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UNDERSTANDING NATURE'S PREFERENCE FOR CELLULOSE I ASSEMBLY: TOWARD A NEW BIOTECHNOLOGY ERA FOR CELLULOSE

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INTRODUCTION

Cellulose is the most abundant macromolecule on earth. As such, it has ignited a tremendous impact upon so many human activities. The anomaly of this impact is that while so much is known about the *utilization* of cellulose, relatively little is fully understood about the structure and biosynthesis of the native allomorph. Yet, progress is being made. Recently, we have achieved the perfection of extended glucan chain polymerization and crystallization into the metastable allomorph, cellulose I using two different assembly pathways. The first involves an *in vitro* assembly with the natural substrate, UDP-glucose and a digitonin-solubilized, product-entrapped enzyme fraction from the plasma membrane. The second entails the use of a highly purified cellulase as the catalyst and cellobiosyl fluoride as the substrate in an acetonitrile/aqueous buffer medium. Although these two reaction pathways are very different, they yield the cellulose I allomorph. A third pathway to cellulose I assembly has been more indirect using the living cell, *Acetobacter xylinum*. Photoisomerization of a dye-altered glucan sheet results in the *ex vivo* assembly cellulose I. Molecular modeling with MM3 energy minimization has yielded new data on the sequence of events of crystallization. This presentation will cover these recent advances in our knowledge of cellulose polymerization/crystallization. From these new data have emerged new concepts and strategies to consider in the fabrication of natural polymer systems with superior strength and valuable new properties.

METHODS

In vitro cellulose assembly from higher plant extracts

We have followed essentially the procedures of Okuda, et al [1] with the exception that the extraction buffer consisted of 50 mM 3-[N-morpholino]-propanesulfonic acid (Mops), pH 7.5, 5 mM EDTA, 0.25 M sucrose, and a combination of protease inhibitors. Membranes were resuspended in a small volume of resuspension buffer consisting of 50 mM Mops, pH 7.5, and 0.25 sucrose. We used a two step digitonin solubilization. We found that the first step using 0.1% digitonin in 50mM Mops differentially extracted the ß-1,4 glucan synthase. The second step of digitonin solubilization followed the first, but employed 1.0% digitonin. Enzymes extracted by this method were preferentially ß-1,3 glucan synthases.

Dye-cellulose interactions and cellulose I recrystallization

Methods followed were essentially those of Cousins and Brown [2]. The photoisomerization reaction was followed both by video and electron microscopy. X-ray diffraction was used to analyze the products.

Molecular modeling of glucan chain sheet formation

We followed the methods of Cousins and Brown [3]. Using an energy minimization program developed by Allinger and tested by Alfred French, we calculated and compared the minimum energies for van de Waals interactions and H-bonding interactions in the formation of ß-1,4 glucan chain sheets. From these calculations we are proposing a mechanism of cellulose I crystallization.

Synthetic cellulose assembly

We have followed the methods of Lee et al [4]. A crude cellulase fraction from Onozuka R10 was purified to homogeneity, yielding a 38 kDa polypeptide as the active component in synthetic cellulose assembly. This polypeptide is an endoglucanase. The synthetic cellulose I assembly has been monitored by sequential video analysis as well as electron microscopy. Electron diffraction analysis was used to determine the crystalline allomorph.

RESULTS

In vitro cellulose assembly

Cellulose assembled by membrane extracts from cotton fibers

The two step digitonin solubilization has greatly improved the yield of *in vitro* cellulose from only 7% of the total *in vitro* product [1] to more than 33% of the total glucan product [5]. With this procedure, most of the cellulose produced is cellulose I. The 37 kDa polypeptide [1] has been confirmed to be enriched with the improved *in vitro* synthesis of cellulose I.

Cellulose assembled from mung bean extracts

Even though these results are only preliminary, they are significant, for in 1984) Callaghan and Benziman published that cellulose was made from extracts of mung bean [6]. These observations were subsequently retracted [7], so we wanted to see if following their procedures and using our CBHI-gold probe, coupled with the acetic acid/nitric acid procedure, cellulose could be detected and identified. We did find cellulose; however, using the improved Mops buffer extraction procedure cited above, we greatly improved the *in vitro* yield of cellulose, almost comparable to the results from cotton [5]. Furthermore, we employed the conditions used by Hayashi, et al [8] for mung bean who claim to have found only ß-1,3 glucans, but we found ß-1,4 glucans in addition to the expected -1,3 glucans. Using a second system such as mung bean has proven that the *in vitro* approaches for generating cellulose are broadly applicable among eukaryotic systems.

Synthetic cellulose production

We have discovered that the progressive purification of a certain cellulase from *Trichoderma* crude fraction results in the production of cellulose I [4]. Furthermore, the enzyme active in synthetic cellulose production is an endoglucanase [9]. Elongated, thin microfibrils are synthesized under these conditions, and they give a characteristic cellulose I ed pattern. Manipulation of the organic solvent ratios and substrate concentrations can affect the outcome of synthetic cellulose production.

Modeling cellulose I assembly

Data from dye-altered cellulose

When *Acetobacter xylinum* (ATCC 23769) is grown in the presence of 250 µM Tinopal LPW, the cellulose produced is in the form of glucan chain sheets which coalesce into sealed tubes. The dye binds to both surfaces of the glucan sheets. Irradiation of the cellulose-dye complex with UV induces photoisomerization of the Tinopal, resulting in a separation of the dye from the cellulose. The sheets then collapse into microfibrils of cellulose I. Video microscopy of cellulose microfibril formation has provided information on the dynamic crystallization leading to cellulose I.

