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A New Thermostable Endoglucanase, *Acidothermus cellulolyticus* E1

Synergism with *Trichoderma reesei* CBH I and Comparison to *Thermomonospora fusca* E_5

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ABSTRACT

A new thermostable endoglucanase, *Acidothermus cellulolyticus* E1, and another bacterial endoglucanase, E_5 from *Thermomonospora fusca*, each exhibit striking synergism with a fungal cellobiohydrolase (*Trichoderma reesei* CBH I) in the saccharification of microcrystalline cellulose. In neither case did the ratio of endoglucanase to exoglucanase that demonstrated maximum synergism coincide exactly with the ratio that actually released the maximum quantity of soluble sugar for a given total cellulase loading. The difference between the two ratios, after significant hydrolysis of the substrate, was considerably larger in the case of *A. cellulolyticus* E1. For both endoglucanase pairings with CBH I, the offset between the ratio for maximum synergism and the ratio for maximal soluble sugar production was found to be a function of digestion time.

Index Entries: *Acidothermus cellulolyticus* E1; *Thermomonospora fusca* E_5; *Trichoderma reesei* CBH I; synergism; endoglucanase.

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INTRODUCTION

Acidothermus cellulolyticus, a bacterium first isolated from mud and fallen-tree wood-splinter samples taken from the acidic hot springs of northern Yellowstone National Park, actively secretes a number of highly thermostable cellulose-degrading enzyme activities (*1*). *A. cellulolyticus* endoglucanase 1 (E1), an enzyme purified (*2*) from the culture filtrate of this organism, has been found to be very active against both carboxymethyl cellulose and (used in conjunction with appropriate exocellulases) against microcrystalline cellulose. Because of its enzymatic properties and its thermostability, *A. cellulolyticus* E1 displays considerable potential for use in high-temperature processes for converting pretreated biomass to fermentable sugars. The fact that E1 is of bacterial origin may be of considerable advantage in the overexpression of the enzyme in a rapidly growing bacterial host.

In the work described here, the activity of *A. cellulolyticus* E1 against microcrystalline cellulose is characterized in terms of its synergistic action with *Trichoderma reesei* cellobiohydrolase I (CBH I) used as a "reference" exocellulase. Parallel studies of the synergistic hydrolysis of microcrystalline cellulose by another thermostable bacterial endoglucanase, *Thermomonospora fusca* E_5 (*3–6*), also acting in concert with *T. reesei* CBH I as reference exocellulase, illustrate the types of differences in enzyme activity that can be delineated by such studies, and the implications of these differences for process development.

The fact that enzymes of various cellulase "complexes" act synergistically was recognized quite early in the study of these enzymes, from the observation that efficient saccharification of cellulose required the concerted action of at least three types of enzymes: endoglucanases, exoglucanases, and β-D-glucosidases (*7*). In recent years, quantitative studies of synergism between two or more purified cellulase-degrading enzymes have emerged as an important approach to the functional characterization and classification of such enzymes (*8–13*). In addition, with the advent of processes involving mixtures of individual enzymes from different organisms, synergism studies have assumed considerable importance in the selection of individual enzymes for cloning and overexpression in suitable hosts.

MATERIALS AND METHODS

Enzyme Purification

Purification of A. cellulolyticus *E1*

Culture supernatant from a 150-L fermentation of *A. cellulolyticus* was concentrated using Amicon 10,000 molecular weight cutoff hollow-fiber filters, followed by clarification by centrifugation prior to purification.

The initial hydrophobic interaction chromatography step was performed by adding ammonium sulfate to the culture concentrate to a final concentration of 1*M* $(NH_4)_2SO_4$. An aliquot of 10 mL of this solution was then loaded onto a Pharmacia HiLoad 16/10 Phenyl Sepharose high-performance column using 20 m*M* Tris buffer, pH 8.0 with 1*M* $(NH_4)_2SO_4$ and eluted with a descending salt gradient. Further purification of E1 by anion exchange chromatography was performed using a HiLoad 16/10 Q Sepharose high-performance column eluted with a linear gradient of 0 to 1*M* NaCl in 20 m*M* Tris, pH 8.0. The final step of purification was size-exclusion chromatography (SEC) using a Pharmacia HiLoad 16/60 Superdex 200 prep grade column in 20 m*M* acetate buffer, pH 5.0.

