Double Labeling of Binding Sites in Cellulosic Substrates Using Endo- and Exoglucanase-Gold Complexes

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ABSTRACT

Thin sections of cellulose fibers were incubated with an endo- and an exoglucanase labeled with gold particles of differing sizes. The hydrolytic sites were then visualized under transmission electron microscopy (TEM). The potential interaction between the β-1, 4-glucan substrates and the endo- and the exoglucanases was investigated using cellulosic and lignocellulosic substrates. The simultaneous visualization was very successful in distinguishing preferred substrates for each cellulase in lignocellulosic substrates. When plant lignocellulose was preincubated with endocellulase, density of the gold labeling greatly increased suggesting that preliminary exposure of lignocellulosic material to endocellulase may have enhanced the accessibility of the substrate to endocellulase and exocellulase. This result provided a plausible explanation for the observed endo/exo cellulase co-hydrolysis.

Key words : cellulose, double labeling, endoglucanase, exoglucanase, gold complex,

INTRODUCTION

Cellulose is a linear polymer of anhydroglucose units joined together by β-1, 4-glucosidic bonds. One of the significant chemical features of cellulose is that the two terminal glucose residues differ in their chemical reactivity. One contains a cyclic hemiacetal structure and is called the reducing end group, whereas the other contains an additional secondary hydroxyl group making this residue, the nonreducing end group (*Morohoshi*, 1991). To date, the mechanisms of the enzymatic hydrolysis of cellulose have been explained in terms of the sequential action of three types of enzymes, i.e., endoglucanase (EC 3,2,1,4), exoglucanase (EC 3,2,1,91), and β-glucosidase (EC 3,2,1,21). It has been said that endocellulases catalyze random hydrolysis of β-1, 4-glucosidic linkages of inner amorphous regions of cellulose chain at nonreducing ends to prepare new reaction sites for exoglucanase who will catalyze hydrolysis of β-1, 4-glucosidic linkages by splitting off cellobiose from the nonreducing end of the crystalline cellulose chain (Bielka *et al.*, 1984; Eriksson and Wood, 1985; Eveleigh, 1987; Jeffries, 1987). However, the mechanisms for the synergistic action of both endo- and exoglucanase are not yet fully understood because so far, only pure cellulose substrates were investigated by several workers (Din *et al.*, 1991; Sprey and Bochem, 1993; Garcia-Kirchner *et al.*, 2002) using only one enzyme at a time. One could question the validity of their findings when applying

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these to plant materials since cellulose microfibrils are surrounded by hemicellulose and lignin. Few investigations were performed to show the effect of various cellulases on the complex structure of native plant cell wall. Elucidation of the enzymatic mechanism for the degradation of more complex lignocellulosics could perhaps be achieved by simultaneous ultrastructural localization of both enzymes in various cellulosic materials.

During the 1990s, a procedure known as enzyme-gold complexes was developed for localization of macromolecules (Bendayan, 1984). The molecular probe was more developed by Benhamou *et al.* (1996) for the in situ detection of β-1, 4-glucan-containing molecules at the ultrastructural level. This probe has direct physical contact with the substrate molecules since the enzyme-gold complex attaches itself to sites on the molecular surface of the substrates that are accessible to the enzyme. The purpose of the present cytochemical study was to use this enzyme-gold technique to simultaneously visualize the specific hydrolytic sites of an endoglucanase and an exoglucanase on different lingocellulosic substrates.

MATERIAL AND METHODS

Plant tissue and cellulose substrate preparation

Three substrates were evaluated: Whatman No1 filter paper, alfalfa (*Medicago sativa* L.) and birch wood (*Betula spp.*). They were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2h at room temperature under a gentle pressure and then samples were placed at 4℃ overnight. After three washes (3×20 min.) in buffer, they were postfixed with 1% osmium tetoxide in the same buffer for 2 h at room temperature. They were then dehydrated in an ethanol series and embedded in Epon 812. Ultrathin sections were collected on Formvar-coated nickel grids and processed for cytochemical labeling.

Purification and properties of enzymes Cel5-CBM6

Endoglucanase IV (Cel5) from *Ruminococcus albus* was expressed in *E. coli* JM109 after gene fusion with the single-cellulose binding domain II (CBM6) of *Clostridium stercorarium* as an affinity tag (*Bae et al.*, 2003). The fusion protein (Cel5-CBM6) was purified in a single step procedure, based on the affinity of CBD II for ball-milled cellulose and for cellobiose. Its activity was tested toward carboxymethylcellulose. The molecular mass of the purified fusion protein was calculated to be 46 kDa, its isoelectric point 4.79 and its optimal activity pH 7.0.

