

Double-Antibody Sandwich Enzyme-Linked Immunosorbent Assay for Quantitation of Endoglucanase I of *Trichoderma reesei*

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A sensitive and specific enzyme-linked immunosorbent assay for endoglucanase I (EG-I) has been developed. The monoclonal antibody a-EG-I 2, directed against an epitope on the core part of the enzyme, was used to capture the antigen in microtiter plate wells. A second, polyclonal antibody against the enzyme was then used to detect and quantitate the bound antigen. The test was specific for EG-I; neither endoglucanase II nor cellobiohydrolase I or II interfered. As little as 20 pg of EG-I protein could be detected. The coefficients of variation were 3.8% within plates and 6% between plates for a diluted *Trichoderma reesei* culture supernatant that contained 31 ng of EG-I per ml. Binding of the antigen to the monoclonal antibody was pH dependent and restricted to values between pH 6.5 and 10.5 with a maximum around pH 9. Standard solutions of EG-I were very stable at concentrations as low as 5 ng/ml when prepared in buffer that contained 1% bovine serum albumin and that was stored at -20°C. After 37 weeks the antigenicity was still 97%. With this test it was possible to monitor the production of EG-I in a cellulase-producing strain of *T. reesei* and to demonstrate the apparent absence of the enzyme in a strain with the *eglI* gene deleted.

The cellulose-degrading enzymes from the filamentous fungus *Trichoderma reesei* are of great biotechnological importance. They are used in starch processing, animal feed applications, grain alcohol fermentation, malting and brewing, and the textile industry (9, 18, 21). New applications are being sought in the pulp and paper processing industry (20).

Present *T. reesei* strains are capable of secreting up to 40 g of extracellular protein, most of which is cellulase (8), per liter. However, the proportions of individual enzyme components (as percentages of the total secreted proteins) and their specific activities remain essentially constant: cellobiohydrolases, ca. 64 to 80%; endoglucanases, ca. 20 to 36%; and β -glucosidases, only ca. 1% (5, 11, 23).

To obtain *T. reesei* strains with optimized compositions of cellulase that were therefore capable of performing desired steps in industrial processes, we started to alter the genetic makeup of this fungus (21). One of the major obstacles to the evaluation of the success rate and to the establishment of new enzyme compositions in these genetically engineered strains is the specific quantitation of these enzyme proteins in the culture supernatants.

The cellulase enzymes exhibit overlapping substrate specificities and synergistic activities (14). This makes it very difficult to assign specific activities and to determine the enzyme protein quantity specifically, especially in such complex mixtures as culture supernatants without previous separation and purification of the components, which inherently involve losses. The standard assay for endoglucanase is the hydroxyethyl cellulose assay (3). However, there are at least two (4, 30), and probably even more (13, 29), different endoglucanases in *T. reesei*. Thus, the published methods for the measurement of endoglucanase and cellobiohydrolase activities (32) are not completely specific and are therefore only of limited value. This is of particular importance in instances in which one wants to assess the deletion of a gene with the subsequent absence of a particular enzyme or the presence of an inactive enzyme.

A way out of this dilemma is the application of immunological tests for the differentiation and quantitation of cellulases, such as endoglucanase I (EG-I), the main endogluca-

nase produced by *T. reesei*. This approach was initially introduced by Fägerstam and Pettersson (10), Nummi et al. (24), Schülein et al. (28), and Oh et al. (25), who used polyclonal antibodies. However, polyclonal antibodies against cellulases show significant cross-reactivity and are not useful for the specific detection and quantitation of single enzyme species (2, 24, 30). Only monoclonal antibodies against the various cellulase enzymes (1, 16, 19, 22, 26) are capable of specifically detecting one single cellulase enzyme in complex mixtures such as culture supernatants.

Recently, the procedures for a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) for the quantitation of cellobiohydrolase I (CBH-I) (27) and different ELISAs for CBH-I, CBH-II, and EG-I (16) were published.

Here I report the development of a highly specific and sensitive double-antibody sandwich ELISA for EG-I with a monoclonal anti-EG-I antibody as the capture antibody. With this ELISA it is possible to specifically and quantitatively measure EG-I protein in complex mixtures such as culture supernatants of *T. reesei* that contain all of the different cellulases as well as other proteins.

