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Thermal Denaturation of *Trichoderma reesei* Cellulases Studied by Differential Scanning Calorimetry and Tryptophan Fluorescence

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ABSTRACT

The thermal denaturation of four purified *Trichoderma reesei* cellulase components, cellobiohydrolase (CBH) I, CBH II, endoglucanase (EG) I, and EG II, has been monitored using a combination of classical temperature/activity profiles, differential scanning calorimetry (DSC), and thermal scanning fluorescence emission spectrometry. Significant correlations were found between the results of enzyme activity studies and the results obtained through the more direct physical approaches, in that both DSC and the activity studies showed EG II (T_m=75°C) to be much more thermostable (by 10–11°C) than the other three enzymes, all three of which were shown by both activity profiles and DSC to be very similar in thermal stability. The temperature dependence of the wavelength of maximum tryptophan emission showed a parallel result, with the three enzymes exhibiting less thermostable activity being grouped together in this regard, and EG II differing from the other three in maintaining a less-exposed tryptophan microenvironment at temperatures as high as 73°C. The DSC results

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suggested that at least two transitions are involved in the unfolding of each of the cellulase components, the first (lower-temperature) of which may be the one correlated with activity loss.

Index Entries: Thermal protein denaturation; *T. reesei* cellulases.

INTRODUCTION

Of the cellulose-degrading enzymes produced by the fungus *Trichoderma reesei,* four major components, including two endoglucanases (EC 3.2.1.4), denoted EG I and either EG II (*1*) or EG III (*2*), and two cellobiohydrolases (EC 3.2.1.91), denoted CBH I (*1*) and CBH II (*3*), have been obtained in pure form and subjected to fairly extensive characterization with respect to both structural and enzymatic properties. Complete peptide sequences have been deduced from gene sequences for all four of the components (*2–4*), with a complete sequence by amino acid analysis also available for CBH I (*5*), the most abundantly produced of the enzymes. The sequences of all four of the enzymes have been shown to share two conserved homologous regions located together at one or the other end of the peptide chain, and generally referred to as the "A" and "B" regions, with interenzyme sequence homology as high as 70% in the A regions (*6, 7*). Sequence homology between the remaining portions of the sequences is limited, although for CBH I and EG I, the degree of sequence homology is as high as 45% for the complete sequences (*8*).

The results of limited proteolysis studies on CBH I, CBH II, and EG II (*9,10*), and of small-angle X-ray scattering studies on both intact CBH I and the larger of two fragments produced by limited proteolysis (*11,12*), indicate that these molecules may be rather asymmetrical (tadpole-shaped), with a protruding "tail" connected to the rest of the molecule (referred to as the core) by the heavily glycosylated B (or hinge) region. Based on studies of the enzymatic activities of the intact CBH I and CBH II and of the core molecules produced proteolytically from each, it has been proposed that the tail region contains a binding site for cellulose (that may also function in disrupting the structure of crystalline cellulose), whereas the hydrolytic, bond-cleaving site is located in the core portion of the protein (*9*). The solution structure of the synthetic cellulose-binding region of the CBH I sequence has been determined by means of two-dimensional NMR (*13*), and an X-ray crystallographic structure of the proteolytically derived core fragment of CBH II has been obtained (*14*), holding out the hope that additional structures may be forthcoming for this quartet of enzymes.

Considerable information is available concerning the disulfide-bonding patterns for CBH I (*5*), and from sequence homologies between CBH I and EG I, disulfide-bridge patterns have been suggested for the latter enzyme (*5,8*). Two disulfide bonds appear to be internal to the A region in

CBH I, and because half-cystines are among the most conserved residues in the various A regions (*6*), this may well be the case for all four of the cellulases. For at least one of the enzymes (CBH I), changes in intrinsic fluorescence emission intensity have been shown to be useful measures of the guanidine-hydrochloride unfolding of the molecule and its subsequent renaturation (*15*).