Purification of T. reesei *CBH I*

The purification procedure used in this study was that developed earlier (*14*) following the general SEC/anion-exchange chromatography protocol described by Shoemaker for the purification of CBH I (*15*).

Purification of Aspergillus niger *β-D-Glucosidase*

A 5.0-mL sample of Novozym 188 Cellobiase, Lot #847 (Novo Laboratories, Bagsvaerd, Denmark) was diluted with 5 mL of 20 m*M* acetate, 100 m*M* NaCl, pH 5.0 buffer, and filtered through a 0.2 μm filter. This material was then loaded onto a Pharmacia Superose 12 prep grade size exclusion column and the protein eluted in 20 m*M* acetate, pH 5.0 at a flowrate of 2.0 mL/min. The fractions were tested for β-D-glucosidase activity using p-nitrophenyl-β-D-glucopyranoside (Sigma Chemical Co., St. Louis, MO). The high-MW fractions containing the β-D-glucosidase activity were pooled, concentrated, and diafiltered in 20 m*M* Bis-Tris buffer pH 5.8, using an Amicon stirred cell and PM10 membranes. The initial SEC step was followed by anion-exchange chromatography using a HiLoad 16/10 Q Sepharose high performance column. The sample was loaded in 20 m*M* Bis-Tris, pH 5.8 and eluted in a linear salt gradient of 0–1*M* NaCl in 20 m*M* Bis-Tris pH 5.8. The final step of purification was SEC on a Pharmacia HiLoad 16/60 Superdex 200 prep grade column in 20 m*M* acetate buffer, pH 5.0. The specific activity of the purified β-glucosidase was found to be 38 U/mg, where one unit is defined as that quantity of enzyme activity that will convert one micromole of cellobiose to two micromoles of glucose/min at pH 5.0 and 50°C.

Purification of T. fusca E_5

The endoglucanase E_5 was originally purified from *T. fusca* broth using DEAE-Sephadex chromatography (*3*). E_5 used in the present study was purified by phenyl Sepharose chromatography of cell lysates from *Streptomyces lividans* TK24 containing the *T. fusca* E_5 gene (*16*).

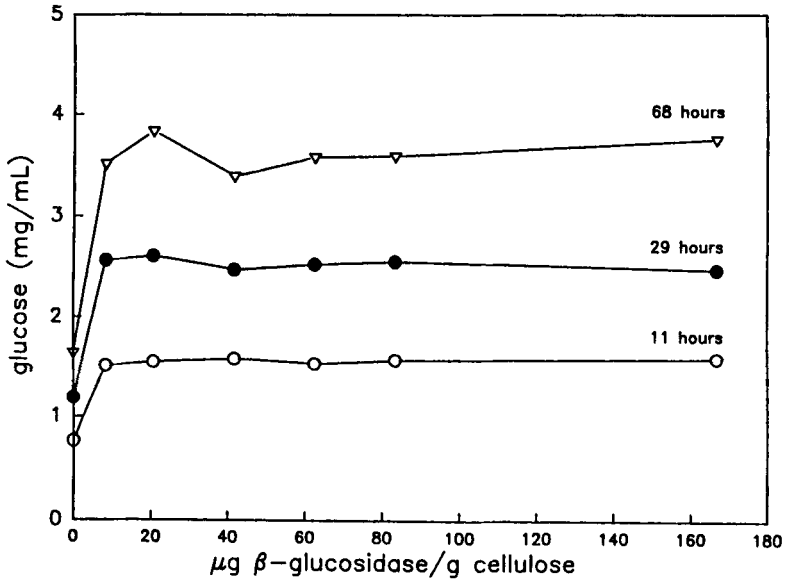

Fig. 1. Demonstration of the sufficiency of the β-glucosidase loadings used to effect conversion to glucose of the cellobiose produced from microcrystalline cellulose by a mixture of 20% *T. fusca* E_5 and 80% *T. reesei* CBH I (0.65 μ*M* total cellulase). The cellulase mixture was incubated with 5% (w/v) Sigmacell, type 20, at pH 5.0, 50°C, with mixing by inversion at 10 rpm, in the presence of different loadings of purified *A. niger* β-glucosidase.Solubilized reducing sugar was measured (as glucose) at the indicated digestion times.