MATERIALS AND METHODS

The enzymes EG-I (EC 3.2.1.4), EG-II (EC 3.2.1.4), CBH-I (EC 3.2.1.91), and CBH-II (EC 3.2.1.91), purified from *T. reesei* and stored frozen at -70°C in 50 mM sodium acetate (pH 5.0) containing 0.02% sodium azide, were obtained from M.-L. Niku-Paavola, Technical Research Centre, Espoo, Finland.

The characteristics of polyclonal antibodies against EG-I produced in rabbits and purified by protein A-Sepharose (Pharmacia, Uppsala, Sweden) affinity chromatography were analyzed previously (1, 2).

For the quantitation of EG-I in culture supernatants, the monoclonal antibody a-EG-I 2 was chosen, primarily because of its high apparent affinity and easy availability. Its production, characterization, and purification on protein G-Sepharose (Pharmacia) were described previously (1).

Alkaline phosphatase-conjugated anti-rabbit immunoglob-

ulin G (IgG; heavy and light chains) produced in swine and alkaline phosphatase-conjugated anti-mouse IgG (heavy and light chains) produced in rabbits were obtained from Orion Diagnostics, Espoo, Finland.

Bovine serum albumin (BSA) was from Sigma, St. Louis, Mo.; diethylamine-magnesium chloride buffer (pH 10) was from Orion; *p*-nitrophenyl phosphate was from Merck, Darmstadt, Germany; and Tween 20 was from Serva, Heidelberg, Germany. All other substances were of the highest purity available and purchased from various suppliers.

The concentration of purified enzyme protein was determined in 96-well flat-bottomed microtiter plates (Immuno-plate, maxisorb; Nunc, Roskilde, Denmark) with a protein assay kit (Bio-Rad, Richmond, Calif.) with bovine IgG as the standard protein as described by Bradford (6). The concentration of purified antibodies was determined by photometry at a 280-nm wavelength with an extinction coefficient of 14 for a 1% solution. Assays in culture supernatants were carried out after the mycelia and insoluble culture medium components were removed by centrifugation. The total protein in the culture supernatant was determined by the method of Lowry et al. (17) with BSA as the standard. Enzyme activity against hydroxyethyl cellulose (mittelviskös; FLUKA, Buchs, Switzerland) was measured as described by Bailey and Nevalainen (3).

The absorbance of solutions in microtiterplate wells was measured in a Multiskan PLUS microtiter plate reader (Labsystems, Helsinki, Finland).

Two ELISA systems were used: (i) an ELISA to measure antibody binding to antigen and (ii) a double-antibody sandwich ELISA to determine antigen. The general procedures were as follows.

(i) One hundred microliters of purified antigen (1 μ g/ml), diluted in 30 mM Tris-HCl buffer (pH 8.5), was incubated in microtiter wells overnight (15 to 20 h) at 4°C. Afterward, the plates were washed with PBST (20 mM sodium phosphate buffer [pH 7.2] containing 150 mM sodium chloride, 0.02% sodium azide, and 0.05% Tween 20) by emptying and filling the plates three times. Then the plates were incubated with 200 μ l of a 1% BSA solution in PBST per well for 1 h at 37°C to block the remaining binding sites on the plastic surface. After the plates were emptied, 100 μ l of antiserum, ascites, hybridoma culture supernatant, or another test solution containing antibodies diluted in 1% BSA in PBST was incubated for 2 h at 37°C and then washed three times with PBST. To detect bound polyclonal antibodies, the plates were then incubated for 2 h at 37°C with 100 μ l of anti-rabbit IgG-alkaline phosphatase conjugate diluted in PBST. To detect bound monoclonal antibodies, the plates were incubated with anti-mouse IgG-alkaline phosphatase conjugate. After three final washes with PBST, 200 μ l of a 1-mg/ml *p*-nitrophenyl phosphate solution in substrate buffer was added to the wells, and the color was developed for 30 min at room temperature. The reaction was stopped by the addition of 50 μ l of 2 N sodium hydroxide, and the A_{405} was measured.