These structurally homologous *Trichoderma* cellulase components have been found to act as a system in the degradation of crystalline cellulose, with the activities exhibiting synergism not only between the EG and the CBH components (*16–18*), but also between the two CBH components (*19,20*). In view of the observed synergism between CBH I and CBH II, it has been suggested (*20*) that, although both enzymes cleave cellobiose units from the nonreducing ends of cellulose chains, each may be specific for one of the two sterically different end-group orientations that occur in crystalline cellulose.

Correlations between structural changes in enzyme proteins and changes in the activity of the enzymes are of great interest, both in terms of fundamental research into the mechanisms of action of native enzymes and in terms of the design of procedures for modifying or enhancing the activity of enzymes by chemical modification, genetic engineering, or a combination of both. The *Trichoderma* cellulase family, presenting as it does a series of significantly homologous (and, quite possibly, evolutionarily connected) enzymes with closely related, but not identical functions, constitutes an interesting subject for studies aimed at relating enzyme structure and function. Furthermore, since these cellulase enzymes are used widely in industrial applications where enzyme lifetime under process conditions is an important issue, studies elucidating the mechanisms of activity loss are essential. We present here the results of a study of the thermal unfolding of purified samples of all four of these cellulase components, using, in addition to classical temperature/activity profiles, two more direct, physical methods of monitoring the disruption of protein structure: thermal scanning fluorescence spectrometry, which as used here measures changes in the environments of certain reporter residues (primarily tryptophan) in the protein molecule, and DSC, which is more general in that it detects any endothermic process (such as the breaking of hydrophobic, ionic, or hydrogen bonds) involving any of the residues in the protein during denaturation.

EXPERIMENTAL

Protein Samples

The four cellulase components used in this study were purified to homogeneity from a Genencor International commercial cellulase (150L), derived from *T. reesei* using the general methodologies employed in the

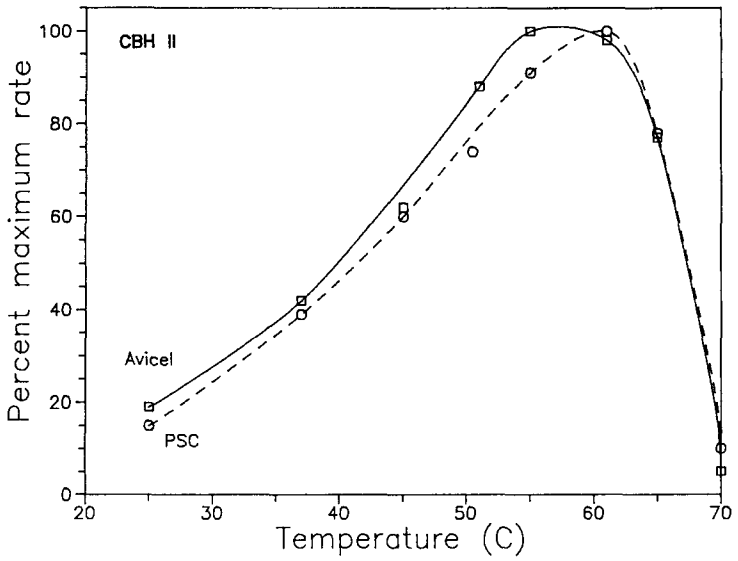

Fig. 1. CBH II (15 μg/mL) was incubated in 50 m*M* sodium acetate buffer, pH 4.5, with (circles) phosphoric acid swollen cellulose (PSC) (approx 0.1 wt%) and (squares) Avicel (1 wt%) in the presence of excess β-D-glucosidase for 30 min at the given temperatures. Glucose production was measured using the hexokinase reagent.

purifications of CBH I and EG I (*1*). All four purified proteins migrated as single, narrow bands on SDS-polyacrylamide gel electrophoresis (not shown). Details of the purifications of CBH II and EG II will be published separately.

Temperature/Activity Profiles

Activities of the different cellulase components were determined at assay temperatures ranging from 22 to 70°C using as substrates microcrystalline cellulose (Avicel), carboxymethyl cellulose (CMC), and phosphoric acid swollen cellulose (PSC), under the specific assay conditions described in the legends to Figs. 1–4.

