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CP/MAS ^{13}C NMR analysis of cellulase treated bleached softwood kraft pulp

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Abstract—Fully bleached softwood kraft pulps were hydrolyzed with cellulase (1,4-(1,3:1,4)-β-D-glucan 4-glucano-hydrolase, EC 3.2.1.4) from *Trichoderma reesei*. Supra-molecular structural features of cellulose during enzymatic hydrolysis were examined by using CP/MAS ^{13}C NMR spectra in combination with line-fitting analysis. Different types of cellulose allomorphs (cellulose I_α, cellulose I_β, *para*-crystalline) and amorphous regions were hydrolyzed to a different extent by the enzyme used. Also observed was a rapid initial phase for hydrolysis of regions followed by a slow hydrolysis phase. Cellulose I_α, *para*-crystalline, and non-crystalline regions of cellulose are more susceptible to enzymatic hydrolysis than cellulose I_β during the initial phase. After the initial phase, all the regions are then similarly susceptible to enzymatic hydrolysis.

Keywords: Cellulose; NMR; Cellulase hydrolysis; Supra-molecular structure; Crystallinity

1. Introduction

Cellulose is the most abundant and renewable biopolymer in nature, biosynthesized largely in the cell wall of higher plants, as well as from bacteria, algae, and some fungi. Although the elementary structure was established 166 years ago by Anselme Payen, the ultrastructure of cellulose remains a field of active study today. Indeed, recent synchrotron X-ray and neutron diffraction studies by Nishiyama and Langan et al.[1–3] have provided some of the most definitive information regarding crystal structure data for cellulose I_α, I_β, and II crystal structures.

Along with celluloses' well established fiber and chemical derivative applications, a growing field of study is the use of cellulose as a chemical resource for the synthesis of innovative chemicals and biofuel applications.[4] Key to these latter chemistries is the depolymerization of cellulose to glucose, either enzymatically or chemically. Enzymatic hydrolysis of cellulose to glucose by cellulases has been the subject of considerable research efforts world wide for past several decades.[5,6] Cellulases in nature are a multicomponent enzyme system composed of a (1,4)-β-glucan endohydrolase (EC 3.2.1.4), a cellobiohydrolase (EC 3.2.1.91), and a cellobiase (EC 3.2.1.21). The biochemistry and mechanism of cellulase action have been studied and modeled in detail.[7–11] Recently, the impact of cellulase treatments on celluloses from various sources, including lignocellulosic materials, has come under active investigation. A comparison of cellulase hydrolyzed cellulose from cotton linters and dissolving grade pulps from fir and eucalyptus indicated that changes in the degree of polymerization of cellulose were dependent of species.[12,13] Analysis of changes in crystallinity accompanying cellulase treatments indicated that the amorphous portion was preferentially hydrolyzed yielding a product with increased order. These changes in crystallinity indexes were shown to be sensitive to the source of cellulose employed.[14] The

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changes in the efficacy of cellulase to hydrolyze cellulosics due to substrate properties were highlighted in a recent review by Mansfield et al.[15] Mooney et al.[16] demonstrated that the fiber size of Douglas fir kraft pulps influenced hydrolysis rates, with the smaller fibers being hydrolyzed more efficiently.

Since the early 1980s, high resolution solid-state NMR has become a powerful tool in the investigation of structural features of cellulose, especially of the crystalline ultrastructure. Atalla and VanderHart[17–19] were among the first to apply the high resolution solid-state CP/MAS ^{13}C NMR technique to the investigation of native cellulose structures and demonstrated resolution of multiple resonances for some of the chemically equivalent carbons in the anhydroglucose units. They proposed that native cellulose was a composite of two different crystalline forms (allomorphs), I_α and I_β.[20–22] The Horii group[23] also studied the crystal structures of cellulose I and found that native cellulose could be classified into two types, cotton–ramie and bacterial-*Valonia*, which are enriched in cellulose I_β and I_α, respectively. The cellulose I_α crystalline form is dominant in bacterial and algal cellulose and I_β crystalline form is dominant in tunicate cellulose and higher plants such as cotton, ramie, and wood.[20,22,24–27] Electron diffraction and CP/MAS ^{13}C NMR studies have revealed that cellulose I_α can be assigned to an allomorph with triclinic unit cells and cellulose I_β can be assigned to an allomorph with two-chain monoclinic units.[25–28]

