Use of Recombinant Cellulose-Binding Domains of *Trichoderma reesei* Cellulase as a Selective Immunocytochemical Marker for Cellulose in Protozoa

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Some unicellular organisms are able to encyst as a protective response to a harmful environment. The cyst wall usually contains chitin as its main structural constituent, but in some cases, as in *Acanthamoeba*, it consists of cellulose instead. Specific cytochemical differentiation between cellulose and chitin by microscopy has not been possible, due to the similarity of their constituent β-1,4-linked hexose backbones. Thus, various fluorescent brightening agents and lectins bind to both cellulose and chitin. We have used a recombinant cellulose-binding protein consisting of two cellulose-binding domains (CBDs) from *Trichoderma reesei* cellulases linked together in combination with monoclonal anticleellulase antibodies and anti-mouse immunoglobulin fluorescein conjugate to specifically stain cellulose in the cysts of *Acanthamoeba* strains for fluorescence microscopy imaging. Staining was observed in ruptured cysts and frozen sections of cysts but not in intact mature cysts. No staining reaction was observed with the chitin-containing cyst walls of *Giardia intestinalis*, *Entamoeba dispar*, or *Pneumocystis carinii*. Thus, the recombinant CBD can be used as a marker to distinguish between cellulose and chitin. Thirteen of 25 environmental or clinical isolates of amoebae reacted in the CBD binding assay. All 13 isolates were identified as *Acanthamoeba* spp. Five isolates of *Hartmannella* and seven isolates of *Naegleria* tested negative in the CBD binding assay. Whether cyst wall cellulose really is a unique property of *Acanthamoeba* spp. among free-living amoebae, as suggested by our findings, remains to be shown in more extensive studies.

Protozoan parasites, which occur in a trophic form, have the ability to protect themselves by forming a cyst wall, which is resistant to environmental stress such as desiccation, lack of nutrients, and variations in temperature and pH. In most pathogenic protozoa studied, chitin is the carbohydrate polymer conveying the required structural toughness to the cyst wall. *Acanthamoeba* spp. are exceptions, as their endocysts are made up of cellulose (6, 35). The exact composition of ectocyst wall is not well defined. In addition to proteins and lipids, the presence of putative carbohydrate components is suggested by lectin binding studies (7, 29).

Cellulose consists of β-D-glucosyl units linked by β-1,4-glucosidic bonds. Chitin is very similar but contains N-acetylglucosamine as the monomer. Both polymers form very similar crystalline macroscopic structures (5). Specific cytochemical differentiation between cellulose and chitin by microscopy has not been possible due to the similarity of the constituent β-1,4-linked hexose backbones. This is especially true for various fluorescent brightening agents, such as calcofluor white, used as a cytochemical marker in microscopic diagnostics of protozoan and fungal infections, such as intestinal microsporidiosis, *Acanthamoeba* keratitis and *Pneumocystis* pneumonia (9, 12, 28).

A two-domain structural organization is often observed in cellulose-degrading enzymes. Most *Trichoderma reesei* cellulases consist of a catalytic domain and a cellulose-binding domain (CBD) joined by a linker. The catalytic domain contains the active site with the amino acid residues responsible for the hydrolytic mechanism. The role of the CBD is binding to the solid cellulose. The ability of CBDs to attach to cellulose can be utilized in various applications. Individual types of CBDs can vary significantly in their properties, such as affinity, preference for crystalline or amorphous cellulose, and cross-reactivity with other similar carbohydrates (25).

Members of the genus *Acanthamoeba* have recently received much attention as potential carriers of pathogenic bacteria, with *Legionella* species being the most intensely studied (14). The present study was initiated as part of our attempts to identify free-living amoebae containing pathogenic bacteria in water samples to which patients with *Legionella* pneumonia had been exposed (J. Winiecka-Krusnell, E. Linder, M. Lundholm, E. Hjelm, and H. Hallander, presented at the Annual Meeting of Swedish Physicians in Gothenburg, Sweden, 1998).

The current identification method for isolates of free-living amoebae, which is based on morphological and biochemical features, is labor-intensive and requires cloning and axenization. Our objective was to develop a rapid method for the specific identification of *Acanthamoeba* in samples containing a mixed population of amoebae. In this study we used a recombinant dimeric CBD fusion protein in indirect immunofluorescence to specifically stain the cellulose and visualize its localization in cyst wall.  

