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Quantitation of *Acidothermus cellulolyticus* E1 Endoglucanase and *Thermomonospora fusca* E_3 Exoglucanase Using Enzyme-Linked Immunosorbent Assay (ELISA)

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ABSTRACT

Two distinct quantitative indirect ELISAs were developed to determine the concentration of recombinant cellulase enzymes in culture filtrates. A monoclonal antibody (E1P7) was used as the primary antibody in developing an ELISA specific for *Acidothermus cellulolyticus* E1 endoglucanase. Likewise, a polyclonal rabbit serum (Ab684) was used to develop an ELISA specific for *Thermomonospora fusca* E_3 exoglucanase. Dose–response curves indicated a dynamic range for both assays between 0.01 and 0.08 μg/mL (1–8 ng/assay) when purified enzymes were used as standards. These assays have been used to estimate concentrations of secreted recombinant E1 and/or E_3 in culture supernatants of *Streptomyces lividans* strain TK24 in which the corresponding genes have been cloned and expressed.

Index Entries: Cellulases; *Acidothermus cellulolyticus; Thermomonospora fusca;* antibodies; ELISA.

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INTRODUCTION

Cellulases hydrolyze cellulose by acting synergistically on crystalline cellulose surfaces. Most fungal cellulase systems, as typified by the *Trichoderma reesei* system, are comprised of exo-β-1,4-D-glucanases (cellobiohydrolases; EC 3.2.1.91), endo-β-1,4-D-glucanases (EC 3.2.1.4), and β-D-glucosidases (cellobiases; EC 3.2.1.21). Endoglucanases cleave β-1,4-D-glucans at random along the polysaccharide chain, whereas exoglucanases cleave cellobiosyl units from either the reducing or the nonreducing end of a cellulose strand. Cellobiases hydrolyze cellobiose to form glucose. Bacterial cellulases are also capable of hydrolyzing cellulose, but the cellulase system of these organisms seems to vary in general, but not fundamentally, from that of fungi (*1–4*). In nature, cellulolytic microorganisms rely on these enzymes to convert cellulose to simple sugars for use as a carbon source for growth. Industrial uses of cellulases include applications in food and animal feed processing, horticulture, textile treatment, and laundry detergents (*5–7*). The US Department of Energy is currently funding a program to develop a process for the conversion of lignocellulosic biomass to ethanol transportation fuel in which enzymatic degradation of cellulose plays a key role.

Although *T. reesei* has long been known as a prolific producer of an effective cellulase system, the limitations of this enzyme preparation constrain process options. Process parameters, such as pH, temperature, specific activity, and sensitivity to feedback inhibition, are among the variables that are being studied for improvement. Using recombinant DNA technology, we are pursuing strategies to develop novel cellulase systems that demonstrate high specific activity and high productivity for hydrolysis of lignocellulosic feedstocks. Key to this strategy is the use of high specific activity, thermotolerant bacterial enzymes, such as those described from *T. fusca* (*8*) and *A. cellulolyticus* (*9*).

To estimate differences in cellulase productivities among different recombinant strains, we must quantitate the particular enzymes of interest. This is normally achieved by assaying enzyme activities (*10*). These techniques are useful, but they do not directly measure enzyme concentration. Complementary methodologies that are effective for quantitation rely on immunotechniques. The use of antibodies for quantitation of specific antigens in complex mixtures of compounds eliminates the effects of interfering biological activities (*11–13*), and with the advent of solid-phase immunosorbent assays, the quantitation of many analytes has become possible (*14*). One of the most accurate and easy-to-use assays for such purposes is the enzyme-linked immunosorbent assay (ELISA). Initial ELISAs were based on polyvalent antisera (*15,16*), but with the advent of monoclonal antibodies (MAb) (*17*), increased sensitivity and stability of the assays were achieved. MAb specific for *T. reesei* cellulases have been instrumental in specific detection of individual cellulase components in crude mixtures (*18–21*). The development of ELISAs to determine several native

Table 1
Streptomyces lividans: Parent and Recombinant Strains

Strain name	Genotype or phenotype	Reference
TK24	str-6	(*26*)
JT46	pro-2, str-6, rec-46	(*27*)
TK24/pIJ702	tsr, mel	This study
TK24/pFD666	neo	This study
TK24/pSZ7	tsr, E_3	This study
TK24/E1-pIJ702	tsr, E1	This study
TK24/E1-pFD666, pSZ7	neo, tsr, E1, E_3	This study (two plasmids)
JT46/E1-pSZ7	tsr, E1, E_3	This study (one plasmid)

cellulases from *Trichoderma* has previously been described (*22–25*). These assays have used polyvalent animal sera and MAb as either primary, secondary, or capture antibodies.

