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Glycosylation of bacterial cellulases prevents proteolytic cleavage between functional domains

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Glycosylated cellulases from *Cellulomonas fimi* were compared with their non-glycosylated counterparts synthesized in *Escherichia coli* from recombinant DNA. Glycosylation of the enzymes does not significantly affect their kinetic properties, or their stabilities towards heat and pH. However, the glycosylated enzymes are protected from attack by a *C. fimi* protease when bound to cellulose, while the non-glycosylated enzymes yield active, truncated products with greatly reduced affinity for cellulose.

Cellulase; Proteolysis; Domain; Glycosylation; (*Cellulomonas*)

1. INTRODUCTION

The bacterium *Cellulomonas fimi* elaborates a complex array of cellulolytic activities [1]. Two cellulases, EngA and Exg (formerly CB1 and CB2, respectively [2]), have been studied intensively. Their genes, *cen*A and *cex*, were cloned in *Escherichia coli* and their DNA sequenced [3–5]. Many microbial glycanases are glycoproteins. EngA and Exg from *C. fimi* share this property [2]. Their counterparts synthesized in *E. coli* are not glycosylated. Here we have compared the properties of enzymes from both sources to assess the importance of glycosyl substitution.

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Abbreviations: EngA, endoglucanase A; Exg, exoglucanase; CMC, carboxymethylcellulose; HBAH, hydroxybenzoic acid hydrazide reagent; PMSF, phenylmethylsulfonyl fluoride; pNPC, *p*-nitrophenyl-β-D-cellobioside

2. EXPERIMENTAL

2.1. *Bacterial strains, plasmids and media*

C. fimi (ATCC 484) was grown on basal medium containing 3% Avicel [2] or 0.1% glycerol. *E. coli* PM191/pUC12-1.1 (PTIS) [6] and JM101/pUC18Ec2 (Guo, in preparation) were grown on LB medium [6] to provide non-glycosylated Exg and EngA, respectively.

2.2. *Assays*

Viscometric assay of CMCase and determination of pNPCase and protein have been described [2]. CMCase was also assayed with HBAH [7] with reference to glucose.

2.3. *Enzymes*

EngA and Exg were purified from *C. fimi* grown with Avicel by concanavalin A affinity chromatography [2], followed by anion-exchange chromatography in 20 mM Tris-Cl, pH 7.0 (Exg) or piperazine, pH 9.8 (EngA), 0–1 M NaCl, on Mono Q resin (Pharmacia). EngA was further purified by gel electrophoresis [2]. EngA and Exg

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were purified from *E. coli* by immunoadsorption [6] followed by anion-exchange chromatography. Culture supernatant from *C. fimi* grown with glycerol provided crude protease essentially free of cellulases (Langsford, in preparation).

2.4. *Antisera*

Purified non-glycosylated Exg was used to raise antiserum in rabbits [3]. Serum was also raised against a synthetic peptide: PTPTPTTPTPTPTTP TPTPTSG, corresponding to the Pro-Thr box of Exg [4], cross-linked to Keyhole limpet hemocyanin [7].

2.5. *Gel electrophoresis*

SDS-gel electrophoresis was as described [2]. Some gels were blotted onto nitrocellulose, reacted with antiserum, and developed with alkaline phosphatase-conjugated goat anti-rabbit IgG. Zymograms were as described [9].

3. RESULTS

3.1. *Catalysis*

Expression of active EngA and Exg by *E. coli* [2] had eliminated an essential role for glycosylation in catalysis. The possibility that glycosylation might modulate activity remained. Kinetic parameters for hydrolysis of CMC and pNPC by glycosylated and non-glycosylated enzymes were determined (table 1). Only minor differences in K_m or V_{max} were found.

3.2. *Stability to pH and heat*

Glycosylation may stabilize some cellulases to physical extremes [10]. We compared the stabilities of purified glycosylated and non-glycosylated EngA. At 60°C, both had half-lives of 33 min. At pH 3.5 and 9.5, the half-lives were 120 and 156 min, respectively. Exg gave similar results.

3.3. *Binding to cellulose*

Many cellulases, including those of *C. fimi* [2], bind tightly to cellulose. The possibility that glycosyl moieties of EngA and Exg might mediate this interaction was tested. Both glycosylated and non-glycosylated forms bound to Avicel. Equilibrium was attained in less than 5 min at 0°C. Avicel, in 50 mM potassium phosphate, pH 7.0, bound 1.3 mg of Exg and 4.6 mg EngA/g. Binding was resistant to further buffer washes.

Table 1

Kinetic parameters for hydrolysis by glycosylated (+) and non-glycosylated (−) cellulases

Substrate	Enzyme	K_m	V_{max}
CMC	EngA, +	0.19 ± 0.1	62.5 ± 1.1
	EngA, −	0.17 ± 0.1	56.6 ± 1.0
	Exg, +	3.18 ± 0.21	42.9 ± 1.5
	Exg, −	3.04 ± 0.23	35.8 ± 1.9
pNPC	Exg, +	0.64 ± 0.03	9.3 ± 0.2
	Exg, −	0.70 ± 0.02	11.4 ± 0.1

Initial velocities at 30°C were determined with HBAH or pNPC, over a substrate range of 0.1–8.0 K_m. Values, ± SE, were derived by weighted linear regression analyses of double reciprocal plots. K_m values are reported as mg/ml for CMC and mM for pNPC; V_{max} values as μmol product/min per mg protein.