Differential Scanning Calorimetry

DSC measurements were carried out using a Microcal MC-2 scanning calorimeter (Microal, Inc., Amherst, MA) with sample and reference cell vol of 1.13 mL. Sample solutions were buffered at pH 4.8 in 50 m*M* acetate (pH adjustment at 22°C). For the data shown, sample protein concentrations ranged from 23.73 μ*M* (1.12 mg/mL) for CBH II to 4.95 μ*M* (0.223 mg/mL) for EG I. Data collection and analysis utilized the DA2 software package (Microcal, Inc.) and an interfaced IBM PC-XT computer. Deconvolutions of the endotherms were based on the assumption that the observed asymmetrical peak shapes arose from the addition of multiple symmetrical (i.e., two-state or van't Hoff-type), overlapping endotherms.

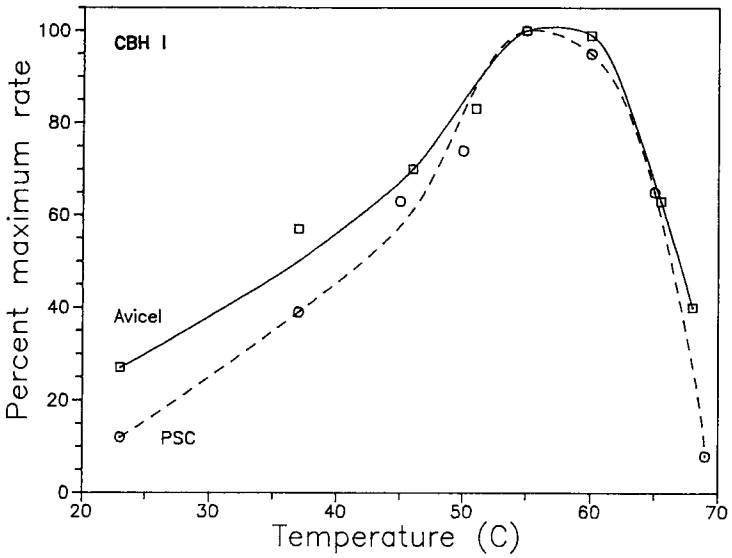

Fig. 2. CBH I (30 μg/mL) was incubated with PSC and Avicel as described in Fig. 1. Labels are the same as above.

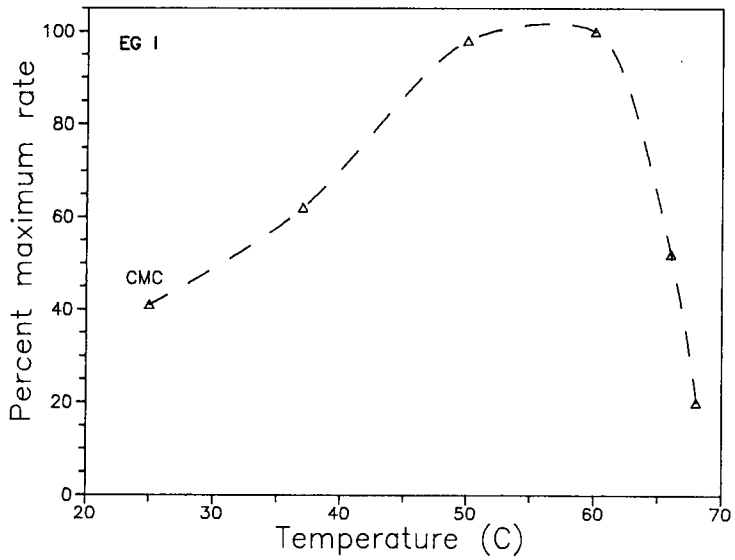

Fig. 3. EG I (20 μg/mL) was incubated in 50 m*M* sodium acetate buffer, pH 5.0, with carboxymethyl cellulose (CMC, 1 wt%). Reducing sugar was measured with dinitrosalicylic acid (DNS) reagent after 30 min.

Although both sequential and independent models were tested for ability to model the data, the enthalpy values given in Table 1 were generated using the assumption that the multiple transitions contributing to the observed endotherms occurred independently rather than sequentially. In all cases, the presumably slightly sigmoidal baseline beneath the endotherms (*21,22*) was approximated by a straight line connecting the low- and high-temperature baselines (*23*).

