Volume 282, number 2, 355–358 FEBS 09687 May 1991

ADONIS 001457939100396G

Structural analysis by circular dichroism of some enzymes involved in plant cell wall degradation

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Received 15 February 1991

Three enzymes which degrade different polysaccharide components of plant cell walls have been characterized by circular dichroism (CD). A bacterial endoglucanase, which in the native state forms part of a multiprotein cellulase complex, showed a tendency to form aggregates as measured by CD. Depending on its degree of aggregation, this enzyme displayed between 50% and 100% helical structure, whereas a bacterial xylanase and a fungal polygalacturonase exhibited more β-sheet structure. The polygalacturonase was apparently devoid of helical structure.

Cellulase; Polygalacturonase; Xylanase

1. INTRODUCTION

The polysaccharide polymers cellulose, hemicellulose and pectin are 3 of the main structural components of plant cell walls. Cellulose consists entirely of β-1,4-linked glucose molecules; the major polymeric component of hemicellulose, xylan, consists of β-1,4-linked xylose residues which may be substituted with variable amounts of other side chain sugars. Pectin is a polymer of β-1,4-linked galacturonic acid containing esterified methyl groups.

Complete degradation of plant cell walls is effected by a repertoire of microbial enzyme activities (bacterial and fungal) which includes endoglucanase, polygalacturonase and xylanase.

Cellulases (endoglucanases, cellobiohydrolases and β-glucosidases) of bacterial and fungal origin, have been the subject of detailed studies. In both classes of microorganism the cellulase system consists of multiple proteins encoded by multiple genes but, apart from primary sequence data, comparatively little is known about the structures of individual proteins. Endoglucanase D, synthesized by *E. coli* containing the cloned *celD* gene from *Clostridium thermocellum*, has been crystallized but the crystal structure is not yet available [1]. Similarly, cellobiohydrolase II from the fungus *Trichoderma reesei* has been crystallized and the structure of the catalytic domain, obtained by treating the native enzyme with papain, has been shown to be a 7-fold α/β barrel [2,3]. This is the first report of this type of 7-fold structure as opposed to the 14 examples of 8-fold α/β barrels (for review see [4]).

Hemicellulases have been less extensively studied but a purified xylanase from *Bacillus pumilus* has been crystallized and preliminary X-ray data indicate a structure containing predominantly β-sheets [5,6].

The polygalacturonase family of enzymes has been investigated in some detail and a gene coding for this activity has recently been cloned [7]. A similar enzyme, pectate lyase, which cleaves pectin by a β-elimination mechanism, has also been crystallized, but no structural information is available [8,9].

Efficient hydrolysis of each of the polysaccharide components of plant cell walls using microbial enzymes would allow more effective use to be made of cellulosic biomass contained in agricultural and domestic wastes, and is an obvious goal for biotechnology. In order to develop the mixtures of enzymes necessary to achieve this goal, we will require knowledge of each of these proteins at the molecular level, including an understanding of their structure/function relationships. It may then be possible to use protein engineering techniques to produce highly active composite enzymes.

In this paper, we describe the biophysical analysis of an endoglucanase from the anaerobic bacterium *C. thermocellum*, a xylanase from the aerobic bacterium *Pseudomonas fluorescens* subsp. *cellulosa* and a polygalacturonase from the fungus *Colletotrichum lindemuthianum*. The first two proteins were purified from cultures of *E. coli* expressing cloned genes; the poly-

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galacturonase is the full-length native enzyme produced by the normal fungal host. The data obtained highlight significant differences between the secondary structures of the endoglucanase and the two enzymes which attack the more amorphous substrates pectin and xylan.

2. EXPERIMENTAL

2.1. *Enzyme purification*

2.1.1. *Endoglucanase E. E. coli* JM 83 containing a truncated version of the *celE* gene encoding residues 4–367 of mature EGE from *C. thermocellum* cloned in pMTL22p [10] was cultured in LB broth containing ampicillin (100 μg/ml). Catalytically active EGE contained in the periplasmic fraction [11] was purified by anion exchange chromatography [12].