Lennholm et al.[29] have developed a statistical model to determine the proportions of cellulose I_α and I_β in different lignocellulosic materials by taking advantage of multivariate data analysis techniques. In later studies, Iversen and Larsson[30,31] investigated the supra-molecular structures of cellulose by non-linear spectral fitting with a combination of Lorentzian and Gaussian functions, and developed a model and method to quantitatively analyze the crystalline allomorphs and disordered domains. By means of non-linear spectral fitting analysis of CP/MAS ^{13}C NMR spectra, they proposed the occurrence of a *para*-crystalline component in cellulose, which is less ordered than crystalline I_α and I_β allomorphs, but more ordered than amorphous domains.[31,32] Wickholm et al.[33] developed a chemometric model to predict the relative amounts of different cellulose forms in complex cellulosic materials by using spectral fitting analysis of the C4-region of CP/MAS ^{13}C NMR spectra. Line-shape spectral fitting analysis of solid-state NMR spectra allows a detailed comparison and characterization of cellulose supra-molecular structures, and has been extensively used to investigate the structural characteristics of celluloses and its derivatives.[28,30–32,34–38]

In comparison with studies of the biochemistry of cellulases, comparatively little work has been done on the structural aspects of cellulose dimorphs (I_α/I_β) after treatment with these enzymes. ^{13}C NMR analysis by Kim and Newman[39] has revealed that cellulose I_α is preferentially degraded in brown rot decay of Korean red pine (*Pinus koraiensis*). Using FT-IR spectroscopy and electron diffraction, Hayashi et al.[40,41] investigated the relative susceptibilities of cellulose I_α and I_β to enzymatic degradation by *Trichoderma viride* cellulase and observed comparable reactivities. For the development of improved cellulase treatments, changes in cellulose ultrastructure need to be further defined with alternative sources of cellulose. This report summarizes changes in cellulose supra-molecular structures as determined by CP/MAS ^{13}C NMR.

2. Results and discussion

A 1% slurry of fully bleached softwood kraft pulp was treated with cellulase (0.13 IU/mg pulp) at 38 °C for 0, 1, 2, 4, 8, and 24 h reaction time. The amounts of cellulosic fibers recovered were determined and these results are summarized in Figure 1. Typical CP/MAS ^{13}C NMR spectral data and cellulose signal assignments are presented in Figure 2. The most informative region

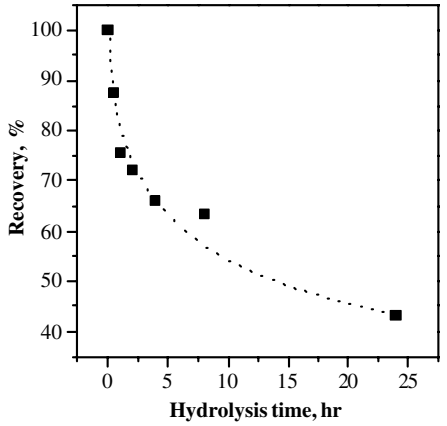

Figure 1. Recovery of hydrolyzed pulp substrates during cellulase hydrolysis.

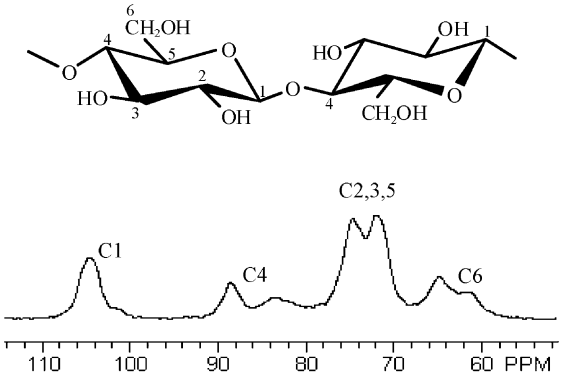

Figure 2. CP/MAS ^{13}C NMR spectrum of control pulp sample.

in the NMR spectrum of cellulose is the signal cluster with a chemical shift distribution between δ 80 and 92 ppm.[17,18,30–32] The fairly sharp signals from δ 86 to 92 ppm correspond to C-4 carbons from crystalline forms together with *para*-crystalline domains, whereas the broader upfield resonance line from δ 80 to 86 ppm is assigned to the amorphous domains.[31,42,43]

