number of carbons in the acyl substituent				
DS	C_3	C_4	C_6	C_{12}	C_{14}
0	80	80	80	80	80
0.001					80
0.002					80
0.04			79		
0.05			65		
0.06			62		
0.10			60		
0.13	77		63		
0.20		67		35	18
0.30					
0.40		55	25		
0.50	54		0		
0.55				0	0
0.60		40			
0.70			0		
0.90			0		
1.0				0	
1.6					
1.7	0				

dation rate and degree of substitution favors the adoption of a multiweek incubation period. However, failure to degrade is safely established after 2 days of incubation.

3. Effect of Substituent Type and DS on Degradability. The relationship between CEB degradability and DS for several cellulose ester types (Figure 6) illustrates that degradability is derivative-specific. Whereas cellulose propionates consistently show the greatest degradability at DS levels rising from 0 to 1.6, cellulose myristates show the lowest degradability in relation to DS (i.e., steepest slope in the relationship between degradability and DS, Figure 6). This observation, although based on a limited number of data points (Table 3), must tentatively be interpreted in relation to the ability of the cellulase enzymes to recognize a cellulose molecule following modest degrees of modification. The higher the values of DS, the smaller the number of unsubstituted AHG sequences available for scission. In addition, esters with larger substituents, e.g., laurate (C_{12}) and myristate (C_{14}) (see Figure 6), do not degrade, even at DS levels of <1.0. The contiguous anhydroglucose unit model (*13, 21*) proposed for alkyl ethers of cellulose with low DS (or moles of substituent, MS) appears to be applicable to the corresponding ester derivatives. Therefore, the sharp drop in biodegradability, and the narrow DS window in which this

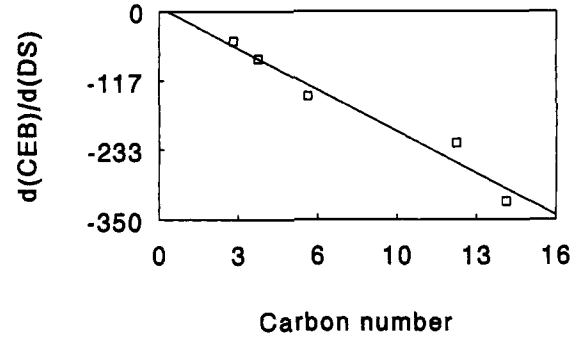

Figure 7. Relationship between the CEB/DS (cf. Figure 6) and substituent size in number of carbons per acyl substituent.

happens, cannot be attributed to the lack of unsubstituted monomer units. Thus, vast differences in degradability between cellulose butyrate (DS = 0.2, Table 3), and laurate and myristate (both DS = 0.2), suggest that the presence of unsubstituted monomer sequences alone is not a necessary and sufficient condition that determines degradability. Enzymatic recognition, and thus cellulolytic biodegradability, is quantitatively related to both DS and substituent size. The latter, the relationship between CEB per unit of DS (e.g., d[CEB]/d[DS]) and substituent size, indicates a significantly linear relationship (Figure 7). It is evident that the larger the substituent of the cellulose ester, the harder it is for the enzyme to recognize the macromolecule and degrade it.

This is the first time a relationship has been established between substituent size and enzyme recognition for cellulose esters. While such a relationship is not surprising, its practical utility for predicting the biodegradability of cellulose esters remains limited. Since cellulose ester degradation seems to proceed in succession with deacylation (at least at DS levels > 1), differential ester hydrolysis rates become another parameter determining the biodegradability of this class of cellulose derivatives.

Questions remain, however, as to the relevance of this closed laboratory assay system to the highly varied and dynamic conditions that are found in the natural environment. In conjunction with physical, chemical, and mechanical processes, living organisms can become involved in a complex series of interactive pathways that degrade even novel materials. Biodegradability is a material characteristic that, in a broad sense, can be answered only following extensive adaptation to a series of complex environmental circumstances. Even with a broad range of activities, the preparations used in this study are not fully representative of all of the enzymes that do or could exist in the natural environment. Recognition of a macromolecule by cellulases (and conversion) provides assurance of biodegradability by recognized pathways; failure in recognition implies that this pathway is inoperative or requires further adaptation before it becomes operative. However, the degradation of a cellulose (derivative) via these existing enzymes should be a good indicator of biodegradability.

Conclusions

(1) A cellulase enzyme-based cellulose derivative degradability assay produces meaningful results after as little as 2 days of incubation. (2) Degradation rate seems to be related to cellulose (derivative) morphology as well as the degree of substitution. (3) The ability of cellulase to degrade a cellulose derivative is significantly related to both the degree of substitution and substituent size. The smaller the substituent, the higher the DS that can be tolerated by cellulolytic enzymes.

Acknowledgment

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