A purified exoglucanase (Cel6B; β-1,4-glucan cellobiohydrolase purified from *Thermomonospora fusna*) was a kind gift from Dr. D. B. Wilson (Cornell University, New York). The isoelectric point of the enzyme was found to be 4.13 and its optimal activity pH around 7-8.

Preparation of the enzyme-gold complexes

The endoglucanase or exoglucanase were complexed to colloidal gold according to Benhamou (1996). Briefly, 0.5 mg of the purified enzymes was mixed with 10mL of colloidal gold (15 nm for endoglucanase and 5 nm for exoglucanase) at pH 4.79 and pH 4.13, respectively. The red pellets were recovered after centrifugation at 4℃ at 25,000 rpm (15 nm) and 35,000 rpm (5 nm) for 1hour, using a Ti-rotor. The dark red sediments were resuspended in 0.01 M phosphate-buffered saline (PBS), pH 7.0, containing 0.02% polyethylene glycol 20000. The stock solutions of the enzyme gold complex were stored at 4℃ until use.

Cytochemical experiment

Grids were first floated on a drop of PBS-PEG, pH 7.0, for 5 min, and then transferred on a drop of one enzyme-gold complex for 1 hour at room temperature in a moist chamber. For double labeling, the above step

was repeated using the second enzyme gold complex after washing the grid in the water. They were then jet-washed with PBS, pH 7.2, rinsed with distilled water, and contrasted with uranyl acetate and lead citrate. Examinations were carried out with a JEOL 1200 EX electron microscope.

Specificity of labeling was assessed by means of the following control tests: (1) incubation with the endoglucanase-gold complex or exoglucanase-gold complex which were previously adsorbed with Carboxymethylcellulose with cellulose substrates (2) incubation with a nonenzymic protein-gold complex such as bovine serum albumin (BSA)-gold complex; (3) incubation with the stabilized gold suspension alone.

RESULTS AND DISCUSSION

Cel5-CBM6 and Cel6B were purified to homogeneity and determined by SDS-PAGE. One band was found for each protein (46 kDa and 60 kDa, respectively), which is in good agreement with their theoretical mass (Fig. 1A). The each purified cellulase