An ELISA that quantitates recombinant cellulases secreted by a heterologous host has not previously been described. Herein we describe the development of two quantitative ELISAs that detect recombinant *A. cellulolyticus* E1 endoglucanase and *T. fusca* E_3 (an exoglucanase), secreted by transformed *S. lividans* strains. The E1 ELISA is based on primary detection by an E1-specific MAb, and the E_3 ELISA by an E_3-specific rabbit serum.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *S. lividans* strain TK24 (*26*) was supplied by D. B. Wilson (Cornell University, Ithaca, NY), and *S. lividans* strain JT46 (*27*) was supplied by Christopher Reeves (Panlabs Inc., Bothell, WA). *Streptomyces* strains were grown in tryptic soy broth (TSB) with or without antibiotics (i.e., thiostrepton: 5 μg/mL in TSB broth, 50 μg/mL in agar plates; kanamycin: 50 μg/mL in liquid and solid media).

Five milliliter seed cultures of *S. lividans* were grown for 48 h at 30°C before transferring into 40 mL of fresh TSB. Five milliliter aliquots were collected at various time-points during growth of this culture for estimation of enzyme activity, cell mass, and antigen concentration.

Plasmid pIJ702 (*28*), a standard *Streptomyces* cloning vector, was purchased from the American Type Culture Collection (Rockville, MD; ATCC # 35287). Shuttle plasmid pFD666 (*29*), which carries an origin of replication from pJV1, was a gift from Ryszard Brzezinski (University of Sherbrook, Quebec, Canada). Plasmids pFD666 and pIJ702 are compatible in *S. lividans* cells.

Plasmid pSZ7, a shuttle plasmid containing a 3-kb fragment of *T. fusca* genomic DNA encoding the E_3 exoglucanase, was supplied by D. B. Wilson. The *A. cellulolyticus* E1 endoglucanase gene was cloned as a 3.7-kb genomic DNA fragment into pIJ702 at the unique *Bgl*II site, after ligation of *Bgl*II linkers, to produce plasmid E1-pIJ702. The same *A. cellulolyticus* DNA fragment containing the E1 endoglucanase was likewise cloned into the unique *Hind*III site of both pSZ7 and pFD666, producing plasmids E1-pSZ7 and E1-pFD666, respectively. Plasmid E1-pFD666 carries only the E1 endoglucanase gene. Plasmid E1-pSZ7 carries both the E1 endoglucanase and E_3 exoglucanase genes.

Estimation of Packed-Cell Volume and MUC Assay

Packed-cell volume (PCV) was determined by centrifuging a 1-mL aliquot of culture broth in a microfuge tube. The aliquots were centrifuged at 1720*g* for 10 min using a Beckman Model TJ-6R tabletop centrifuge (Beckman Instruments, Palo Alto, CA). Cell volume was estimated with calibration marks on microfuge tubes.

Unlike the *T. fusca* E_3, the *A. cellulolyticus* E1 endoglucanase can hydrolyze the fluorogenic substrate 4-methylumbelliferyl-β-D-cellobiopyranoside (MUC). This substrate has been used to screen transformants for expression of the E1 endoglucanase in transformed *S. lividans* strains, as well as to estimate the amount of E1 activity present in culture filtrates of transformed *S. lividans*. Supernatants from *S. lividans* strains transformed with the E1 gene were diluted 50-fold in TSB, and 25.5 μL were used in each 100-μL reaction containing 1 m*M* MUC and 20 m*M* Bis-Tris buffer (pH 5.8). Reactions were incubated at 60°C for 30 min and were terminated by adding 100 μL of 0.15*M* glycine-sodium hydroxide (pH 10.3). Fluorescence owing to formation of 4-methylumbelliferone (MU) by E1 was quantitated in a Model 7620 Microplate Fluorometer (Cambridge Technology, Inc., Watertown, MA) with excitation at 360 nm and emission at 460 nm. Activity is expressed as fluorescence units (FU).

Purification of Enzymes

Culture supernatant from recombinant *S. lividans* strain TK24 that contained the *A. cellulolyticus* E1 endoglucanase was concentrated and purified as previously described (*30*). Purified recombinant E1 endoglucanase (*S. lividans* host) was obtained for use as an ELISA standard at a concentration of 87 μg/mL. *T. fusca* E_3 exoglucanase was supplied by D. B. Wilson, after purification according to Irwin et al. (*31*). E_3 was diluted from a 117 μg/mL working stock to prepare ELISA standards.