The analysis method is based on a non-linear least-square fitting of CP/MAS ^{13}C NMR spectra enabling determination of the relative amounts of cellulose I_α, cellulose I_β, *para*-crystalline cellulose, and celluloses at accessible and inaccessible surfaces.[30–32,43] Line-shape spectral fitting analysis of cellulose C-4 regions was performed by using Lorentzian lines applied for the signals from the crystalline domains, I_α, I_β, and $I_{\alpha+\beta}$, and Gaussian lines for the signals from the remaining domains (including *para*-crystalline cellulose, accessible fibril surface, and inaccessible fibril surfaces). In Figure 3, the spectral fitting is shown for the cellulose C-4 region of CP/MAS ^{13}C NMR spectrum of the control pulp sample. Assignments of the signals in the C-4 region of the spectrum obtained from the control sample are shown in Table 1.

Sugiyama et al.[27] have characterized the two crystalline allomorphs I_α and I_β, and reported that the monoclinic two-chain cellulose I_β is structurally denser and thermodynamically more stable than the triclinic one-chain cellulose I_α in the heat-annealing treatment. Figures 4 and 5 show the relative proportions for cellulose I_α and I_β allomorphs from line-fitting analysis after cellulase treatment. Obviously, the enzyme discriminates between the two crystalline forms I_α and I_β. Cellulose I_α has a decreased relative proportion as the enzymatic hydrolysis proceeds, while cellulose I_β has an increased

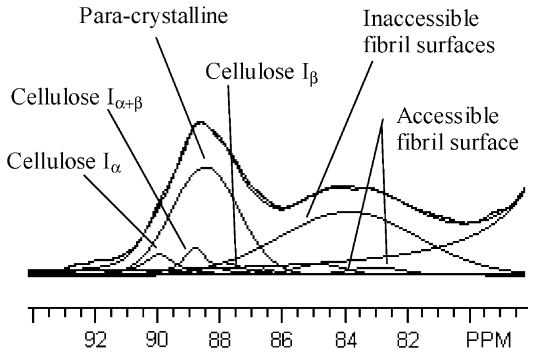

Figure 3. Spectral fitting for the C-4 region of CP/MAS ^{13}C NMR spectrum of the control pulp sample.

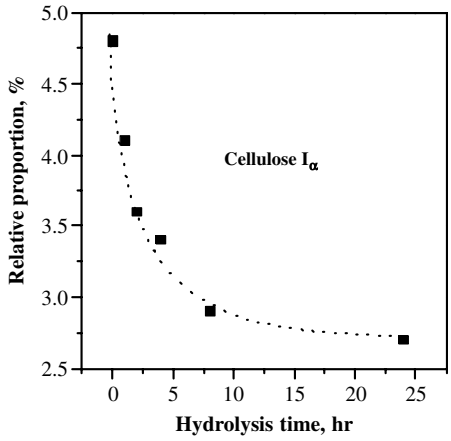

Figure 4. Relative proportions of cellulose I_α for the control and cellulase treated pulp samples as determined by line-fitting analysis of CP/MAS ^{13}C NMR spectra.

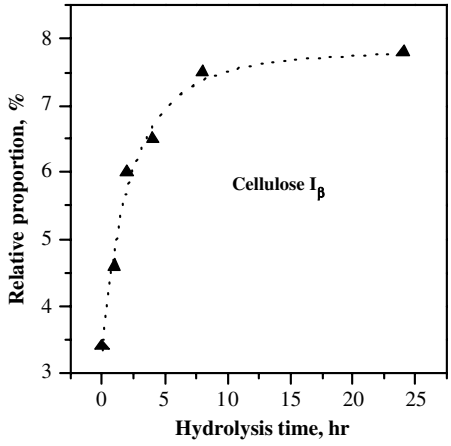

Figure 5. Relative proportions of cellulose I_β for the control and cellulase treated pulp samples as determined by line-fitting analysis of CP/MAS ^{13}C NMR spectra.