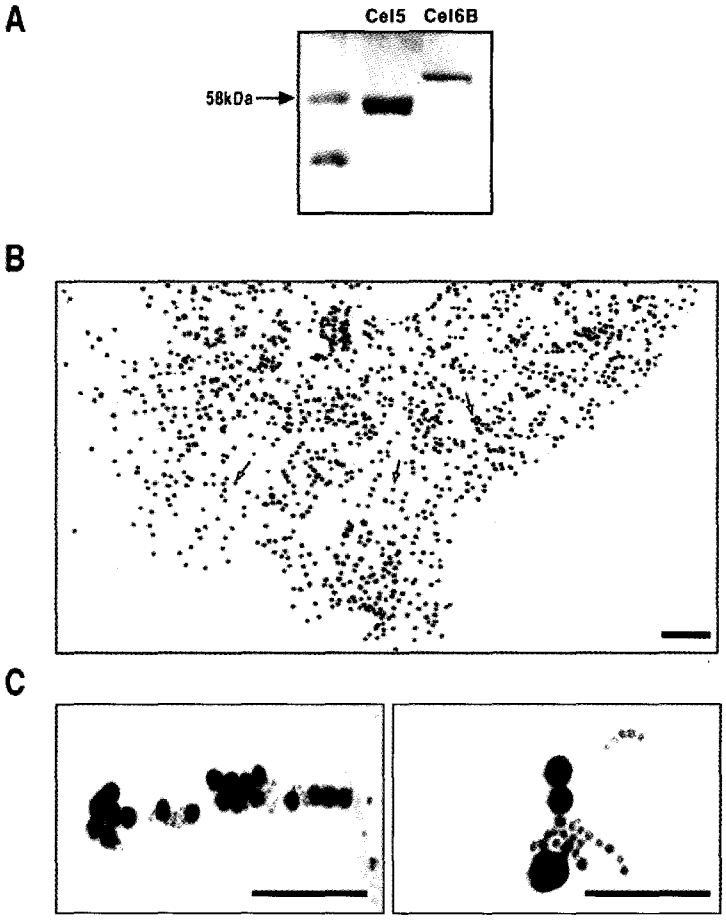

Fig. 1. SDS-PAGE of purified endo- and exoglucanase and double labeling of endo- and exoglucanase-gold complexes on the cellulose microfibrils in the Whatman filter paper. (A) purified Cel5-CBM6 and Cel6B. Lane 1, molecular weight marker; Lane 2, endoglucanse (Cel5-CBM6); Lane 3, exoglucanase (Cel6). (B) Double labeling of endo- and exoglucanase-gold complexes on the cellulose microfibrils in the Whatman filter paper (bar = 500nm). (C) High magnification of double labeling. FP: filter paper. Bar=100 nm.

was directly complexed to colloidal gold and the cellulase linked colloidal gold and tested with various cellulosic materials. The enzyme-gold complex procedure helped to visualize the binding sites on specific substrates (Baldan *et al.*, 2001). Incubation of ultrathin sections of filter paper samples with the endoglucanase-gold complex showed a regular distribution of gold particles over the cellulosic substrate and endoglucanase-gold complex which is mainly found on the cellulose surface of cellulosic filter paper (data not shown). Observation of filter paper with exoglucanase-gold complex revealed particles localization over the cellulose substrate. Following a double incubation with the endo- and exo-gold complex, small and large gold particles were irregularly distributed over cellulose fiber of filter paper even though there is no uniformity of gold labeling (Fig. 1B). It can be observed that endoglucanase-gold particles mostly occurred in terminal areas of cellulose microfibril (Fig1Ca) and that exoglucanase-gold particles attached themselves to all regions of cellulose microfibrils (Kim *et al.*, 1997; Mayer, *et. al.*1987) (Fig. 1Cb). The present electron microscopic work does not indicate whether cellulase-gold complexes preferentially attacked the amorphous or the crystalline cellulose. However, our enzyme-gold complex work clearly showed that endo- and exoglucanase were associated with the cellulose microfibrils, mainly along the surface of disrupted cellulose microfibrils.

Examination of ultrasections of alfalfa plant revealed that they were composed of fibres, tracheids, and vessels. The sequential incubation of alfalfa samples with Cel5-CBM6 and Cel6B-gold complexes (15 nm and 5 nm respectively) produced intense labeling. Difference in pattern of labeling of both cellulases occurred over the alfalfa cell wall (Fig. 2). In alfalfa, endoglucanase-gold particles (15 nm) were mainly associated with the secondary cell wall while the compound middle lamella (CML) and intercellular spaces (IS) were virtually devoid of them (Fig. 2A). By comparison, exoglucanase-gold particles (5 nm) were observed over the entire cell wall although the secondary wall was irregularly labeled (Fig 2B). No exocellulase was seen in cell cytoplasm. Such preference in labeling distribution may reflect physiological difference in cellulose and lignin content or microfibril angle in the plant cell wall. The specificity of the labeling was tested against several