Cellulose Digestions

Digestion mixtures contained as substrate 5% (w/v) Sigmacell Type 20 (Sigma Chemical Co.) suspended in 50 m*M* acetate, pH 5.0, containing 0.004% (w/v) sodium azide. Total cellulase loadings (endoglucanase plus exoglucanase) were held constant at 1.8 μ*M* in the 1.0-mL reaction mixtures. Sufficient purified *A. niger* β-D-glucosidase (4.17 μg/mL of digestion mixture, equal to 0.61 U/mL or 12.2 U/mg cellulose) was added to the reaction mixtures to eliminate the problem of cellobiose inhibition. The "sufficiency" of the β-glucosidase loading was established, for the individual enzymes and for representative mixtures, by means of experiments in which reducing-sugar output was measured in the presence of different loadings of β-glucosidase, to determine loadings above which further increases in loading produced no further increases in yield of reducing sugar. Typical results of such experiments are illustrated by Fig. 1, which presents data for a mixture of 20 mole% *T. fusca* E_5 and 80 mole% *T. reesei* CBH I. For loadings at or above 8.33 μg β-glucosidase/g of cellulose (0.417 μg/mL of digestion mixture), which was the lowest nonzero loading tested, the concentration of reducing sugar in the liquid phase was found to be essentially independent of β-glucosidase loading. A loading of 83.3 μg

β-glucosidase/g cellulose, or 10 times the minimum loading in Fig. 1, was found to be more than adequate for this and the other cellulase combinations, and was therefore adopted as the standard β-glucosidase loading for all of the digestion experiments reported here.

The miniaturized apparatus used to economize on purified enzyme utilized 1.5-mL Wheaton autoinjector vials as reaction vessels. The enzyme mixtures for the different digestion mixtures were first placed in the vials, and substrate was added to initiate the reaction. The vials were sealed with aluminum crimp-caps (PTFE-faced silicone septa, Kimble Glass, Inc., Vineland, NJ), then placed in a rotator head immersed in a 50°C water bath and continuously mixed by inversion at 10 rpm. Periodically, aliquots (0.04 mL) were withdrawn, diluted to 2.0 mL with deionized water, and centrifuged to remove all solid substrate. Reducing-sugar content of the supernatant was then determined (as glucose) using the bicinchoninic-acid method of Doner and Irwin (*17*). In the withdrawal of samples for determination of sugar concentrations, every effort was made to withdraw "representative" samples from well-mixed digestion mixtures, in order to avoid perturbing the solid/liquid composition of the mixtures and thus compromising the validity of later samples. The sampling techniques used were those that have been found in preliminary studies not to produce significant differences between the results for digestion mixtures sampled once at the end of a 120-h digestion, and the 120-h results for mixtures that were sampled as many as 5 times prior to withdrawal of the 120-h samples.

Synergism ratios were calculated by dividing the reducing-sugar production of the mixture of endoglucanase and exoglucanase by the sum of the productions in parallel digestion mixtures containing the endoglucanase and exoglucanase separately.

RESULTS AND DISCUSSION

It should be noted at the outset that both of the endoglucanases studied here are capable of effective catalysis at temperatures higher than the 50°C digestion temperature employed here (*1–3*). The 50°C digestion temperature was chosen to accommodate the lesser thermal stability of *T. reesei* CBH I, which at pH 5.0 begins to lose structural integrity (and activity) at approx 55°C (*14,18,19*).

Figure 2(A,B) utilizes data from relatively long-term digestions (118 h for E1/CBH I; 124 h for E_5/CBH I) to illustrate the dependence on endoglucanase/exoglucanase ratios of both the synergism ratio for solubilization of microcrystalline cellulose by mixtures of each of the two endoglucanases with *T. reesei* CBH I, and the actual production of soluble reducing-sugar (as glucose) by these mixtures. The 118–124 h digestion periods were