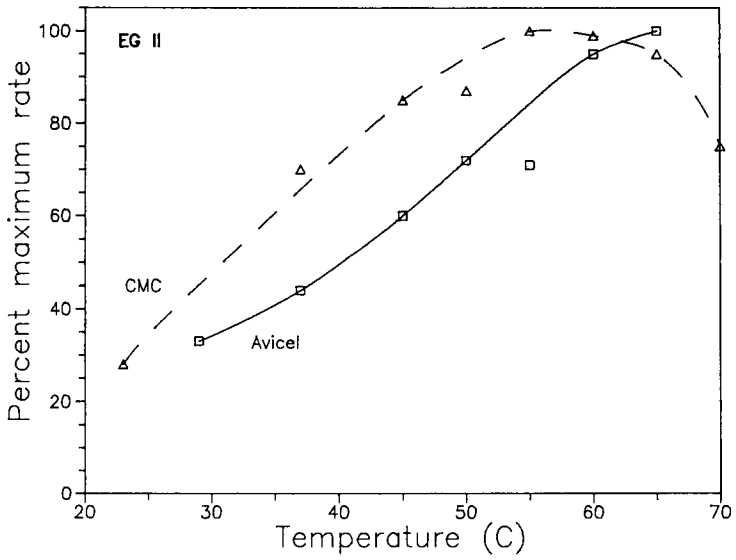

Fig. 4. EG II (1.9 μg/mL) was incubated in 50 m*M* sodium acetate buffer, pH 4.5, with (triangles) carboxymethyl cellulose (CMC) and (squares) Avicel. Excess β-glucosidase was present only with Avicel. Reducing sugar (DNS reagent) or glucose (hexokinase assay) was measured when CMC or Avicel was the substrate, respectively, after 60 min.

Table 1
Experimental and Calculated Values from DSC of *T. reesei* Cellulases

	Overall values			Deconvoluted[a] transitions	
Enzyme	T_m °C	ΔH_{cal}, kcal/mol	$\Delta H_{V\cdot H}$, kcal/mol	T_m, °C	$\Delta H_{cal} = \Delta H_{V\cdot H}$, kcal/mol
CBH I	64.35	365.4	203.7	62.95	167.4
				64.43	231.2
CBH II	64.05	356.2	148.3	61.54	139.3
				64.34	207.0
EG I	64.55	289.7	215.1	63.07	210.5
				64.96	256.5
EG II	75.05	469.9	231.9	73.64	206.6
				75.32	286.4

[a]Deconvolution analysis described in Experimental.

Thermal Scanning Fluorescence

The temperature dependences of the intrinsic fluorescence emissions of the purified cellulase components were determined using a FLUOROLOG-2 fluorescence spectrometer (Spex Industries, Edison, NJ) equipped with a Spex DM1B Laboratory Coordinator for data collection and analysis. A water-jacketed sample cell holder was installed in the spectrometer, and

the temperature of the holder was varied using an external circulating water bath. Temperature of the sample, which was magnetically stirred during the experiment, was measured by means of a thermocouple (model 115 TC, Omega Engineering, Stamford, CT) immersed in the sample solution. Protein concentrations in the samples, which were buffered at pH 4.8 in 50 m*M* acetate, were between 0.1214–0.0400 mg/mL. Emission spectra were collected at intervals of 2°C by setting the excitation monochromator at 285 nm and scanning the emission monochromator from 300 to 450 nm.

RESULTS AND DISCUSSION

Temperature/Activity Profiles

The activities of the various purified components were measured at temperatures from 23 to 70°C, using as substrates soluble CMC and suspensions of PSC (0.1 wt%) and Avicel (1.0 wt%), all in acetate buffer. The temperature/activity profiles were quite similar for three of the enzymes. CBH I, CBH II, and EG I reach maximal activity against all substrates between 55–60°C, and all three show sharp decreases in activity as the temperature is increased above 60°C (Figs. 1–4). The fourth component, EG II, although assayed for a longer period, which would tend to increase the effect of concurrent enzyme denaturation upon the endpoint product values, retains a much higher percentage of its maximal activity at temperatures > 60°C. In fact, the activity of this enzyme against the microcrystalline substrate Avicel has not yet reached maximum and is still increasing at 65°C.