2.1.2. *Xylanase A (XYLA).* XYLA from *P. fluorescens* synthesized by *E. coli* JM83 harbouring pRS16 [11] was purified form cell-free extract as described previously [12]. As a consequence of post-translational processing by *E. coli*, XYLA produced by this method consisted of the catalytic domain and had an M_r of 39 kDa (c.f. 60 kDa for the full-length protein).

2.1.3. *Polygalacturonase.* Polygalacturonase secreted by *C. lindemuthianum* was purified as described previously [13].

2.2. *Circular Dichroism*

Stock solutions of EGE and XYLA were prepared in 10 mM Tris-HCl buffer, pH 8.0. Polygalacturonase was dissolved in 50 mM sodium acetate buffer, pH 4.8. Samples for circular dichroism (CD) were prepared by diluting these stock solutions to a final protein concentration of approximately 0.1 mg/ml. Absolute protein concentrations were determined from the stock solutions by amino acid analysis. Far-UV CD spectra (260–190 nm) were measured using a Jasco J600 spectropolarimeter, and data were recorded on-line using an IBM personal computer. Spectra presented are the average of 4 scans, recorded at 10 nm/min, using silica quartz spectrophotometer cells generally of 1 mm pathlength. The concentration dependence of the CD spectra of EGE was measured using cells with a range of pathlengths. An instrument sensitivity of ± 20 mdeg full scale was routinely used, along with 4 s time constant [14].

Secondary structure was deduced by analysing CD spectra recorded with protein solutions of known concentration, using the CONTIN program [15] on a VAX computer. This program fits the experimental data with the CD spectra of 16 proteins of known secondary structure. The results of the best fit were output as fractions of the 3 structural components, α-helix, β-sheet and random coil. Instrument calibration was regularly checked during the course of the work using ammonium d-10-camphorsulphonate [16] and d-(−)-pantalactone [17].

3. RESULTS AND DISCUSSION

Far-UV CD spectra obtained for EGE as a function of concentration are shown in Fig. 1. The spectra are normalised with respect to concentration since they are plotted in terms of $\Delta\varepsilon$ and this highlights their concentration dependence. At 0.1 mg/ml the spectrum is intense and typical of a protein containing high levels of helix in its structure. It has been shown that CD can predict an helical structure with a correlation coefficient of 0.97 if the correct statistical methods are applied [18]. The spectrum showed distinct minima in the 218 and 208 nm regions and a positive peak at less than 200 nm. The intensity of this latter peak is a little low for helix but this may be due to excessive light absorption. At 0.05 and 0.02 mg/ml the intensity in the sub 200 nm region is more typical of helix but the intensity of the minima is reduced. The results of analysis of these spectra using the CONTIN programme are shown in Table I. The table shows how the enzyme is apparently moving from a large proportion of helical structure at high concentrations to a much more evenly balanced situation at lower concentrations with equal amounts of helix and sheet. It is well known that the cellulase system ('cellulosome') of *C. thermocellum* is an ordered complex containing 14–18 polypeptides with endoglucanase activity. It is now possible to dissociate and reassociate the cellulosome components [19] and the possibility that the EGE catalytic domain used in our experiments is reassociating spontaneously cannot be excluded. This may have the effect of apparently increasing the ordering of the system such that it appears