Production of Antibodies and Western Blotting

An MAb specific for *A. cellulolyticus* E1 was produced according to established protocols (*20*). Approximately 50 μg of purified E1 were used

per inoculation, and subsequently, hybridomas were screened with indirect, nonquantitative ELISAs. Candidate hybridomas were tested for antigenic specificity using Western blots. A hybridoma cell line specific for E1 was dilution cloned, stabilized, and designated "E1P7." Ascites fluid was purified by Protein G affinity chromatography (Pharmacia, Piscataway, NJ), and stocks of E1P7 at 340 μg/mL were used for experiments. E_3 polyvalent antisera (Ab684) were produced as described previously (*31*). A 2.17 mg/mL stock of Ab684 serum was used for the experiments. Supernatants from untransformed (control) and transformed *S. lividans* strains were tested with specific antibodies using Western blots. Samples to be analyzed were placed in 2X SDS-PAGE sample buffer, boiled for 5 min, and 10 μL of sample were applied to each well of a precast 8–16% gradient polyacrylamide gel (Novex, San Diego, CA). Electrophoresed proteins were transferred to nitrocellulose using a Novex Blot Module according to the vendor's protocol. After transfer, the nitrocellulose membranes were blocked with PBS SuperBlock blocking buffer (Pierce, Rockford, IL) for 15 min and washed three times with TEN (50 m*M* Tris-HCl, 150 m*M* NaCl, 1 m*M* EDTA, pH 7.2) buffer containing 0.05% v/v Tween 20. The nitrocellulose membrane was submerged in a solution containing either E1- or E_3-specific antibody for 1 h and then washed three times as described above. After 1 h of exposure to an alkaline phosphatase species-specific goat antibody (Pierce) (1:1000 dilution in TEN buffer), the nitrocellulose was washed and exposed to 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium tablets in water (Sigma Chemical Co., St. Louis, MO) as suggested by the manufacturer. Enzymes and antibody concentrations were determined using the BCA Protein Assay (Pierce, Rockford, IL).

Development of an ELISA Specific for *T. fusca* E_3

All assays were performed in triplicate and in conjunction with purified standard proteins. Initial experiments were conducted to investigate the optimal parameters for adsorption of purified E_3 to Falcon PVC (Becton-Dickinson, Lincoln Park, NJ) or Dynatech microtiter plates (Dynatech, Chantilly, VA). E_3 was diluted in 0.05*M* sodium carbonate/bicarbonate buffer (pH 9.6) in concentrations ranging from 0.63 to 640 ng/mL. Unless otherwise noted, 100 μL of each E_3 dilution were added to wells and incubated at 37°C for 2 h. Plates were washed with 50 m*M* TEN buffer (pH 7.6) using a Transtar 96-well pipeter (Costar, Cambridge, MA). E_3 polyclonal antibody Ab684 (2.17 mg/mL) was diluted over the range of 1:4000–1:20,000 in 0.1*M* sodium phosphate buffer containing 0.05% Tween 20 (pH 7.0), and 100 μL of antibody solution were applied to the wells. After incubating for 1 h at room temperature and washing, goat antirabbit horseradish peroxidase-labeled antibody at a dilution of 1:5000 was added to each well. After 1 h of incubation at room temperature, the plates were washed and developed with tetramethylene blue for 5 min. The color reaction

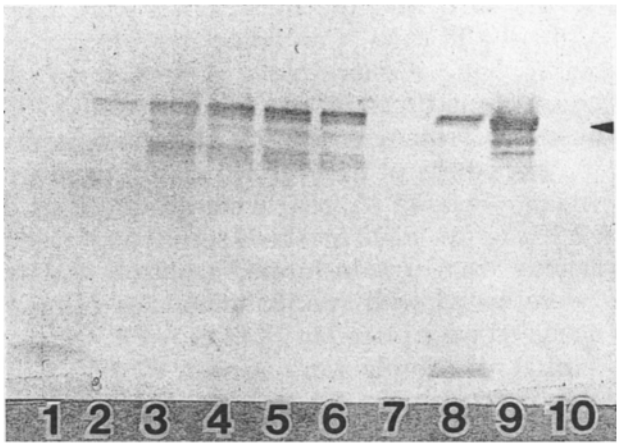

Fig. 1. Western blot demonstrating specificity for E_3 by Ab684 in culture supernatants of transformed *S. lividans* strains. Lane, 1 TK24/pFD666; lane 2, JT46/E1-pSZ7; lanes 3 and 4, two independent isolates of TK24/E1-pFD666, pSZ7 (orientation 1); lanes 5 and 6, two independent isolates of TK24/E1-pFD666, pSZ7 (orientation 2); lane 7, TK24/E1-pIJ702; lane 8, TK24/pSZ7; lane 9, purified *T. fusca* E_3 (1 μg); lane 10, *A. cellulolyticus* crude culture supernatant (10-fold diluted).