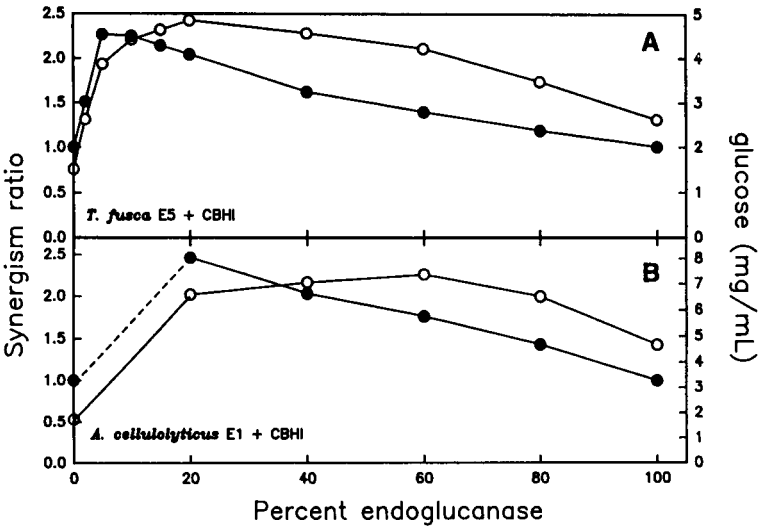

Fig. 2. Synergism ratios (—•—•—) and glucose production (—○—○—) as a function of endoglucanase percentage (molar basis) for digestion of 5% (w/v) Sigmacell-20 by cellulase mixtures at 0.65-μM total cellulase concentration, supplemented with purified *A. niger* β-glucosidase (83.3 μg/g cellulose). A: *T. fusca* E_5 and *T. reesei* CBH I after 124 h at 50°C and pH 5.0, with mixing by inversion at 10 rpm. B: *A. cellulolyticus* E1 and *T. reesei* CBH I after 118 h digestion under the same conditions.

chosen to get all of the digestions past the 2.0–2.5% of "very easily hydrolyzed material" and into the more recalcitrant cellulose, without generating such high concentrations of the final product that glucose inhibition became a factor. The two endoglucanase/CBH I pairs are seen to be alike in that for both *A. cellulolyticus* E1 and *T. fusca* E_2, the maximum synergistic effect, as measured here, occurs at quite low endoglucanase percentages in the constant-molarity cellulase loading: i.e., at 20% or less endoglucanase. For *T. fusca* E_5, maximum synergism is observed at 5–10% endoglucanase. The mixture composition yielding maximum synergism for *A. cellulolyticus* E1 has not been determined with equal precision, inasmuch as the synergism ratio still appears to be increasing with decreasing endoglucanase percentage at an endoglucanase percentage of 20, which was the lowest percentage evaluated. The composition of E1 and CBH 1 resulting in maximum synergism should therefore be described as occurring "near or below 20% endoglucanase." The curve (Fig. 2B) connecting the point for 20% endoglucanase with that for "zero endoglucanase" has been shown as a broken line to indicate this uncertainty.

Despite the lesser precision of synergism optimum determination for E1/CBH I as compared with that for E_5/CBH I, it is clear from Fig. 2(A,B) that in both cases the enzyme ratio resulting in optimum synergism does not coincide exactly with that resulting in the highest production of soluble sugar. The offset between the two maxima is considerably greater for E1/CBH I (60% endo vs 20% or below) than for E_5/CBH I (20% vs 5–10%).

In each case, the synergism ratio continues to increase with decreasing percentage of endoglucanase over a range of endoglucanase percentage in which the soluble-sugar output has begun to *decrease* with decreasing percentage of endoglucanase. The high values of the synergism ratio seen at low percentages of endoglucanase do not mean that the enzyme mixtures are especially effective at these low ratios of endoglucanase to exoglucanase; what the high synergism values mean is that the sum of the soluble-sugar productions of the two enzymes acting alone is decreasing even faster (with decreasing percentage of endoglucanase in the mixture) than is the soluble-sugar production by the two enzymes acting in concert. Given these two different ways of presenting the data, it is likely that basic researchers attempting to classify enzymes and understand their mechanisms of action will be more interested in synergism values, whereas application-oriented researchers will tend to focus somewhat more heavily on the actual performance of the enzymes in combination.

The offsets between the enzyme ratios for maximum synergism and those for maximum soluble-sugar output are not constant over the course of the digestions described here (0 to approx 120 h), but instead are quite large for both enzyme pairs in the early stages of the digestion and then tend to decrease as the digestion progresses. For both enzyme pairs, and at all sampling times investigated here, the maximum synergism was produced by mixtures having 20% or less endoglucanase (data not shown). The fairly large changes observed in the offsets are therefore primarily the results of shifts in the positions of the respective sugar-solubilization maxima. Figures 3(A,B) and 4(A,B) track the sugar production for the different endo/exo mixtures as a function of digestion time. Figure 3 shows the primary data (actual reducing-sugar concentrations in the liquid phase) for both enzyme pairs, whereas Figure 4 presents "normalized" forms of the same data sets shown in Fig. 3. In this "normalization" process, the reducing-sugar values for each curve in Fig. 3 have been divided by the maximum of the values, and the resulting processed data have been plotted in Fig. 4(A,B) as "relative glucose production." The reason for the processing of the data as displayed in Fig. 4 is to highlight shifts in the positions of the sugar-output maxima; the reason for the parallel presentation of the primary data in Fig. 3 is to facilitate keeping in touch with the trends of the actual (not relative) soluble-sugar productions in the different samples.