MATERIALS AND METHODS

D-CBD and anti-CBD antibodies. The CBD used in this work is a recombinant fusion protein consisting of the 38-amino-acid CBD from *T. reesei* cellulohy-
TABLE 1. Reference protozoan species used in the study

| Parasite                  | Origin                        | Cyst wall constituent (reference) |
|---------------------------|-------------------------------|----------------------------------|
| Acanthamoeba spp.         |                               | Cellulose (35)                   |
| Acanthamoeba castellanii  | Human cornea                  |                                  |
| Acanthamoeba rhyodes      | Human cornea                  |                                  |
| Acanthamoeba sp. strain V 38 | Tissue culture              |                                  |
| Hartmannella vermiformis  | ATCC (ATCC 50256)             |                                  |
| Giardia intestinalis HPI88 | Human stool (19)              | Chitin (38)                      |
| Entamoeba dispar          | ATCC (ATCC 30931)             | Chitin (10)                      |
| Pneumocystis carinii      | Human sputum                  | Chitin (37)                      |

* Typed at Public Health Laboratory Service, Bath Avon, England.
* ATCC, American Type Culture Collection.
* No information available.

We showed that cellulose can easily and efficiently be stained and detected by immunofluorescence using T. reesei CBDs in combination with anti-CBD and fluorescein-labeled antibodies. We further showed that this staining is applicable to the detection of cellulose in the inner cyst wall of Acanthamoeba spp. The reference strains of Acanthamoeba spp. and 13 Acanthamoeba strains isolated from water and from keratitis pa-

TABLE 2. Isolates of free-living amoebae belonging to the genus *Acanthamoeba* which tested positive for the presence of cellulose in the cyst wall

| Isolate | Source                  |
|---------|-------------------------|
| Ac 3172 | Human cornea, Sweden    |
| Ac 896  | Human cornea, Sweden    |
| MTC 4a  | Tap water from eye shower, Sweden |
| SMI 2   | Tap water from ice machine, Sweden |
| I 3     | Tap water from hospital, Sweden |
| BMH18   | Tap water from hospital, Germany |
| B 6     | Biowaste, hospital, Germany |
| E 46    | Tap water, Nicaragua    |
| B 77    | Tap water, Nicaragua    |
| B 99    | Well, Nicaragua        |
| E 52    | Well, Nicaragua        |
| I 10    | Geyser, Iceland        |
| I 4     | Geyser, Iceland        |

* Staining was performed on cryosections.

TABLE 3. Free-living amoebae of the genera *Hartmannella* and *Naegleria* which tested negative for the presence of cellulose in the cyst wall

| Isolate | Source                  |
|---------|-------------------------|
| Hartmannella |                          |
| U1       | Tap water, hospital, Sweden |
| U3       | Tap water, hospital, Sweden |
| HSVA 997 | Tap water, hospital, Sweden |
| MTC 2b   | Tap water, eye shower, Sweden |
| I 8.2    | Geyser, Iceland        |
| Naegleria |                          |
| I 8.1    | Geyser, Iceland        |
| SMII     | Tap water, ice machine, Sweden |
| E 14     | Tap water, Nicaragua    |
| E 12     | Well, Nicaragua        |
| E 51     | Well, Nicaragua        |
| E 55     | Well, Nicaragua        |
| E 57     | Well, Nicaragua        |

* Staining was performed on cryosections.
FIG. 1. (A) Cellulose of *Acanthamoeba castellanii* stained by immunoﬂuorescence using recombinant *T. reesei* D-CBD in combination with monoclonal mouse anti-CBD antibodies and FITC-labeled anti-mouse immunoglobulin antibodies. (A) Strong reaction with two ruptured amoeba cysts but not with three intact cysts. (B) Corresponding area seen by phase-contrast microscopy. (C and D) Double ﬂuorescence staining for cellulose in *A. castellanii* in suspension using the CBD procedure (C) and calcoﬂuor (D). Note the similar distribution of cyst wall staining with the two methods and absence of staining of the excysted trophozoite. (E) The same area seen by phase contrast illumination. (F) Control CBD staining of cotton ﬁbers showing cellulose-speciﬁc ﬂuorescent labeling. (G) Corresponding area seen by phase-contrast illumination. (H) The CBD assay fails to detect *P. carinii* organisms (arrows) in bronchoalveolar lavage ﬂuid from patient with pneumonia. The red dots represent nuclear material visualized with DAPI. (I) *P. carinii* cysts are readily detected after double staining with calcoﬂuor. Bar, 10 μm.
FIG. 2. Staining for cyst wall cellulose in frozen sections of different protozoan cysts using the D-CBD procedure. (A) Control sections of *A. castellanii* show a distinct linear cyst wall reaction, whereas *G. intestinalis* (B), *E. dispar* (C), and *H. vermiformis* (D) fail to react. (C’/H11032 and D’/H11032) The latter two organisms shown by phase-contrast illumination. Bar, 10 μm.
patients reacted in a D-CBD–anti-CBD immunofluorescent test (Table 2). All Naegleria and Hartmanella isolates (Table 3) gave negative reactions. The strongest reaction was obtained with empty cyst shells of Acanthamoeba spp. left after exsuction, while trophozoites were negative (Fig. 1). In contrast to the D-CBD, calcofluor bound to both cellulose-containing Acanthamoeba spp. (Fig. 1D) and chitin-containing P. carinii (Fig. 11). Calcofluor and related optical brightening agents with broad absorption and emission spectra have been used for diagnostic purposes to detect not only Pneumocystis but also Entamoeba, Chilomastix, and Microsporida (9). Calcofluor does stain both cellulose and chitin, making it impossible to distinguish between them, and the putative chitin-specific fluorescent probe Fungalase-F-FITC (Anomerics, Inc.) was shown also to react with cellulose (34). Both cellulose and chitin are recognized by N-acetylgalactosamine-specific lectins such as wheat germ agglutinin and tomato lectin (20, 38). Furthermore, in a carefully performed study using other fluorescein-conjugated CBDs (family II CEX from T. reesei and CBHII from T. reesei single domains), it was not possible to distinguish between cellulose and chitin (34).