was stopped by adding 50 μL of 2*M* H_2SO_4. Absorbance of solutions in microtiter wells was read at 450 nm in a variable-wavelength microtiter plate reader (Molecular Devices, Menlo Park, CA).

Development of an ELISA Specific for *A. cellulolyticus* E1

Incubation times, temperatures, and solutions for the E1 ELISA were identical to those for the E_3 ELISA, except as noted below. E1 enzyme was diluted in concentrations ranging from 5 to 250 ng/mL, and 100 μL were applied to microtiter wells. After washing, MAb (E1P7) was diluted 1:2000 and 1:4000, and was added to the wells. After incubating with the antibody for 1 h and washing, goat antimouse peroxidase-labeled antibody at a dilution of 1:5000 was added to each well.

RESULTS

Specificity of Polyclonal and Monoclonal Antibodies

Analysis of the antibody specificity was accomplished using Western blots. Figures 1 and 2 demonstrate the specificity of Ab684 for E_3 and of E1P7 for E1. Figure 1 shows that the anti-E_3 polyvalent antibody, Ab684, recognizes E_3, but not E1 or any other antigen in crude *A. cellulolyticus* culture supernatant or native *S. lividans* control culture (Lane 1). Purified native E_3 strongly reacts with Ab684, as does a recombinant protein of equivalent molecular weight expressed by several *S. lividans* strains carrying

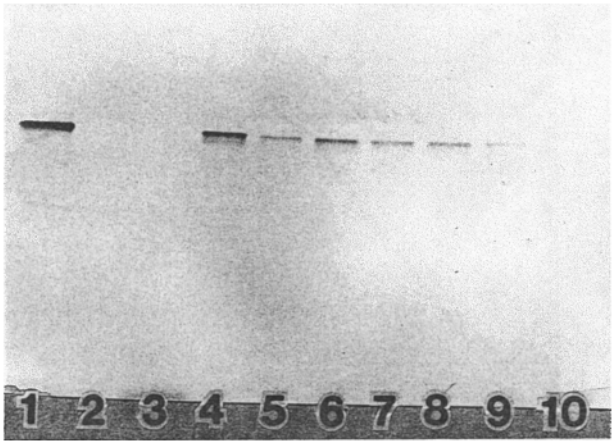

Fig. 2. Western blot demonstrating specificity of MAb E1P7 for E1 in culture supernatants of transformed *S. lividans* strains. Lane 1, *A. cellulolyticus* crude culture supernatant (10-fold diluted); lane 2, purified *T. fusca* E_3; lane 3, TK24/pSZ7; lane 4, TK24/E1-pIJ702; lanes 5 and 6, two independent isolates of TK24/E1-pFD666, pSZ7 (orientation 1); lanes 7 and 8, two independent isolates of TK24/E1-pFD666, pSZ7 (orientation 2); lane 9, JT46/E1-pSZ7; lane 10, TK24/pFD666.

plasmids with the E_3 gene. Lower-mol-wt proteolytic fragments of E_3 enzyme are also detected in these recombinant cultures. Figure 2 shows the specific recognition of *A. cellulolyticus* E1 by E1P7. E1 protein is the only protein detected by the antibody when *A. cellulolyticus* culture supernatant is analyzed. Culture supernatants from recombinant strains of *S. lividans* carrying plasmids with the E1 gene inserted are also shown. The E1P7 MAb does not crossreact with E_3 or any other antigen in the control *S. lividans* culture supernatant (Fig. 2, Lane 10).