(ii) One hundred microliters of purified monoclonal antibodies (10 μ g/ml), diluted in phosphate-buffered saline (PBS) was incubated in microtiter wells at 4°C overnight (15 to 20 h). Then the plates were washed three times with PBST. Blocking was performed as in ELISA system i. After the plates were emptied, 100 μ l of an antigen-containing sample or a standard antigen, diluted in 0.5 M Tris-HCl buffer (pH 8.5) and containing 1% BSA and 0.05% Tween 20, was added to each well. The plates were incubated for 2 h at 37°C and

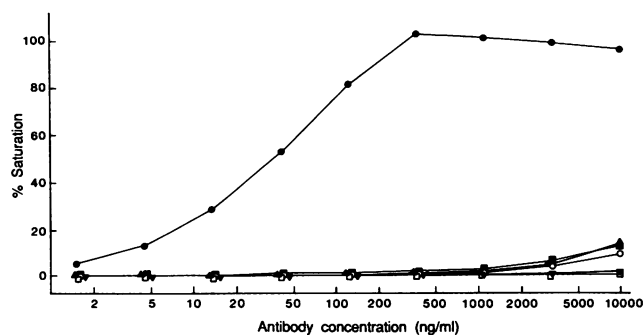


FIG. 1. Specificity of the monoclonal antibody a-EG-I 2 (purified from ascites fluid by affinity chromatography on protein G-Sepharose). The ELISA system i was used. Plates were coated with EG-I (●), EG-II (▼), CBH-I (▲), CBH-II (■), BSA (○), or bovine IgG (□). Only with EG-I was highly specific binding observed. The points are average values of duplicate measurements.

then washed three times with PBST. To detect bound antigen, 100 μ l of a second, polyclonal antibody (5 μ g/ml), diluted in 1% BSA in PBST was added and incubated for 2 h at 37°C. To detect the bound second antibody, the plates were washed three times with PBST, 100 μ l of anti-rabbit IgG-alkaline phosphatase conjugate diluted in PBST was added to the wells, and the plates were incubated for a further 2 h at 37°C. The subsequent steps were identical to those in procedure i.

The analytical detection limit is defined as the concentration at the intersection of a standard dilution curve with the mean blank A_{405} plus 4 standard deviations. In general, there were six blank values per plate and one standard curve.

The following buffers were used to determine the pH dependence of the antigen binding to the monoclonal capture antibody: pH 10.5 sodium borate-NaOH; pH 9.5 and 8.5 Tris-HCl; pH 7.5, 6.5, and 5.5 sodium phosphate; pH 4.5 and 3.5 sodium acetate; and pH 2.5 sodium citrate-HCl. All of these buffers were 0.2 M and contained 1% BSA, 0.05% Tween 20, and 0.02% sodium azide.

Liquid cultivation of *T. reesei* was performed in 250-ml shake flasks (250 rpm) at 30°C in cellulose-containing medium. The pH was initially 5.2. It decreased during the culture to ca. pH 3.

RESULTS

Specificity of the monoclonal antibody. In addition to the criteria for specificity described previously (1), the specificity of the monoclonal a-EG-I 2 antibody was tested by using ELISA system i. a-EG-I 2 binds only to plates coated with purified EG-I antigen and not to plates coated with other closely related enzymes (EG-II, CBH-I, and CBH-II) or to the unrelated proteins BSA and bovine IgG (Fig. 1). A slow increase in nonspecific binding to the other proteins was observed with antibody concentrations above 10 μ g/ml, whereas specific binding to EG-I reached saturation already at antibody concentrations below 400 ng/ml, with a value at 50% of 38 ng/ml.

Optimization of the double-antibody sandwich system. The microtiter plates were coated with the monoclonal antibody a-EG-I 2 in PBS. Figure 2a shows the effect of coating the plates with various concentrations of a-EG-I 2. A concentration of 10 μ g of a-EG-I 2 per ml was determined to be optimal. It gave a final optical density at 405 nm (OD_{405}) of ca. 2 at saturation, a value that did not increase with higher

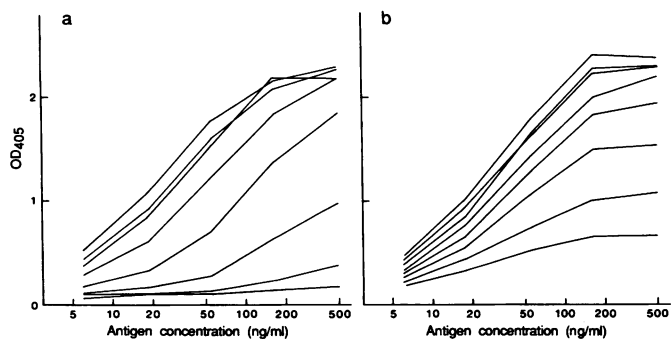


FIG. 2. Optimization of the double-antibody sandwich ELISA for EG-I. The ELISA system ii was employed. (a) Determination of the concentration of purified monoclonal antibody a-EG-I 2 for optimal coating. The concentrations ranged from 45 ng/ml (lowest curve) to 100 µg/ml (highest curve) in threefold increments. An optimal concentration of 10 µg/ml was determined. (b) Determination of the optimal concentration of a second, polyclonal a-EG-I antibody (purified by affinity chromatography on protein A-Sepharose). The concentration of second antibody ranged from 250 ng/ml (lowest curve) to 16 µg/ml (highest curve) in twofold increments. An optimal concentration of 5 µg/ml was determined.