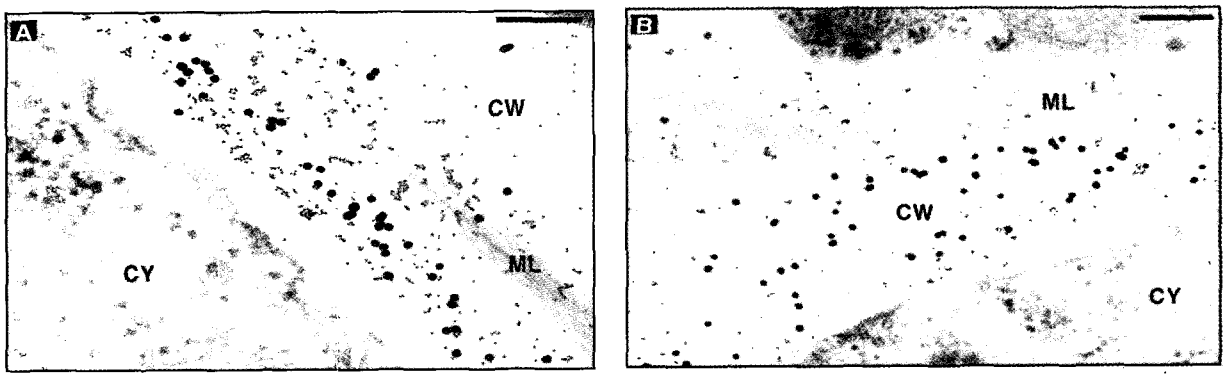

Fig. 2. Electron micrographs showing double labeling experiments with Cel5-CBM6 (15 nm gold particles) and Cel6 (5 nm gold particles) gold complex in alfalfa. ML: middle lamella, CW: cell wall, CY: cytosol. (A) Numerous gold particles of endoglucanase are presented with the S1 layer of the secondary wall, close to which the primary wall. Labeling of exoglucanase is observed over the entire cell wall. (B) Large gold particles (Cel5-CBM6) are occurred mostly to the secondary wall layer but very weak to middle lamella and cell coner but the small ones (Cel6) are observed the compound middle lamella and the external layer of the secondary walls. bar = 300 nm.

controls. Only low level of non specific labeling over sections was obtained.

Next, we examined whether a prior treatment of the birch wood cellulose substrate with an endocellulase, can increase binding of endo-and exocellulase to the microfibril. Birch was pre-treated with endocellulase, and subsequently labeled the endo- or exoglucanase-gold complexes resulted in a significant increase in the number of gold particles in tissues of birch wood (Fig 3). This increase could be attributed to the greater availability of binding sites following the hydrolysis of cellulose by endocellulase. The specificity of the labeling was tested against several controls. Only low level of non specific labeling over sections was obtained. There are several advantages of the technique presented as double labeling with enzyme-gold complexes. As demonstrated here, one is its usefulness in discriminating between two enzymes' binding sites to substrates. The ability to localize each of the enzymes' substrate may prove useful for distinguishing targeted substrates. Another advantage of this method is its ease of use. It does not require antibody or long preparation. This technique is presently being tested on a larger variety of lignocellulosic materials in our lab. The potential interference of the binding domain of the endoglucanase (fusion protein)-gold complex will be determined.

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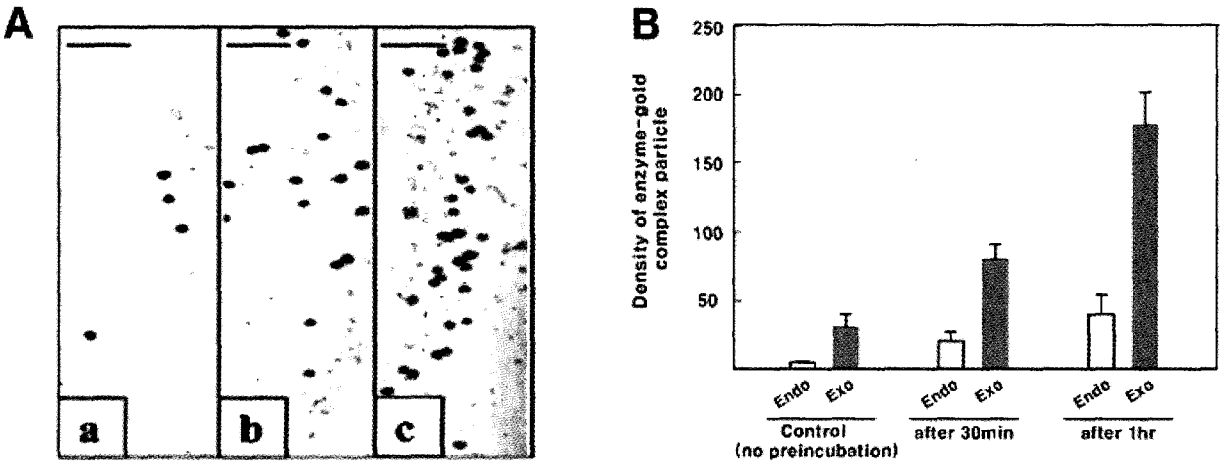

Fig. 3. Double labeling of endo-exoglucanase-gold complexes on the cellulose sample in birch.
(A) Birch, wood sample was pre-treated with endocellulase, and subsequently labeled the endo- or exoglucanase-gold complexes a. no pre-incubation, b. 30 min incubation, c. 1hour incubation. Bar = 200 nm., (B) Density of cellulase-gold complex particles after digestion of birch with Cel5-CBM6 (# gold particles per square micrometre of the wood cell wall). Data is expressed as a mean of 5 fields.

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