Differential Scanning Microcalorimetry Studies of Enzyme Unfolding

Figure 5 shows the thermal unfolding of the four purified components, as measured by DSC. Three of the enzymes (CBH I, CBH II, and EG I) have virtually identical values of T_m, which is the temperature of maximum differential power input (sample cell vs reference cell containing buffer alone), and which for relatively simple processes approximates the temperature at which the denaturation of the protein is half complete. T_m values for these three components are clustered within a very narrow range from 64.0 to 64.6°C. The T_m for EG II, however, is considerably higher, at 75.05°C (Table 1). The observation that the two cellobiohydrolases have virtually identical denaturation temperatures (Table 1) is particularly interesting in light of the considerable differences that have been found between the amino acid sequences of the two proteins, such as the numerous individual-position differences between the respective core regions (*3,4*), the location of the combination of A and B segments at the carboxyl terminus of CBH I, but at the amino-terminus of CBH II, and the

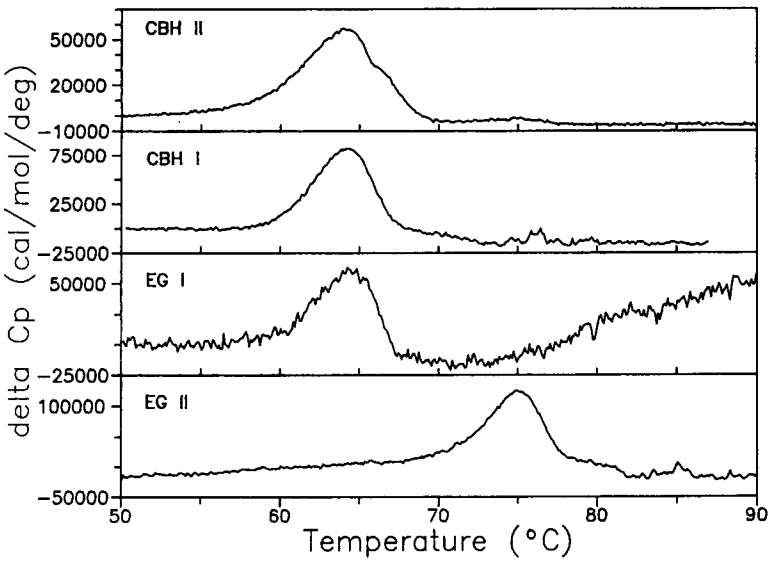

Fig. 5. Coplot of the differential scanning microcalorimetry (DSC) of the four cellulases. Concentration of protein was about 1 mg/mL in cell.

doubling of the B segment in CBH II (*6*). The close similarities between the thermal stabilities of CBH I and CBH II may well represent yet another testimony to the great ability of proteins of similar function to maintain homologous tertiary structures in the face of numerous evolutionary amino acid substitutions (*24*). From the fact that the ratios of the overall calorimetrically measured enthalpy to the effective, or van't Hoff, enthalpy are substantially greater than unity for all of the proteins tested (Table 1), it is evident that the unfolding of these enzyme molecules involves more than one transition, and thus, more different conformational states than simply a "native" and a "denatured" state.

After each of the thermograms shown in Fig. 5 was recorded, the sample and reference cells were cooled and rescanned over the same temperaature range. In no case were any further endotherms detected during the second scan, indicating that, whether the actual unfolding steps are reversible or not, the overall process, including the cooling step, is irreversible.