antibody concentrations but decreased substantially with lower concentrations. No difference was found between coating in sodium carbonate (pH 9.6) and PBS. PBS was chosen for convenience.

BSA (1% in PBST) completely blocked any free sites remaining on the plastic surface after coating and resulted in OD_{405} values below 0.1 and no unspecific binding (see Fig. 4).

Figure 2b demonstrates the dependence of the final OD values on the concentration of the second, polyclonal antibody. A plateau at an OD_{405} of ca. 2 was reached with polyclonal antibody concentrations of 4 µg/ml and above. Lower concentrations resulted in lower OD_{405} values. A second antibody concentration of 5 µg/ml was chosen as optimal. The binding of the second antibody to the antigen (already bound to the monoclonal antibody on the plate) was the same at pH 8.5 (100%) as at pH 7.5 (96%). PBST (pH 7.5) was chosen because the same buffer was already used for blocking.

The binding of EG-I to the monoclonal antibody a-EG-I 2 was highly dependent on the pH of the incubation buffer (Fig. 3). The binding of antigen was restricted to pH values above 6.5 and below 10.5, with maximal binding between 8.5 and 9.5. Binding at pH 7.5 was only 30% of the binding at pH 8.5. The pH dependence was similar for the purified EG-I and for the EG-I in *T. reesei* culture supernatants. Culture medium alone (incubated for the same time period as a *T. reesei* culture) served as a control and did not result in elevated OD_{405} values. Because the culture supernatants were often highly acidic (down to pH 3), 0.5 M Tris-HCl buffer at pH 8.5 was chosen as the diluent (binding buffer) to maintain the optimal pH for antigen binding.

A conjugate dilution of 1:200 in PBST resulted in a maximal OD_{405} of ca. 2 at antigen and antibody saturation without giving excessive blank values (OD , <0.1).

The incubation times and temperatures were not specifically optimized. The protocol as it emerged (1 day with overnight coating) proved reasonably practical.

Specificity of the double-antibody sandwich ELISA. The specificity of the test relies on the specificity of the monoclonal antibody used to coat the plates (1; this study). The

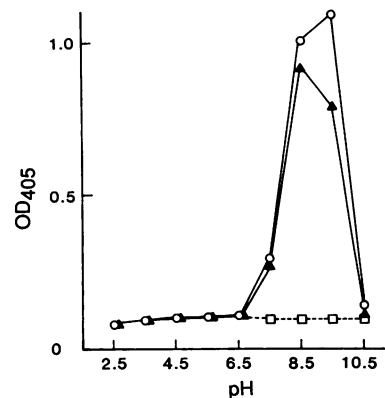


FIG. 3. pH dependence of antigen binding: purified EG-I, 60 ng/ml (○); diluted *T. reesei* VTT-D-79125 culture supernatant containing 40 ng of EG-I per ml (▲); medium A alone (□). The ELISA system ii was used. The buffers are listed in Materials and Methods. The points are average values of duplicate measurements.

second, polyclonal antibody against EG-I is not specific but also detects all other investigated cellulases, i.e., EG-II, CBH-I, and CBH-II (2). Therefore, I also tested all of these purified enzymes. Only EG-I was detected with this ELISA; the others gave background values comparable to those of BSA (Fig. 4). If a constant amount of EG-I (15 ng/ml, resulting in an OD_{405} of 0.78 [100%]) was challenged with increasing amounts of EG-II, CBH-I, or CBH-II in the same assay, an up to 1.8-µg/ml (120-fold) excess of competing antigen did not alter the result (EG-II OD_{405} , 0.76 [97%]; CBH-I OD_{405} , 0.74 [95%]; CBH-II OD_{405} , 0.76 [97%]). However, higher excesses (above 300-fold) resulted in an OD_{405} increase (not decrease) due to unspecific binding of these large amounts of antigens.