Table 1. Assignments of signals in the C4-region of CP/MAS ^{13}C NMR spectrum of the control pulp sample

Assignments	Chemical shift (ppm)	FWHH[a] (Hz)	Intensity (%)	Line type
Cellulose I_α	90.0 (0.04)[b]	116 (8)	4.8 (0.4)	Lorentz
Cellulose $I_{\alpha+\beta}$	88.8 (0.04)	91 (6)	5.0 (0.5)	Lorentz
para-Crystalline cellulose	88.5 (0.05)	254 (12)	37.1 (0.8)	Gauss
Cellulose I_β	87.9 (0.03)	135 (6)	3.4 (0.3)	Lorentz
Accessible fibril surface	84.9 (0.15)	148 (6)	2.7 (0.2)	Gauss
Inaccessible fibril surface	83.9 (0.24)	525 (14)	44.8 (0.7)	Gauss
Accessible fibril surface	83.4 (0.14)	67 (8)	2.2 (0.2)	Gauss

[a] FWHH: Full width at half-height.
[b] Values in parentheses are the standard errors.

relative proportion. This indicates that the I_α phase in the pulp cellulosic material is more susceptible to enzymatic degradation than cellulose I_β and hydrolyzed preferentially. Hayashi et al.[40,41] investigated the enzymatic susceptibility of cellulose crystallites and microfibrils and found that cellulose I_α crystal component was selectively degraded with *T. viride* cellulase with respect to the cellulose I_β. It is well known that the cellulose crystalline allomorph I_α is metastable and can be converted into the cellulose I_β by various solvents and hydrothermal treatments.[28,34,44] These conditions are usually not relevant to cellulase treatments that occur typically at 48 °C or less. The enzymatic enrichment of cellulose I_β is attributed to the low susceptibility of cellulases to hydrolyze the more ordered and relatively stable crystal structure of I_β.

The total crystalline allomorphs in the cellulose are composed of three Lorentzian lines from the line-fitting analysis, I_α, $I_{\alpha+\beta}$, and I_β. Figure 6 shows the relative proportions of total crystalline forms in the recovered celluloses during enzymatic hydrolysis. As enzymatic hydrolysis proceeds, the relative proportion of total crystalline components increases. The increase of total crystalline cellulose consists of two steps, a rapid phase during the first 8 h of enzymatic treatment and a subsequent slow phase. After the first 8 h of hydrolysis, the total crystalline cellulose exhibits only a 0.6% units increase of relative proportions for the subsequent 24 h of enzymatic treatment.

Figure 7 shows the changes of relative proportions for *para*-crystalline form of celluloses during enzymatic hydrolysis. Larsson and Iversen[31] established the existence of *para*-crystalline component in celluloses by using line-fitting analysis of CP/MAS ^{13}C spectra of cellulosic materials. *para*-Crystalline cellulose has been suggested to be less ordered and more mobile than crystalline cellulose I_α and I_β allomorphs, but more ordered

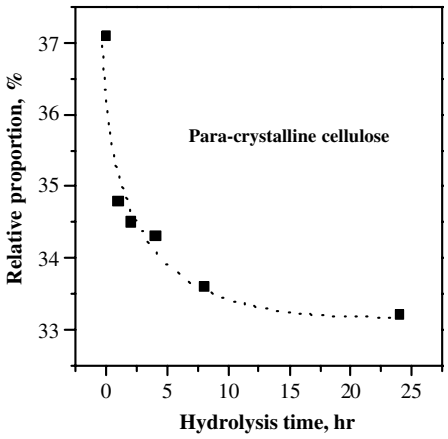

Figure 7. Relative proportions of *para*-crystalline allomorph for the control and cellulase treated pulp samples as determined by line-fitting analysis of CP/MAS ^{13}C NMR spectra.

than amorphous domains of cellulose.[31,32] It can be seen from Figure 7 that the relative proportions of *para*-crystalline component decreases during enzymatic hydrolysis and this process is also characterized by a two-phase reactivity pattern. The enzymatically hydrolyzed pulp samples exhibit a decrease of 3.5% units in relative proportions of *para*-crystalline form over the first 8 h of enzymatic hydrolysis, and only an additional 0.4% units for the subsequent 24 h hydrolysis period.