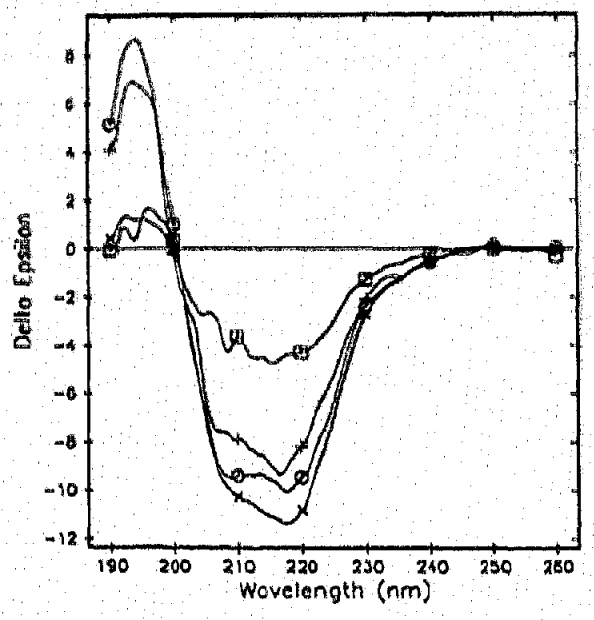

Fig. 1. Concentration dependence of the far-UV circular dichroism spectra of endoglucanase. The protein concentration was (×), 0.1 mg/ml; (○), 0.05 mg/ml; (+), 0.02 mg/ml and (□), 0.01 mg/ml. The symbols are shown to assist in curve identification and do not represent individual data points.

Table I

Results from a computer programme which analyses circular dichroism spectra and attributes secondary protein structural elements statistically.

Endoglucanase conc. (mg/ml)	Secondary structure (%)		Random structure (%)
	α	β	
0.1	100(0.15)	–	–
0.05	76.9(3.0)	23.1(3.70)	–
0.02	42.6(4.8)	57.4(4.8)	–
0.01	35.3(3.6)	32.4(5.3)	32.3

Figures in brackets are the standard deviations.

that there is a preponderance of helix. Concentration-dependent changes in peptide and protein structure have been reported previously. The bee venom peptide, melittin, is perhaps one of the best documented. Circular dichroism studies of this peptide reveal a transformation from a largely random structure to a highly helical structure with increasing concentration [20]. This structural change only occurs under certain buffer conditions but the transformation correlates with the formation of a tetrameric aggregate as the peptide concentration is increased. Aggregation phenomena in several proteins are accompanied by an increase in structure in the region of the interface between monomers in the aggregate. Dimer formation of β-lactoglobulin B is induced by raising the pH from 2 to 3. Infra-red and circular dichroism measurements have identified that dimers are stabilized by the formation of an intermolecular β-sheet formed by hydrogen bonding between surface β-strands of the monomers at the junction zone [21]. Our hypothesis is that the concentration dependent helix formation that we observe with EGE is linked to the formation of aggregates by this protein. It would seem likely that the interface between endoglucanase monomers in these aggregates is stabilized by interactions between helix motifs (most probably hydrogen bonds [22], which form at the junction between neighbouring monomers). The structure at interfaces between subunits of dimeric and tetrameric proteins has been recently reviewed [22]. Proteins that form aggregates by helix-helix packing include citrate synthase, cytochrome c′, phosphofructokinase and lactate dehydrogenase.

The other alternative is that increasing the concentration of the truncated enzyme favours the folded conformation and as the concentration is decreased denaturation increases with apparent loss of conformation.

Far-UV CD spectra obtained from xylanase and polygalacturonase are shown in Fig. 2. Again the spectra show helix-like features but their intensity is much reduced compared with the endoglucanase spectra. The results of the CONTIN analysis of these spectra are shown in Table II and indicate a more stable structure for these enzymes in solution. In particular the xylanase gives a figure for β-sheet of around 60% which is similar to the figure of ca. 57% proposed for *B. pumilus* [6] xylanase (based on X-ray diffraction). The information for polygalacturonase indicates around 60% of sheet and a very low amount of α-helix.