Precision of the assay and stability of standard solutions. Standard EG-I antigen solutions were prepared in binding buffer at concentrations from 5 to 1,500 ng/ml. They were stored at 4°C and at -20°C. The coefficients of variation within one plate ($n = 6$) were 1.3% at 500 ng/ml, 2.6% at 50 ng/ml, and 5.4% at 5 ng/ml. The coefficients of variation between the plates (standard solutions stored at -20°C, six plates analyzed during 37 weeks) were 8.5% at 500 ng/ml, 8.9% at 50 ng/ml, and 9.6% at 5 ng/ml. A *T. reesei* culture supernatant (which was stored at -20°C, and dilutions of 1:40,000, equivalent to 31 ng/ml in the microtiter plate well, were prepared on the day of assay) was measured over the same time period in comparison with these standard antigen

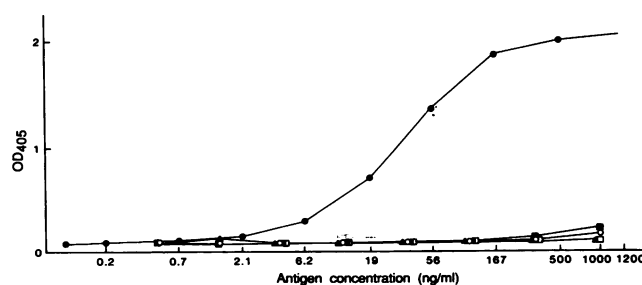


FIG. 4. Specificity of the double-antibody sandwich ELISA for EG-I (ELISA system ii). Only EG-I (●) was specifically detected. EG-II (○), CBH-I (▲), CBH-II (■), and BSA (□) did not react. The points are average values of duplicate measurements.

TABLE 1. Recovery of purified EG-I added to culture medium and to a supernatant of a culture of a genetically manipulated strain of *T. reesei* (A2874) that lacks the *egll* gene and thus does not produce EG-I

EG-I added ($\mu\text{g/ml}$)	Recovery ^a (%)	
	Medium	<i>T. reesei</i> Δegll culture supernatant
75.0	105	116
25.0	106	105
8.3	101	124
2.8	107	124
0.9	83	83

^a The addition of EG-I to binding buffer served as a control (100% recovery).

solutions. In this case coefficients of variation of 3.8% within plates ($n = 6$) and 6.0% between plates ($n = 6$ over 37 weeks) were determined.

In 14 separate experiments, standard EG-I was serially diluted until the OD_{405} approached blank values to determine the analytical detection limit (see Materials and Methods). It was on the average 0.21 ± 0.14 (67%) ng/ml and ranged from 0.08 to 0.55 ng/ml.

At 4°C the standard solutions were not stable. Less than 40% of the antigenicity was left after 37 weeks (half-life of 15 weeks). Storage at -20°C resulted in considerably better stability. Even when the sample was thawed and refrozen six times, the loss was only ca. 15% (extrapolated half-life of more than 3 years). If the sample was not thawed and refrozen, the antigenicity was still 97% after 37 weeks.

EG-I quantitation in culture supernatants. Since the composition of growth medium is very different from that of the buffer used for storage of purified EG-I, I added the purified enzyme to the buffer, growth medium, and a supernatant of a genetically manipulated strain (A2874 [31]) of *T. reesei* that lacks the *egll* gene and thus is incapable of producing EG-I. I then compared the recoveries. With as little as 2.8 μg of EG-I added per ml, all of the added enzyme was detected (Table 1). However, at lower concentrations some unknown material in the medium seems to quench the recovery. In addition, the shape of the sample dilution curves was identical to that of the standard curve.

The release of EG-I into the medium of a culture of a cellulase-producing *T. reesei* strain (VTT-D-79125 [3]) in comparison with other parameters measured is shown in Fig. 5. Between days 3 and 7 of culture the EG-I fraction of total protein released essentially remained the same (14.7% on average), but by day 11 it dropped to 10.4%, indicating more secretion of proteins other than EG-I (i.e., EG-II). In a strain with the *egll* gene deleted (strain A2874), the amount of EG-I was below the detection limit; i.e., no EG-I was found.