Development and Optimization of E_3 and E1 ELISAs

Investigation of the initial ELISA parameters indicated that the best adsorption for E_3 and E1 standards was obtained on Dynatech microtiter plates that had been incubated at 37°C for 2 h (data not shown). To determine the best antibody dilutions to use in these assays, we tested decreasing antibody dilutions against various antigen concentrations and analyzed them over the linear portion of the curve. The dose–response curve for Ab684 at dilutions ranging from 1:2000 to 1:20,000, using E_3 concentrations ranging from 1.3 to 5000 ng/mL (0.13 to 500 ng/well), showed that this antibody could be diluted up to 20,000-fold and still produce good dose–response curves (data not shown). To conserve antibody while preserving reasonable assay response, Ab684 was used at a dilution of 1:20,000 in further work. Similarly, the E1 dose–response curve indicated an optimal antibody dilution for E1P7 of 1:4000 to develop the assay (data not shown).

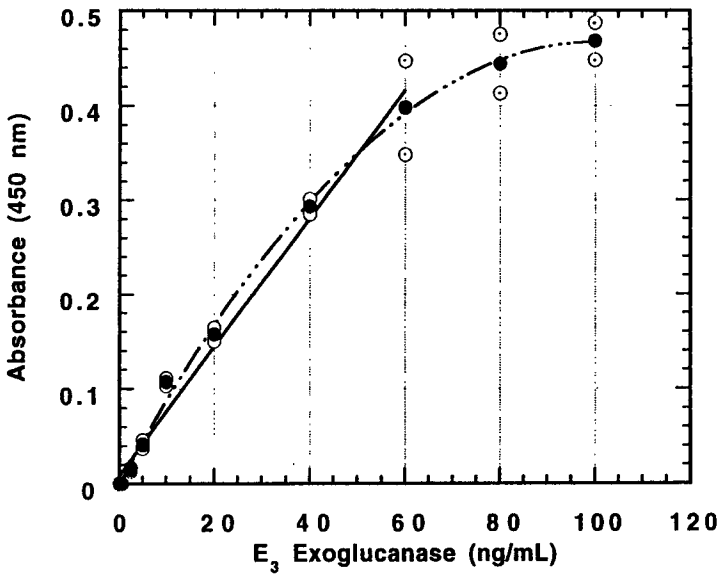

Fig. 3. A narrow range of E_3 concentrations is analyzed to establish the linear range of the E_3 ELISA. The working range of the E_3 ELISA lies between 10 and 60 ng/mL. The filled circles represent the mean values calculated for two independent assays. These data were used to calculate both a third-order polynomial curve fit (dashed line) and a least-squares linear fit (solid line).

To establish the linear working range of both assays, we analyzed small increments of antigen concentrations using the chosen antibody dilutions as stated above. Linear response is apparent at very low concentrations of E_3, with nonlinear behavior becoming evident at 60 ng/mL (6 ng/well). Figure 3 establishes that the operating range of the E_3 ELISA based on duplicate data was 10–60 ng/mL. Linear least-squares fit to E_3 assay data for the 10–60 ng/mL range produced a correlation coefficient (R) of 0.9939.

E1 ELISA response characteristics were more reproducible than those of the E_3 ELISA. E1 assay saturation was found at 300 ng/mL (data not shown), and the linear working range of the E1 ELISA (10-80 ng/mL) is shown for quadruplicate data in Fig. 4. Figure 4 clearly shows an enhanced working range of the E1 ELISA (relative to the E_3 ELISA) and the distinctly linear working assay curve in this range, i.e., R = 0.9992.

Correlation of PCV and MUC Activity with ELISA Response

At selected times, 5-mL aliquots of culture broth were removed from cultures of transformed *S. lividans* grown in TSB. These samples were clarified by centrifugation and analyzed for cell mass (as packed-cell volume) and MUC activity, as described in the Materials and Methods. Aliquots from the same time-points were serially diluted and analyzed using either the E1 or E_3 ELISA, or both, depending on the strain.