These results indicate that there may well be some difficulty in obtaining reliable structural data for the catalytic domain of EGE. The general impression one can gain is that at some concentrations the enzyme may be at the 'molten globule' stage of folding [23] and the extent of the gene truncation may have to be modified to obtain a stable molecule. However the indications are that the truncated form seems to have a minimum conformation of equal quantities of sheet and helix

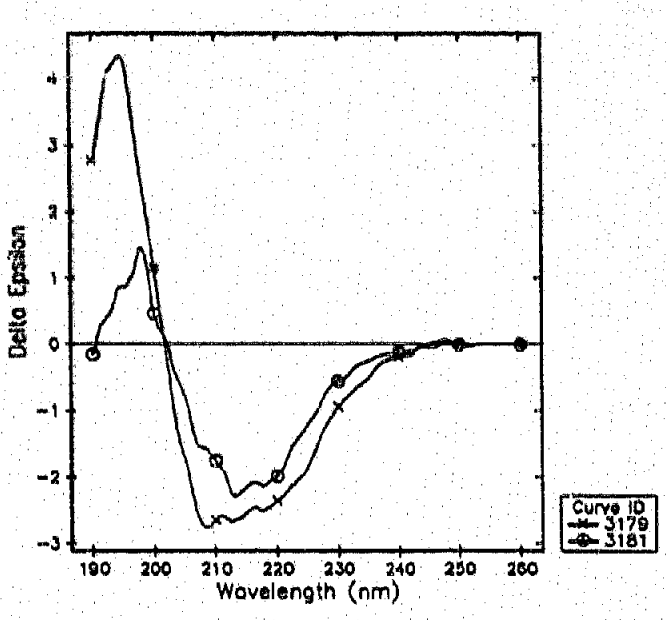

Fig. 2. Far-UV circular dichroism spectra obtained from (×), xylanase and (○), polygalacturonase samples. The spectra were recorded at a concentration of 0.1 mg/ml. The symbols are shown to assist in curve identification and do not represent individual data points.

which is similar to the structure proposed for cellobiohydrolase II. The xylanase and polygalacturonase are clearly mainly sheet structures with little if any helical structure in the polygalacturonase.

The results reported here are the first comparison of the structures that are responsible for the degradation of cellulose, hemicellulose and pectic materials. The catalytic protein which degrades cellulose is different in secondary structure to the enzymes degrading the more amorphous hemicellulose and pectic materials. However this should not be taken to mean that there will be great differences in the active site regions of these enzymes. Attempts to crystallize other intact cellulases rather than cellobiohydrase II from *T. reesei* and endoglucanase D from *C. thermocellum* have failed and it appears to be very important to prepare fragments of the endoglucanase which contain the active site region only [3]. The evidence we provide is that

Table II

Results from a computer programme which analyses circular dichroism spectra and attributes secondary protein structural elements statistically

Protein and conc.	Secondary structure (%)		Random structure (%)
	α	β	
Xylanase (0.1 mg/ml)	29.5(2.7)	65.9(3.1)	4.6(5.1)
Polygalacturonase (0.1 mg/mol)	5.4(3.1)	58.8(3.5)	35.8(5.9)

Figures in brackets are the standard deviations.

truncated endoglucanase enzymes from *C. thermocellum* are still able to aggregate. For workers trying to crystallize these enzymes, our results highlight the importance of examining them by techniques such as CD, which can identify aggregation before proceeding to crystallization trials; this is particularly relevant in the case of known aggregating enzymes such as EGE from *C. thermocellum*. We used an endoglucanase fragment from 4–467 (362 amino acids) compared to the fragment 83–447 of cellobiohydrase II (365 amino acids) used by others [23] and would have expected similar crystallization results However, it has not proved possible to obtain crystals of EGE thus far using the methods of Rouvinen [3]. This is almost certainly because the protein aggregates as it increases in concentration in the hanging drop. Further constructs will be made to try and reduce the tendency for aggregation in this protein as measured by CD.

Attempts to crystallize xylanase have met with more success and preliminary crystallization data will be published separately.

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