Data from computational molecular modeling

Our studies with the dye-altered glucan chain sheets have led to implementation of molecular modeling of cellulose crystallization. Using the MM3 energy minimization program developed by Allinger, we probed glucan sheet formation either by modeling H-bonding or Van der Waals forces. We found that the most stable energy state in glucan chain sheet formation is through Van der Waals interactions. The implications of this model for cellulose I crystallization allow an understanding of the dye-glucan chain interactions as well as the photoisomerization reactions leading to the dynamic crystallization of cellulose I. Furthermore, these data give new insight into how a functioning terminal complex pore of *Acetobacter* can generate glucan chain sheets which subsequently stack to generate the minicrystal of cellulose I.

DISCUSSION

Conditions necessary for crystallization of extended glucan chains

If, during synthesis, the glucan chains are not spatially adjacent to one another in a parallel orientation, they will undergo chain folding and collapse to form the cellulose II allomorph which is the more thermodynamically stable state [10]. Thus, conditions for the assembly of the metastable cellulose I crystalline allomorph require that some initial ordering be provided. In the case of *in vivo* synthesis of cellulose I, this ordering originates from the association of the subunits of the enzyme complexes known as terminal complexes or TCs [11]. In *Acetobacter*, this ordering begins within a single TC which is a basket-shaped receptacle for promoting unidirectional extrusion of parallel glucan chain sheets. The pore structure of the TC may influence the degree of interaction of the glucan chain sheets as they stack to form the crystalline microfibril. We have recently explored through site directed Tn*phoA*/Kan^{r}GenBlock insertion mutagenesis of the various genes of the operon of the *Acetobacter* cellulose synthase complex, conditions which promote cellulose II formation [12]. Apparently the acsD gene is involved in the crystallization, for if this gene is mutated, cellulose II is produced in shaking cultures. When this gene is restored, cellulose I assembly in shaking culture is restored. These studies have revealed that the crystallization of the cellulose product itself may influence the ordering of the catalytic sites, thereby promoting the overall crystallization of glucan chains into cellulose I.

That some ordering is a prerequisite for cellulose I formation is supported by the synthetic cellulose studies in which the endoglucanase itself is not ordered, but becomes oriented in a micelle complex. In this case, we may have generated an artificial TC or ordered complex which has the capacity to direct the polymerization favoring cellulose I formation. Both synthetic and natural *in vitro* systems provide evidence that initial ordering of multiple catalytic sites is very important in the determination of the final outcome of the crystalline allomorph produced. In the evolutionary history of cellulose, it is possible that the most primitive form is cellulose II. Among prokaryotes, only in the more primitive bacterium, *Sarcina*, is cellulose II synthesized *in vivo*. The most advanced bacteria, known as the purple bacteria, of which *Acetobacter* is a member, generate cellulose I *in vivo* [13].

Toward a new biotechnology era for cellulose

Up until now, the major uses of cellulose products have been based on the simple assumption that from various sources of trees and plants, we can "take what we get"; however, it is now clear that certain characteristics of the cellulose might be genetically modified, thus giving new control over the direction of cellulose harvesting. In addition to the traditional sources of cellulose from plants, it has become apparent that microbial cellulose could have a significant impact as a source for highly pure cellulose with properties superior to celluloses from conventional sources. Even more attractive is the longer range prospect to develop *in vitro* systems to assemble cellulose of desired crystallinity and molecular weight. At the present, this is only a dream; however, as we gain insight into the mechanisms which govern polymerization and crystallization, it should become more feasible to develop strategies for *in vitro* systems.

Using genetic engineering approaches with the broadest possible sources for cellulose it should be possible in the near future to genetically engineer into living systems the desired traits which govern cellulose assembly. This would facilitate the development of superior varieties of cotton and forest trees for desired products ranging from

textiles to specific pulps and papers and structural components.

The future for developing genetically superior cellulose-producing crops looks bright, indeed. As the fundamental research characterization of cellulose assembly progresses, we should know much better what to clone and how and when (in a developmental sense) to activate specific cellular processes. An underlying fundamental polymerization mechanism using nucleotide sugars seems to be universal for cellulose-producing organisms. On the other hand, the regulatory systems for the polymerization catalysts may have evolved into rather specialized domains, and in this regard, the field is ripe for continuing fundamental research. At yet another level of organization which includes cellulose microfibril orientation, complex interactions with the cell's microtubule and microfilament systems undoubtedly have important functions in the final cellulose product. The recipe for "improved" cellulose production needs to be more properly defined in terms of which changes will affect specified fundamental properties such as molecular weight, crystalline allomorph, degree of crystallinity, water absorptivity, microfibril size, shape, etc. Once these properties are clearly understood, then changes can be attempted which could modify the cellulose in question. For instance, it may be desirable to have a mixture of cellulose I and cellulose II in certain systems where greater water retention is desired, yet a strong never-dried material is desired. In other systems, it may be desirable to produce only low molecular weight oligosaccharides of ß-1,4 glucans, or it may be useful to have the living cell directly modify the cellulose backbone via substitutions to produce novel cellulose derivatives. All of these options are open for the future as we expand our horizons on the cellulose biosynthesis front. Traditionally, this has been a rather stagnant field, due largely to the extreme difficulty of extracting enzymes with sufficient activity to synthesize *any* cellulose, let alone cellulose I *in vitro*. These problems are now behind us, and with the expanding knowledge, we should be able to tackle these exciting challenges with great vigor and enthusiasm.

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