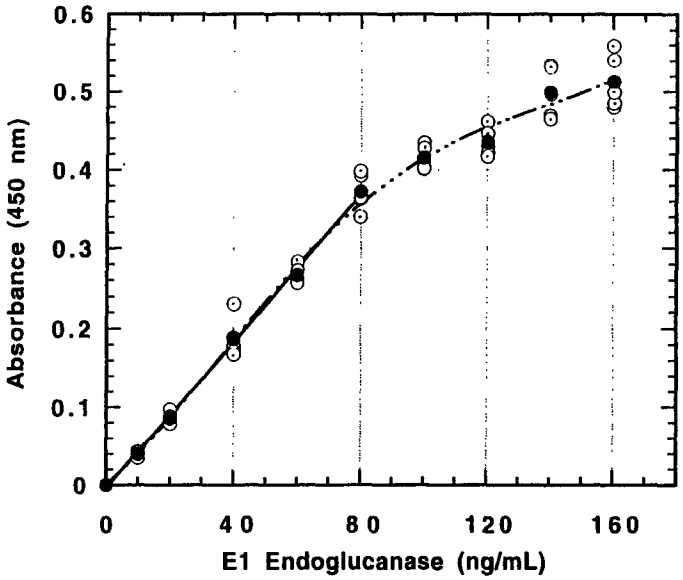

Fig. 4. A narrow range of E1 concentrations is analyzed to establish the linear range of the E1 ELISA. The working range of the E1 ELISA lies between 10 and 80 ng/mL. The filled circles represent the mean values calculated for four independent assays. These data were used to calculate both a third-order polynomial curve fit (dashed line) and a least-squares linear fit (solid line).

Figure 5 illustrates the correlation between packed-cell volume, MUC activity owing to expression of E1 endoglucanase, and the E1 and E_3 ELISAs as a function of time in culture for four different recombinant strains expressing one or both of the foreign cellulase genes. Figure 5A shows good correlation among PCV, MUC activity, and E1 ELISA response for strain TK24/E1-pIJ702, although E1 antigen clearly lags behind MUC activity and cell mass accumulation. In Fig. 5B, cell mass clearly precedes detection of E_3 antigen by at least 24 h. In strain TK24/pSZ7, E_3 accumulates rapidly between 23 and 30 h in culture, but as can be observed, MUC activity is undetectable. Productivity of E_3 is well above that of E1 in these single gene constructs.

In Fig. 5C, cell mass accumulates prior to detection of either MUC activity or the E_3 antigen. In strain TK24/E1-pFD666, pSZ7, E1 expression is extremely low in comparison to strain TK24/E1-pIJ702 (compare Fig. 5A and C). This is probably because of an extremely low copy number for the E1-pFD666 plasmid in this strain (data not shown). E_3 expression is also lower in TK24/E1-pFD666, pSZ7 than in TK24/pSZ7 (compare Fig. 5B and C). Figure 5D demonstrates that the JT46/E1-pSZ7 strain grows poorly, but with time, MUC activity does accumulate. By contrast, the E1 ELISA is not able to detect any antigen, but the E_3 ELISA is able to detect low levels of E_3 in this culture. It is evident that the concentration of E1 in this dual-gene construct is low. At this range of E1 concentration, precision and accuracy of both assays are limited. Therefore, a linear relationship