DISCUSSION

A very sensitive and specific immunoassay for EG-I has been developed. The chosen format, a double-antibody sandwich ELISA with a monoclonal antibody against EG-I specifically to capture EG-I, has a particular advantage, because all of the reagents used are optimized to be in excess over the enzyme (EG-I) to be measured. Specifically capturing the enzyme with a monoclonal antibody allowed a definite improvement in sensitivity and accuracy over those of an immunoassay described earlier (16), in which the enzyme to be analyzed is bound unspecifically directly to the plastic surface of the microtiter plate and then binding is

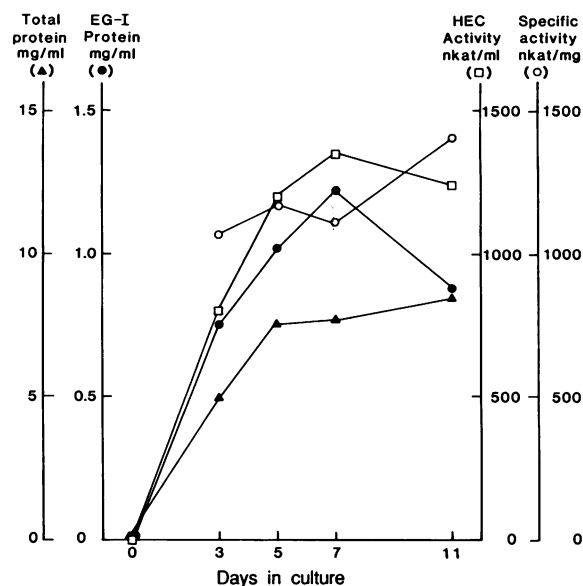


FIG. 5. EG-I secretion into medium compared with several other parameters over 11 days of culture of *T. reesei* VTT-D-79125 in 250-ml bottles of cellulose-containing medium. HEC, hydroxyethyl cellulose.

quantitated with a monoclonal antibody. An analytical detection limit of 0.2 ng/ml (20 pg of EG-I per microtiter well) on the average and an optimal measuring range between 5 and 500 ng/ml compare favorably with the optimal measuring range of 100 to 1,000 ng/ml reported previously (16).

Of particular importance was the pH dependence of the antigen binding to the monoclonal antibody. By increasing the pH and the concentration of the antigen dilution buffer it was ensured that EG-I could also be as easily detected in culture supernatants of high acidity (down to pH 3 is not uncommon) as in cultures kept at nearly neutral pH.

The specificity of the test is determined by the capture antibody, which is a monoclonal antibody directed against an epitope located on the core structure of the EG-I molecule (1). This part of the molecule is known to be less conserved on exo- and endoglucanases (15), and indeed the antibody does not detect any of the other investigated cellulases (1; this study). In contrast, the tail region of the cellulases seems to be very antigenic (1, 2) and much more conserved among the cellulases (15). This leads to polyclonal antibodies that are mainly directed at epitopes on the tail structure and that are not specific for different cellulases but recognize all of them with similar avidity (2). To avoid losses in specificity and sensitivity, we therefore chose a monoclonal antibody with high apparent affinity for the core structure as the capture antibody and a polyclonal antibody against EG-I, which mainly detects structures completely unrelated to the epitope recognized by the monoclonal capture antibody, as the detection antibody.

Particular emphasis was put on the development of stable antigen solutions that can be stored at working dilutions. This ensures the fewest variations in the tests done at different times by avoiding manifold dilutions of concentrated stocks of standard antigen. The addition of 1% BSA to the binding buffer and the storage at -20°C assured a measured shelf life of at least 9 months, a time that certainly can be extended.

With this highly sensitive and specific double-antibody sandwich ELISA, it is thus possible to measure EG-I spe-

cifically in complex culture supernatants not only of *T. reesei* cultures, where milligram amounts of the enzyme per milliliter are secreted, but also in other systems, e.g., heterologous gene expression in other organisms where perhaps only small amounts of even inactive enzyme are secreted. In addition, the test is useful for ascertaining the absence of the enzyme in EG-I deletion mutants or for detecting spurious contaminations in purified enzyme preparations. This is of particular interest because the substrate specificities and synergistic activities of these enzymes overlap (14) and therefore the purity of an enzyme preparation is of utmost importance to elucidate its functional properties.

Together with ELISAs of identical format to quantitate CBH-I and CBH-II as well as EG-II developed in our laboratory (7), a tool is available for easy monitoring of the proportions of these cellulases in existing and new genetically engineered *T. reesei* strains for industrial use (12).

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