The amorphous regions of cellulose derived from the line-fitting analysis consist of Gaussian lines from δ 80 to 86 ppm, that is, accessible and inaccessible fibril surfaces.[31,32] The relative proportions of amorphous regions of pulp cellulose during hydrolysis are given in Figure 8. During the first 8 h of cellulase treatment, the amorphous components of pulp cellulose decreased by 3.9% in relative proportions with respect to the control material. However, after 8 h of enzyme treatment,

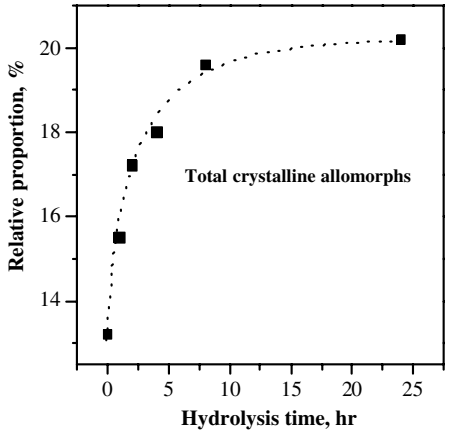

Figure 6. Relative proportions of total crystalline allomorphs for the control and cellulase treated pulp samples as determined by line-fitting analysis of ^{13}C CP/MAS NMR spectra.

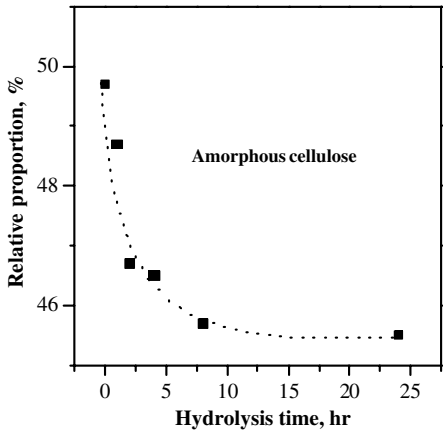

Figure 8. Relative proportions of amorphous cellulose forms for the control and cellulase treated pulp samples as determined by line-fitting analysis of CP/MAS ^{13}C NMR spectra.

virtually no further change in amorphous components of cellulose is observed.

The crystallinity index (CrI) has been considered to be an important factor for monitoring the structural features of the cellulosic substrate. CP/MAS ^{13}C NMR has been used to determine the CrI and proved to be consistent with the FT-IR method.[29,45–47] The crystallinity index was measured as the ratio of the integration area between δ 86.0 and 92.0 ppm to the area between δ 80.0 and 92.0 ppm according to the published method.[29,45] Figure 9 shows the crystallinity results as determined by CP/MAS ^{13}C NMR spectra. The CrI value increases in the initial phase, and then remains almost constant in the second phase, in accordance with the above total crystalline allomorphs results.

Previous studies indicated that enzymatic hydrolysis of cellulose was characterized by a rapid decline in the reaction rate. This slow reaction rate is considered to be one of the major factors affecting the commercial application of enzymatic hydrolysis.[48] The results in Figures 6–9 indicate that cellulase hydrolysis of the fully bleached softwood kraft pulp consists of two phases, a reactive phase, in which the *para*-crystalline and amorphous cellulose components are readily hydrolyzed yielding a cellulose product with progressively increasing amounts of crystalline cellulose allomorphs. After this reactive phase, the *para*-crystalline and amorphous celluloses are enzymatically hydrolyzed at similar rates as the crystalline allomorphs. This change in hydrolysis could be attributed to changes of the cellulose supramolecular composition along with possible changes in enzyme performance and product inhibition.

Crystalline regions of celluloses were normally considered to be more difficult to degrade than amorphous domains, due to chains tightly held by intermolecular hydrogen bonding. Several researchers demonstrated increased crystallinity during enzymatic hydrolysis, and concluded that the loosely structured amorphous regions were hydrolyzed more rapidly than the crystalline domains,[14,49–52] while other studies have observed little changes of crystallinity of cellulose after enzymatic hydrolysis.[53–57] In our study, while the degree of crystallinity was increased during the initial rapid phase of hydrolysis, once the hydrolysis entered the slow phase, the cellulose crystallinity degree remained relatively unchanged. The preferential hydrolysis of cellulose I_α observed in this report is consistent with a recent study by Hayashi et al.,[58] in which they demonstrated that enzymatically hydrolyzed *Cladophora* microcrystalline cellulose was composed mainly of short crystalline elements of cellulose I_β.