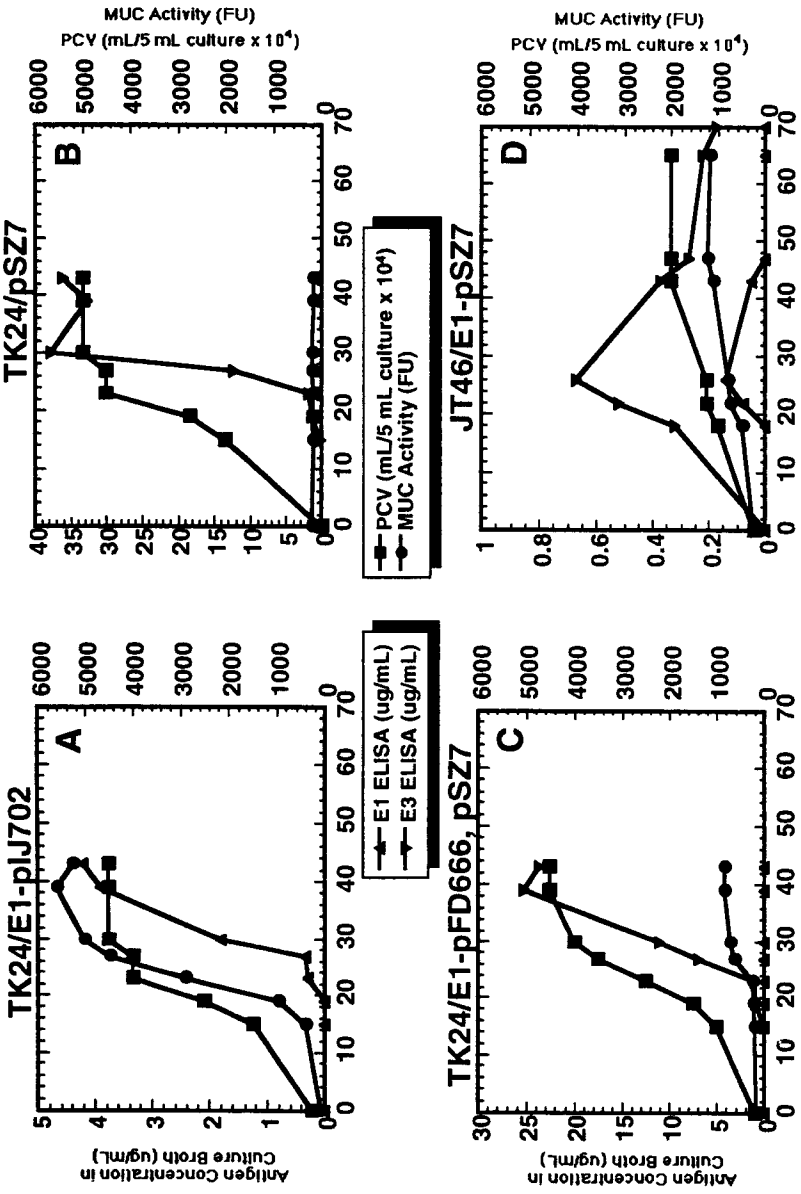

Fig. 5. Comparison of PCV and MUC activity owing to E1 endoglucanase action with results from E1 and E_3 ELISA assays as a function of culture time in recombinant *S. lividans* cultures. ELISA assay results have been adjusted to account for supernatant dilutions used to arrive at concentrations of E1 and E_3 in crude culture supernatants. 5A, strain TK24/E1-pIJ702; 5B, strain TK24/pSZ7; 5C, strain TK24/E1-pFD666, pSZ7; 5D, strain JT46/E1-pSZ7.

between antigen and MUC activity may not be expected. In the two gene constructs, it may be assumed that the presence of two genes is a metabolic burden on the transformants. Clearly, the strains carrying more than a single heterologous gene cannot express them at as high a level as when the genes are cloned individually in separate strains.

These results illustrate how ELISAs can be used to determine relative levels of expression for cloned genes in genetically engineered *S. lividans*. For enzymes such as E_3, which have no easily demonstrable enzymatic activity, this ELISA provides a reliable method for estimating relative levels of expression in recombinant strains.

DISCUSSION

Although enzyme assays exist for analysis of the various cellulases in microbial systems, the hydrolytic characteristics of each enzyme may overlap, making distinctions between enzymes difficult. By having specific immunological probes, antibodies become useful in the analysis of individual enzymes. One of the major applications of antibodies (especially MAb) is their use as clinical tools for quantitation of acute and convalescent medical conditions. This same technique has been applied to cellulases. Several *S. lividans* strains transformed with plasmids containing one or both of two different cellulase genes have been analyzed for the level of expression and secretion of *T. fusca* E_3 and *A. cellulolyticus* E1.

Because only one specific antibody or serum for each enzyme employed in this study was available, antigen ELISAs were the method of choice. Our assays and that of Kolbe and Kubicek (*23*) were based on indirect ELISAs. These studies indicated similar working ranges (1–10 ng/sample). A double-antibody assay developed by Buhler reported the quantitation of *T. reesei* EG I with a detection level of 20 pg/sample (*25*). The difference in detectability may be owing to the type of assay developed. As the number of immunological probes increase in a particular assay, so does the sensitivity. Because of the polyclonal nature of Ab684, development of the E_3 ELISA was more difficult than the E1 ELISA, which relied on an MAb. The presence of multiple antibodies having differing affinities for a particular epitope or antigen, as seen in serum, causes slight variabilities in the day-to-day use of such assays.

Quantitation of an enzyme in solution, using ELISA, is independent of any knowledge regarding specific activity of the recombinant proteins. These assays allow for the quantitation of total E1 or E_3; however, not all the antigen being measured is necessarily enzymatically active. The availability of various well-characterized MAb enabled Kolbe and Kubicek to quantitate truncated and native enzymes in crude culture broths (*23*). In a situation where two recombinant genes show interference in terms of biochemical activity, ELISAs could provide a reliable means for determining the relative amounts of each enzyme in a mixture.

Results from the E_3 and E1 ELISAs indicate that the working range of both assays is very similar. The use of purified enzymes and optimized antibody dilutions showed that quantitation of these recombinant proteins is possible at low levels. We will employ these assays with updated recombinant expression systems involving the E1 and/or E_3 genes. With the development of an E_3-specific MAb, a more sensitive and consistent assay should be possible.