It can be seen in all four thermograms that the baseline is negative in the temperature region above the denaturation peak. Such an observation is indicative of one or more exothermic processes, such as, for example, the (intermolecular) coagulation of unfolded protein molecules (*21,25,26*). Because a significant proportion of the protein in the samples is found in fact to be coagulated upon removal of the samples from the cells after postscan cooling, it is likely that coagulation is the exothermic process involved. It is also quite likely that this coagulation is the reason for the overall irreversibility of the process (i.e., the fact that no protein-unfolding peaks are observed during the second and subsequent scans), and further likely that the release of energy by the coagulation process, simultaneous with the uptake of energy by bond breaking during the

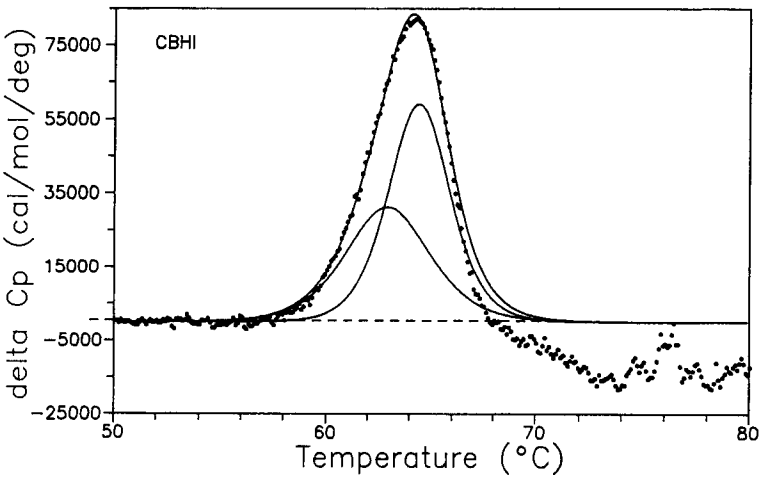

Fig. 6. Superposition of actual experimental data for the thermal denaturation of CBH I (shown as small circles), the two elements (curves) generated by deconvolution using the DA2 software (assuming multiple independent, reversible, two-state transitions), and the sum (largest curve) resulting from these two elements.

denaturation of the last of the protein sample, may distort the shape of the endothermic peak at its high-temperature edge.

Other observations, however, strongly suggest that the above-mentioned distortion is not serious. For all of the enzymes except EG I, DSC scans have been carried out with ranges of protein concentrations differing by factors of 2–4. In no case has the position of T_m been found to depend on protein concentration, as would be expected were the process significantly affected near T_m by an intermolecular process, such as coagulation (*27*). For CBH I and CBH II, the enzymes tested so far at more than one scan rate, the position of T_m was also found to be essentially unaffected by a twofold variation in scan rate (0.5°/min and 1.0°/min). Scan-rate independence, or the absence of kinetic control of the unfolding, has been cited as an indication of reversible unfolding even in cases, such as the one described here, in which the endotherms cannot be reproduced upon a subsequent rescan after cooling of the sample (*28–31*). These observations support the view that the shape of the denaturation peak, at least up to 1 or 2°C above T_m, is largely unaffected by the postunfolding coagulation, and, more fundamentally, in the case of CBH I and by a tentative extension to the other three enzymes, that the overall irreversibility of the process may be due to coagulation subsequent to the unfolding represented by the endothermic peak, and not to irreversibility of the actual unfolding step(s) itself. Consistent with this view is the fact that the DSC peaks for all four of these enzymes can be modeled quite well by algorithms that assume reversible, equilibrium unfolding processes. If the deconvolution process is restricted to that portion of each peak occurring at temperatures below a point 1.5–2.0°C above the T_m, the fits obtained are even better. Figure 6 illustrates the process for CBH I. Here the observed

unfolding peak is represented as the sum of the two independent, reversible transitions, with T_m values of 62.95°C and 64.43°C, respectively. The two transitions do not differ greatly in midpoint temperatures, but the smaller of the two component peaks, which is broader because it represents a process with a less dramatic temperature dependence, dominates the low-temperature edge of the overall profile to a much greater extent than it would were the two peaks of the same half-value width. Because these enzymes (CBH I, CBH II, and EG I) begin to show significant losses of activity at temperatures below 59°C, a temperature at which the major (64.43°C) transition has not taken place to any significant extent, it is possible that the loss of enzymatic activity may reflect an irreversible step depending on, and subsequent to, the smaller, lower-temperature thermal peak. The larger, higher-temperature peak may then be seen as representing the destruction of residual structure in an already inactivated enzyme molecule.