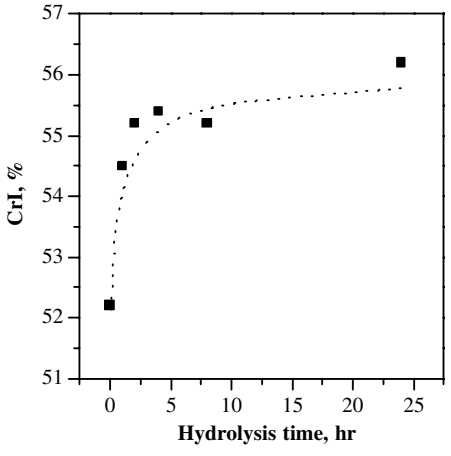

Figure 9. Crystallinity index (CrI) of the control and cellulase treated pulp samples as determined by CP/MAS ^{13}C NMR spectra.

3. Experimental

3.1. Materials

A laboratory prepared fully bleached softwood kraft pulp derived from southern USA pine chips was used for all the studies reported.[59] The carbohydrate composition (w/w) of pulp was determined[60] to be arabinan 0.54%, galactan 0.32%, xylan 8.91%, mannan 6.84%, and glucan 80.44%. The bleached kraft pulp was Soxhlet extracted with acetone overnight, air dried, and then Wiley milled with a 40-mesh grid. The pulp powder was then stored in plastic bags at room temperature.

3.2. Enzymatic hydrolysis

Cellulase (1,4-(1,3:1,4)-β-D-glucan 4-glucano-hydrolase, EC 3.2.1.4) from *Trichoderma reesei* was purchased from Aldrich–Sigma and used as received. The enzyme activities were 0.17 IU/mg measured with Sigmacell® cellulose as substrate at pH 5.0 at 37 °C (IU: international units). The pulp (0.200 g) was suspended in 50.00 mM phosphate acetate buffer adjusted to pH 6.8 with hydrochloric acid at a consistency of 1% (w/v). Enzyme (0.157 g) was added into the suspension and the mixture was incubated at 38 °C under continuous agitation. After hydrolysis, the mixture was frozen and processed according to the literature methods.[9,61] Control pulp was prepared under similar conditions, without enzyme addition.

3.3. CP/MAS ^{13}C NMR spectroscopy

The solid-state CP/MAS ^{13}C NMR experiments were performed on a Bruker Avance-400 spectrometer operating at frequencies of 100.59 MHz for ^{13}C. All the experiments were carried out at ambient temperature using a Bruker 4-mm MAS probe. The control and enzymatic hydrolyzed samples were treated with 2.5 M HCl at 100 °C for 4 h to remove hemicelluloses to facilitate NMR analysis, as described in the literature.[31,35,43] The

pulp samples (~25% moisture) were packed in 4-mm ZrO rotors and spun at 5 kHz and ^{13}C CP/MAS data were acquired following the literature methods[32,33,62] with 8000 scans accumulated/sample. Glycine was used for the Hartman–Hahn matching calibration procedures and as an external standard for the calibration of the chemical shift with the maximum intensity of the carbonyl carbon resonance assigned to 176.0 ppm.[33,42]

3.4. Spectral fitting

Spectral fitting was performed according to the model and method developed by Iversen et al.[30–33,35] The obtained spectral data were transferred from the spectrometer to a personal computer by means of local area network and WS_FTP LE file transfer software (version 5.08 from Ipswitch, Inc). The line-fitting analysis of spectra was performed using NUTS NMR Data Processing software (Acorn NMR, Inc).

4. Conclusions

Different types of cellulose allomorphs (cellulose I_α, cellulose I_β, *para*-crystalline) and amorphous regions were hydrolyzed to a different extent by the enzyme used. Also observed was a rapid initial phase for enzyme hydrolysis followed by a slow hydrolysis phase. Cellulose I_α, *para*-crystalline, and amorphous regions of cellulose are more susceptible to enzymatic hydrolysis than cellulose I_β allomorph during the initial phase. After the initial phase, all the domains are comparably susceptible to hydrolysis in the subsequent slow phase and hydrolyzed at similar rates.

Acknowledgments

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