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REFERENCES

1. Coughlan, M. P. (1985), *Biotechnol. Genet. Eng. Rev.* **31,** 39–109.
2. Puls, J. and Wood, T. W. (1991), *Biotechnology* **36,** 15–19.
3. Woodward, J. (1991), *Bio/Technol.* **36,** 67–75.
4. Rapp, P. and Beerman, A. (1991), in *Biosynthesis and Biodegradation of Cellulose,* Haigler, C. H. and Weimer, P. J., eds., Marcel Dekker, New York, pp. 535–597.
5. Coughlan, M. P. (1985), *Biochem. Sci. Trans.* **13,** 405–416.
6. Mandels, M. (1985), *Biochem. Sci. Trans.* **13,** 414–416.
7. Grohmann, K., Wyman, C. E., and Himmel, M. E. (1992), in *Emerging Technologies for Materials and Chemicals from Biomass,* ACS Symposium Series 476, American Chemical Society, Washington, DC, pp. 354–392.
8. Wilson, D. B. (1988), *Methods Enzymol.* **160,** 314–323.
9. Tucker, M. P., Mohagheghi, A., Grohmann, K., and Himmel, M. E. (1989), *Bio/Technology* **7,** 817–820.
10. Wood, T. M. and Bhat, K. M. (1988), *Methods Enzymol.* **160,** 87–135.
11. Perlmann, P. and Kaltenbach, J. C. (1957), *Expt. Cell Res.* **12,** 185–188.
12. Oudin, J. (1958), *J. Immunol.* **81,** 376–388.
13. Hitzig, W. H., Schiedegger, J. J., Butler, R., Gugler, E., and Hassig, A. (1959), *Helv. Med. Acta.* **26,** 142–151.
14. Butler, J. E., ed. (1991), *Immunochemistry of Solid Phase Immunoassay,* Chemical Rubber Company, Boca Raton, FL.
15. Berson, S. A. and Yallow, R. S. (1959), *J. Clin. Invest.* **38,** 1996–2016.
16. Ekins, R. P. (1960), *Clin. Chim. Acta* **5,** 453–459.
17. Kohler, G. and Milstein, C. (1975), *Nature* **256,** 495–497.
18. Riske, F., Labudova, I., Miller, L., Macmillan, J. D., and Eveleigh, D. E. (1987), in *Biomass Conversion Technology, Principles and Practice,* Moo-Young, M., ed., Pergamon, New York, pp. 167–176.

19. Mischak, H., Hofer, F., Messner R., Weissinger, E., Hayn, M., Tomme, P., Esterbauer, H., Kuchler, E., Claeyssens, M., and Kubicek, C. P. (1989), *Biochim. Biophys. Acta* **200,** 643–649.
20. Nieves, R. A., Himmel, M. E., Todd, R. J., and Ellis, R. P. (1990), *Appl. Environ. Microbiol.* **56,** 1103–1108.
21. Aho, S., Olkkonen, V., Jalava, T., Paloheimo, M., Bühler, R., Nikv-Paavola, M.-L., Bamford, D. H., and Korhola, M. (1991), *Eur. J. Biochem.* **200,** 643–649.
22. Oh, T. K. and Kim, S. H. (1986), *Biotechnol. Lett.* **8,** 403–406.
23. Kolbe, J. and Kubicek, C. P. (1990), *Appl. Microbiol. Biotechnol.* **34,** 26–30.
24. Riske, F. J., Eveleigh, D. E., and Macmillan, J. D. (1990), *Appl. Environ. Micro.* **56,** 3261–3265.
25. Buhler, R. (1991), *Appl. Environ. Microbiol.* **57,** 3317–3321.
26. Chater, K. F., Hopwood, D. A., Kieser, T., and Thompson, C. J. (1982), *Curr. Top. Microbiol. Immunol.* **96,** 69–95.
27. Tsai, J. F.-Y. and Chen, C. W. (1987), *Mol. Gen. Genet.* **208,** 211–218.
28. Katz, E., Thompson, C. J., and Hopwood, D. A. (1983), *J. Gen. Microbiol.* **129,** 2703–2714.
29. Denis, F. and Brzezinski, R. (1992), *Gene* **111,** 115–118.
30. Baker, J. O., Adney, W. S., Nieves, R. A., Thomas, S. R., Wilson, D. B., and Himmel, M. E. (1994), *Appl. Biochem. Biotechnol.* **45/46,** 245–256.
31. Irwin, D. C., Spezio, M., Walker, L. P., and Wilson, D. B. (1993), *Biotechnol. Bioeng.* **42,** 1002–1113.