The failure of the D-CBD to react with mature, intact cysts of Acanthamoeba spp. is apparently due to lack of access of the D-CBD to cellulose present at the inner aspect of the cyst wall. No reaction was seen with a H. vermiformis reference strain or with G. intestinalis, E. dispar, or P. carinii cysts known to contain chitin (10, 37, 38).

The D-CBD used in the present study was shown previously by other methods to have some affinity for chitin but not for other insoluble carbohydrates, such as mannan or xylan (23), or any significant binding to soluble carbohydrates. The reason for the observed selective staining of cellulose in the present study is consistent with previous observations on the behavior of different variants of the CBD. The CBHII CBD alone is not very tightly bound to cellulose and does not significantly bind to chitin (23, 26). Because it is easily washed away from cellulose, it is not by itself useful as a probe, as noted previously (22, 34). However, the D-CBD is, mainly due to the CBHII CBD, tightly bound to cellulose (22), although it is easily washed away from chitin (8). The D-CBD has the additional benefit that it can be produced heterologously rather efficiently in E. coli. Detection of chitin using antichitin antibodies seems to be possible (20, 37). In analogy with the demonstrated cellulase-based immunocytochemical detection of cyst wall cellulose, the possibility of detecting chitin with chitinase is obvious. Chitinases occur widely in nature and appear to be efficient anti-fungal agents (16). Interestingly, a highly sensitive assay to detect alpha-chitin has been developed based on the binding of a unique protein from Streptomyces olivaceoviridis, which interacts specifically with crystalline alpha-chitin (39).

Lack of D-CBD binding to the chitinous cyst walls however, is not a definite proof of cellulose specificity or of lack of chitin reactivity, since some intact Acanthamoeba cysts failed to stain. Apparently mature cysts pose a diffusion barrier. Such a masking of cellulose by noncellulose materials has also been suggested in previous studies (17, 35). We tried trypsinization prior to incubation with D-CBD and anti-CBD antibody without success. Drastic chemical treatment may be necessary, as shown for the chitinous layer of Neurospora crassa hyphae (17), but preliminary experiments using 1 M sodium hydroxide caused severe morphological damage and nonspecific background staining. We therefore prepared frozen sections, which yielded reproducible results (Fig. 2). A positive staining reaction was seen only with Acanthamoeba cysts. No reactivity of D-CBD was seen on sections of G. intestinalis, E. dispar, or H. vermiformis.

Free-living amoebae represent a large, nontaxonomic group comprising various genera which commonly inhabit all types of aquatic environments. Several different species can be detected in drinking water distribution systems (15). Three frequently found genera, which also have medical importance, were included in our study. H. vermiformis has been identified as the most dominant amoeba in water distribution systems, often associated with the presence of Legionella pneumophila (31, 32), while Acanthamoeba and Naegleria, in addition to supporting growth of pathogenic bacteria, are potential human pathogens (4, 18). There is very little information on the constituents of cyst walls of free-living amoebae. Among the genera tested here, D-CBD binds only to cysts of Acanthamoeba, suggesting that the others do not contain cellulose. However, if the presence of cellulose is a unique characteristic of Acanthamoeba spp., distinguishing this genus from other free-living amoebae will require further investigation of other species in this group.

The described method has potential as a diagnostic tool for the detection of cellulose-containing protozoa, which also seems to be useful for tissue sections, as shown recently (27). The problems encountered in studies on environmental samples were (i) exposing the cyst wall cellulose in order to make it accessible to the CBDs, (ii) getting rid of nonspecific background fluorescence, and (iii) making CBDs readily available to interested workers in the field.

We are addressing these points in ongoing studies. It should be possible to solve the problem of exposure of cellulose by using different extraction methods eliminating the barrier formed by the outer cyst membrane. A novel D-CBD, produced as a hydrophobin I fusion protein in the homologous host T. reesei, which can be easily purified by two-phase separation at production levels of grams per liter (24), also selectively recognizes cellulose. The problem of nonspecific background fluorescence due to autofluorescence of protozoan cyst walls can be eliminated by using immunocytochemical staining methods for light microscopy. Furthermore, direct labeling of the D-CBD obviously should be a practical improvement.

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