Given the strong indications from studies utilizing small-angle X-ray scattering, limited proteolysis, or both that the *Trichoderma* cellulases have a bilobed, tadpole shape (*10,11*), and should it be shown that the two transitions are completely independent, it might be tempting to suggest that the larger of the two contributing transitions represents the unfolding of the head or core segment of the protein, whereas the smaller transition represents the unfolding of the smaller tail segment. We have found, however, that for at least one of the *Trichoderma* cellulases (CBH I) the asymmetrical DSC endotherm displayed by the intact enzyme is duplicated upon DSC scanning of the proteolytically derived core fragment, and that the isolated tail fragment shows no detectable DSC endotherm at all over the range from 12 to 95°C (*32*). The data presented in Figs. 5 and 6 can, moreover, be fitted almost as well by models assuming two sequential, rather than independent transitions. (The independent-transition model is presented in Fig. 6 for reasons of representational simplicity and clarity—in the sequential model the overall peak is not simply the sum of the two component peaks). The observation that the locations of the two apparent component peaks for the highly thermostable EG II are displaced virtually the same distance on the temperature scale with respect to their corresponding component peaks in the three less-thermostable enzymes is actually easier to understand in terms of a sequential model than in terms of a model involving independent transitions.

Thermal Scanning Fluorescence Studies of Enzyme Unfolding

The wavelength of maximum fluorescence emission for tryptophan residues in proteins (λ_{flr}^{max}) is a sensitive function of the polarity of the microenvironment surrounding a given residue—the more polar the environment, the longer the wavelength of maximum emission (*33*). This

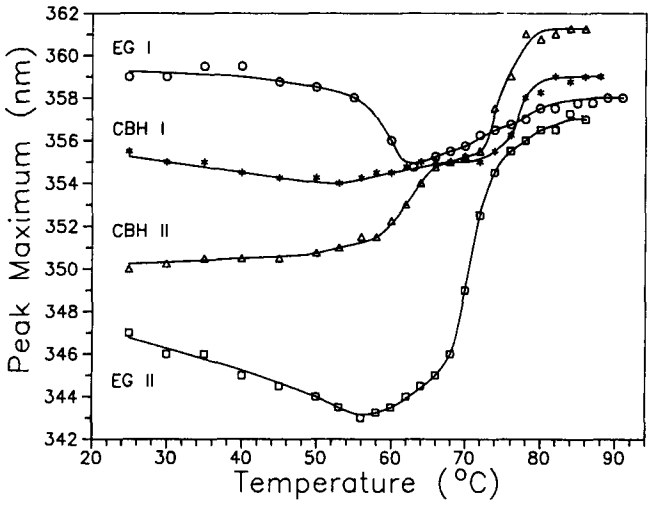

Fig. 7. The temperature dependence of the fluorescence emission maxima for the four purified cellulase enzymes.

principle is used to monitor the transfer of tryptophan side chains from a relatively nonpolar (hydrophobic) environment in the interior of a tightly packed globular protein to an essentially aqueous environment as the protein is unfolded to an open, random-coil structure.

Figure 7 plots the dependence on temperature of λ_{flr}^{max} for each of the *Trichoderma* cellulase components in this study. In its details, this plot almost certainly is governed by other influences in addition to the aforementioned sensitivity to environmental polarity of λ_{flr}^{max}. For instance, because the tryptophan fluorescence of a protein containing multiple tryptophan residues will be the sum of the emissions of the individual residues, an increase in solution temperature may well produce a change in the wavelength of maximum intensity for the overall fluorescence envelope, even in the absence of any substantive change in average protein conformation. This can come about for at least two reasons related to differential quenching of the fluorescence of different residues: first, because an increase in solution temperature, and therefore in the average velocity of solvent molecules and of other quenchers dissolved in the solvent (such as molecular oxygen), can result in increased quenching of the fluorescence of tryptophan residues already exposed on the surface (*33–35*), and second, because at higher temperatures, the protein undergoes increasingly large thermally driven oscillations about its average conformation, without major changes in the *average* position. This flexing of the protein molecule results in increased "dynamic quenching" of partially exposed residues owing to quenching species that penetrate the structure during the fluctuations. This differential quenching, constituting a change in the intensity, rather than in the wavelength, of the emission from surface residues, can