In the case of *T. fusca* E_5 (Figs. 3A and 4A), the loading response curve is a very broad peak early in the digestion (at 5 h), but begins to build toward a peak at the low end of the endoglucanase-loading scale, with the maximum sugar yield shifting from 60 to 80% endoglucanase (5-h curve) to 40% by 21–50 h, and then to 20% endoglucanase at the 92- and 124-h samplings. The loading response curve for *A. cellulolyticus* E1 (Figs. 3B and 4B) is likewise broad at early stages of the digestion, with the very earliest curve appearing to show the greatest production for the 80%-endoglucanase mixture at the high end of the mixture scale. (Data for the

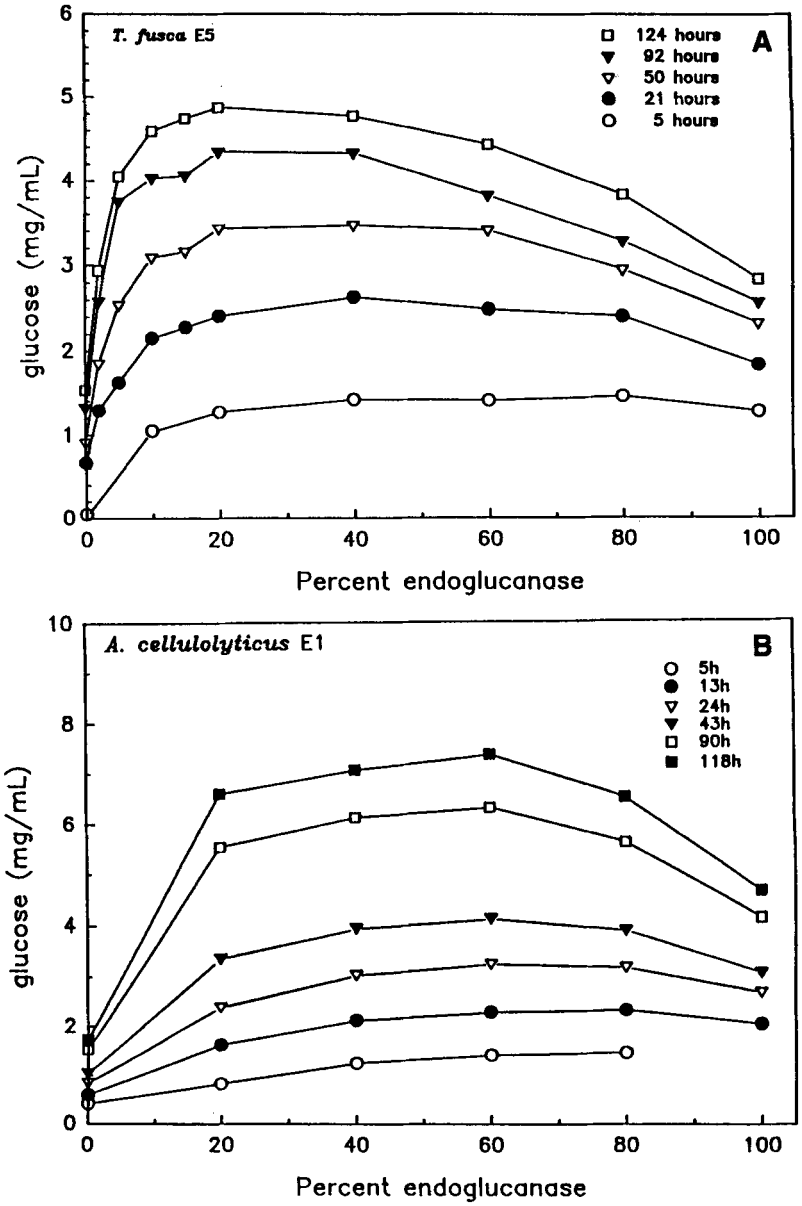

Fig. 3. Cumulative glucose production from Sigmacell-20 as a function of endoglucanase percentage in mixtures with *T. reesei* CBH I. Conditions as in Fig. 2. A: *T. fusca* E_5; B: *A. cellulolyticus* E1.