result in increased domination of the overall shape of the envelope by more buried and, hence, less quenched residues, thereby shifting the overall minimum to lower wavelengths. The decrease in maximum emission wavelength seen for EG II over the range from 25 to 56°C may be an example of such an effect on emission from tryptophans in an extremely stable protein structure.

Even with such considerations taken into account, however, it is apparent that, over the temperature range from 55 to 80°C, over which range these cellulases are known from kinetic and DSC studies to undergo profound structural alterations, there are also striking changes in the wavelength of maximum emission. The most interesting observation in the present case is that, between the temperatures of 60–70°C, over which range CBH I, CBH II, and EG I are known to undergo major loss of folded structure, these three enzymes have virtually identical tryptophan environments, as reflected in the wavelength of maximum emission. In contrast, the much more thermostable EG II, which does not denature until the temperature exceeds 70°C (DSC results), has a maximum emission wavelength much shorter (for temperatures near 65°C) than the value shared by the three less thermostable enzymes, indicating less solvent-exposed average environments for the tryptophans in EG II in this temperature range. EG II does, however, show significant changes in λ_{flr}^{max} over the range from 56 to 66°C, as do CBH II and EG I (Fig. 7).

Structural interpretations based on comparisons between the fluorescence response of different proteins, such as the comparisons made in the preceding paragraph, must be made with great caution. Because the intrinsic fluorescence of proteins depends as heavily as it does on the fluorescence response of a single type of amino acid side chain, namely that of tryptophan, the response and its interpretation are extraordinarily sensitive to the location of the reporter (tryptophan) groups within the molecule. An obvious example is provided by the case in which structurally analogous regions of related proteins differ by including or not including tryptophan residues. In the first instance, the unfolding of the region in question can be expected to result in a large change in the fluorescence spectrum; in the second, the unfolding of the region may be essentially undetectable as far as fluorescence measurements are concerned. Given certain additional information about the protein, such as a reasonably high-resolution three-dimensional structure providing the location of the tryptophan residues, much more definite conclusions may be drawn. Although such information is available for the major (core) segment of CBH II (*14*), a three-dimensional structure is not yet available for EG II. What the present fluorescence results reveal is that thermal unfolding of EG II results in a very strong signal in terms of λ_{flr}^{max} and can thus be expected to be an especially profitable subject for fluorescence studies once the three-dimensional structure is available.

CONCLUSIONS

The thermal inactivation behavior of the four major cellulase components from *Trichoderma reesei* appears to be closely related to the thermal unfolding of the protein structure as monitored by DSC and thermal scanning fluorescence spectrometry. This study revealed that the component enzyme that exhibits the most stable activity at elevated temperatures (EG II) is also the protein shown by the DSC and fluorescence studies to maintain its native, folded conformation at temperatures high enough to disrupt the structures of the other three enzymes studied.

DSC in particular appears to be well suited as a companion technique to activity assay in studies of structure–function relationships in this enzyme system. Protein intrinsic fluorescence can serve as a probe of structural changes in these enzymes (and a very sensitive probe in the cases of EG I, EG II, and CBH II), and can yield information that complements that obtained from activity and DSC studies. Because of the complexity of the structural influences on emission spectra, however, it is probably best that monitoring of conformational changes by fluorescence be based on more than one approach, i.e., residue mobility studies (fluorescence depolarization) and tyrosine-tryptophan energy transfer, used in conjunction with studies based on residue microenvironmental polarity as used in the present study. With the interpretational limitations of fluorescence studies kept in mind, it would appear that studies seeking correlations between activity, calorimetric, and fluorescence approaches to the monitoring of protein conformational changes can be expected to be synergistic, yielding more valuable information than the sum of that obtained by employing these methods separately.

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