100%-E1 digestion mixture was lost at this sampling.) Like the response curve shown for E_5 in Figs. 3A and 3B, the maximum of the glucose production curve for E1 shifts downscale with further digestion, but appears to stabilize at 60% endoglucanase by 24 h digestion and to stay there until the end of this experiment at 118 h, rather than approaching the position of the synergism ratio maximum as closely as does the corresponding glucose-production curve for E_5.

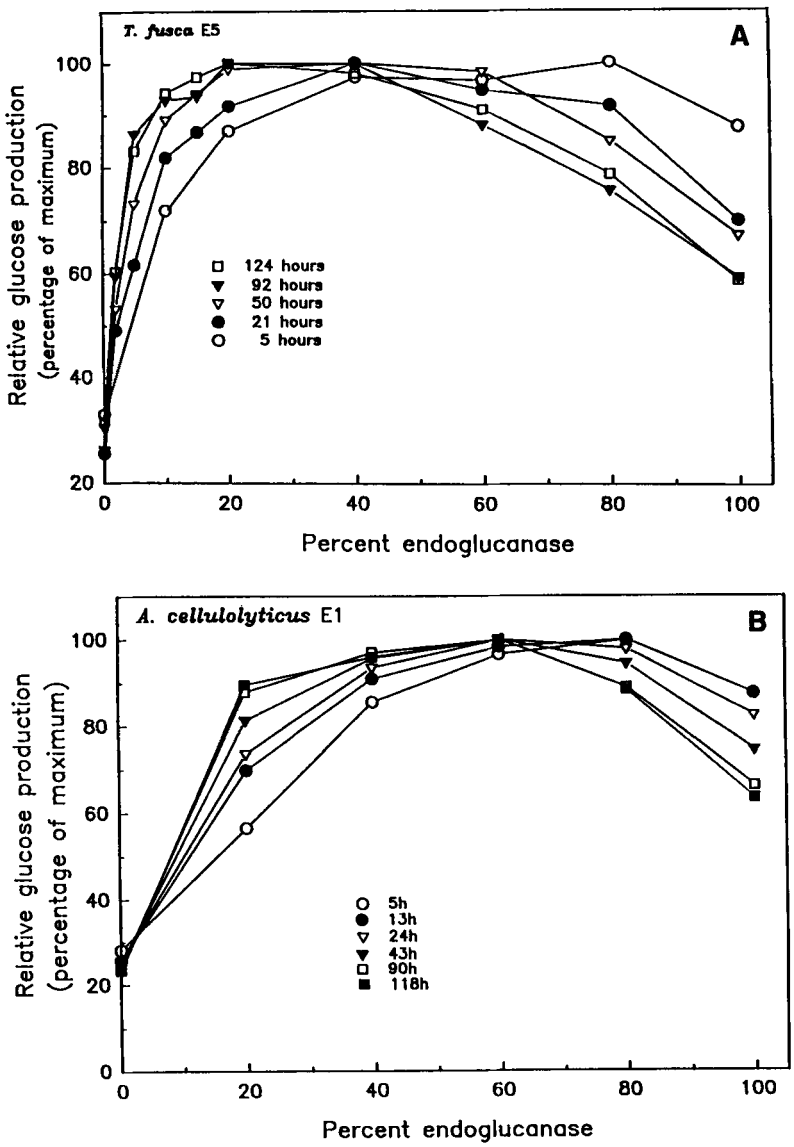

Fig. 4. Normalized presentation of the glucose-production data in Fig. 3. For each curve, the ordinate values are shown as a percentage of the maximum value.

With increasing digestion time, the normalized glucose-production curves for both of the enzyme pairs show clearly the emergence of a second prominence (a shoulder, in this case) located below the position of the current output maximum on the endoglucanase-loading scale. In both cases, the emerging prominence appears to be in the vicinity of the mixture proportions yielding maximum synergism (Fig. 2, the values being 5–15% endoglucanase for E_5 and "near or below 20% endoglucanase" for E1). Even though the glucose output maximum appears to be stalled at

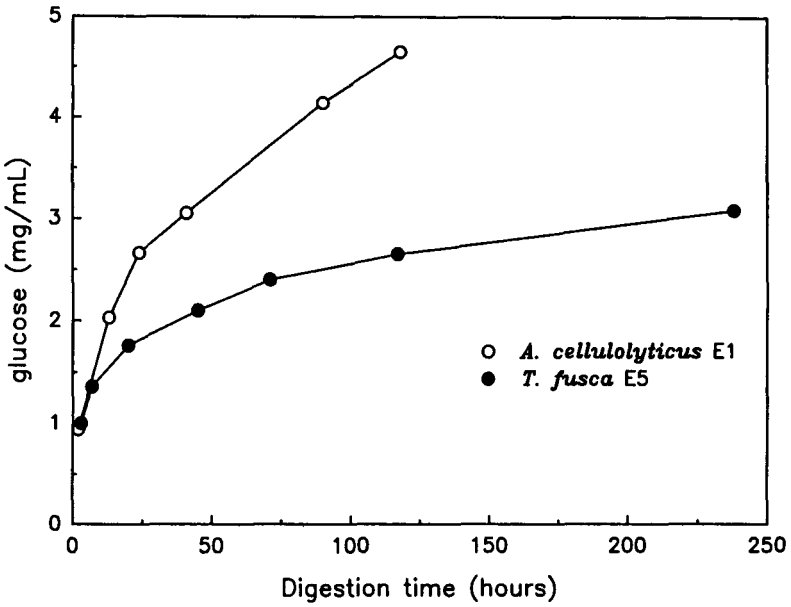

Fig. 5. Glucose production from 5% w/v Sigmacell-20 by *A. cellulolyticus* E1 and *T. fusca* E_5, each acting alone at a concentration of 0.65 μM. Conditions as in Fig. 2.

60% endoglucanase for E1 at the longest digestion times evaluated here, it does not appear to be too unreasonable to suggest that the output curves for *both* enzyme pairs may be building toward a shape with glucose-output and synergism maxima closely superimposed. In this view (which, we wish to reemphasize, is based on unsupported projections of the observed trends to longer digestion times) the significant difference between the digestion patterns shown by the two enzyme pairs would lie in the much slower approach by the E1/CBH I pair to the "final" state in which the synergism and glucose-output maxima coincide.

The differences observed in the digestion patterns for the two endoglucanases bring to mind intriguing questions concerning possible explanations for the observations in terms possible differences in the specific modes of attack by the two enzymes. For example, do the observed different relationships between synergism and absolute glucose output reflect differences in ranges of substrate specificity, or can they be explained in terms of hydrolysis rates that are proportionally higher, across the board against the same array of reaction-site types, for one endoglucanase as compared with the other? (The data shown in Fig. 5, in which each of the two endoglucanases hydrolyzes Sigmacell without the assistance of exoglucanase, would seem to indicate that in the digestion of the most easily-hydrolyzed material, represented by generation of the first 1.4 mg/mL or so of glucose, the two enzymes are virtually identical in ability, whereas E1 seems to be better suited to the hydrolysis of the more recalcitrant material left after the initial rapid hydrolysis.)

Answers to questions about specific mechanisms of attack by the two endoglucanases will require more extensive digestion data than that presented here, plus additional types of data, such as (but not limited to) careful analysis of soluble products released in the absence of added β-glucosidase and studies of binding equilibria for individual enzymes during the digestion. What can be reasonably proposed at this point is that, leaving aside questions about specific mechanisms, there appear to be two digestion processes operating in parallel in each of the digestions studied here. One process appears to be heavily dependent on synergism between endoglucanase and exoglucanase, and manifests itself as the increasing relative rates of glucose production seen (Fig. 4) at low endoglucanase/exoglucanase ratios near those required for maximum synergism. The second process appears to be less tightly coupled to synergism (at least to the type of synergism represented by the synergism-ratio peaks at 20% and lower endoglucanase) and may have a larger contribution from the enzymes acting independently. With regard to characterization of the two endoglucanases, the interesting question is why this second process, displayed as the high initial rates for mixtures with high (60–80%) proportions of endoglucanase, persists as a major contributor so much farther into the digestion of the substrate for E1/CBH I than for E_5/CBH I. Questions such as this present challenging subjects for future studies.

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