3.4. *Susceptibility to protease*

C. fimi secretes a serine protease when grown on basal medium plus either CMC, cellulose, xylan or glycerol ([1] and Langsford, in preparation). We have argued that proteolysis contributes to the multiplicity of cellulases seen in *C. fimi* cultures but that binding to cellulose affords protection [1]. Therefore, we examined the effect of crude *C. fimi* protease on EngA and Exg, bound to Avicel. Active products of EngA were analysed on a zymogram (fig.1). The zymogram was intentionally over-exposed to reveal the presence of any minor components. Products of Exg were detected on a Western blot probed with antiserum to Exg (not shown). No changes were observed in the behaviour of glycosylated EngA (fig.1, lane B) or Exg following prolonged incubation. In contrast, protease treatment of the non-glycosylated enzymes resulted in the formation of discrete products. EngA (48 kDa) gave a 30 kDa fragment which retained enzymic activity (fig.1, lanes K and O). Exg (46.5 kDa) produced a 39 kDa immunoreactive fragment (not shown). Cleavage was not seen in the absence of protease and was inhibited by PMSF. The appearance of the 30 and 39 kDa fragments in reaction supernatants (fig.1, lane O, and not shown) indicates a reduction in

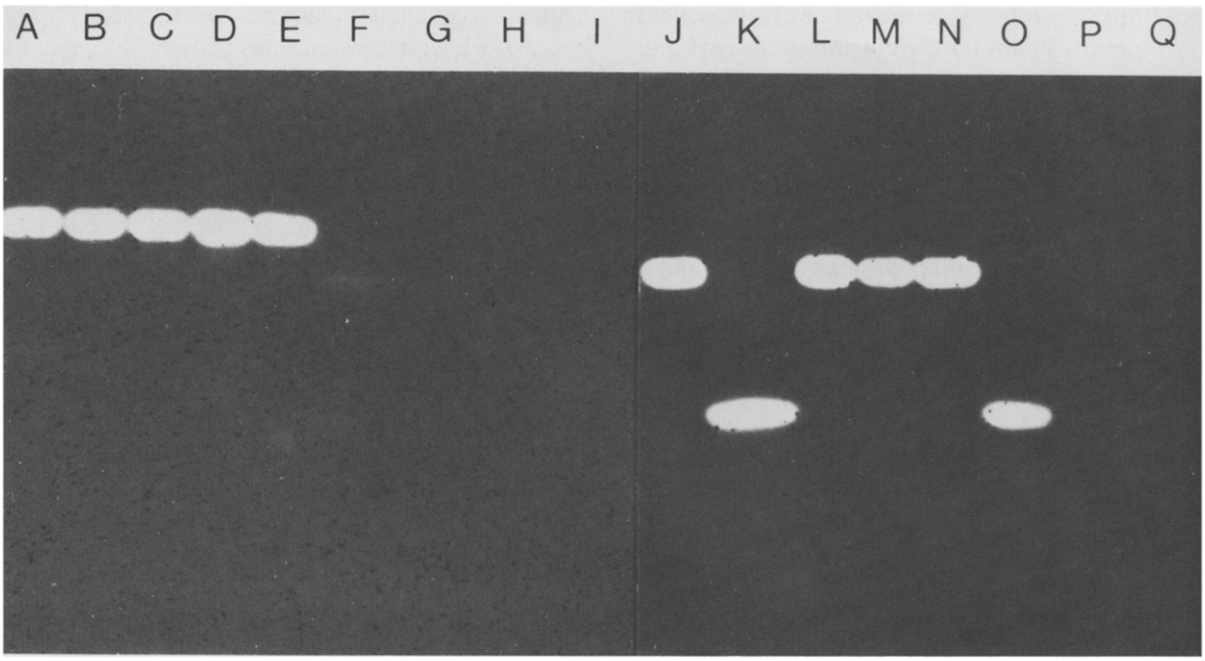

Fig.1. Zymogram of glycosylated (A–I) and non-glycosylated (J–R) EngA after incubation with *C. fimi* protease. Cellulases, bound to Avicel, were incubated with protease or control buffer for 72 h, at 30°C, then centrifuged to give cellulose-bound (A–E, J–N) and supernatant (F–I, O–Q) fractions. Products were separated on a SDS gel, replicated onto CMC-agarose and developed with Congo red [9]. A,J, buffer control (4°C incubation); B,F,K,O, protease; C,G,L,P, protease + PMSF control; D,H,M,Q, buffer control; E,I,N, buffer + PMSF control.

their affinities for cellulose. Since pNPCase activity could be detected in the supernatant of Exg plus protease, the 39 kDa product also retained enzymic activity. Residual 30 and 39 kDa material associated with the cellulose fraction was still detectable following centrifugation of the reaction mix (e.g. fig.1, lane K). However, this material

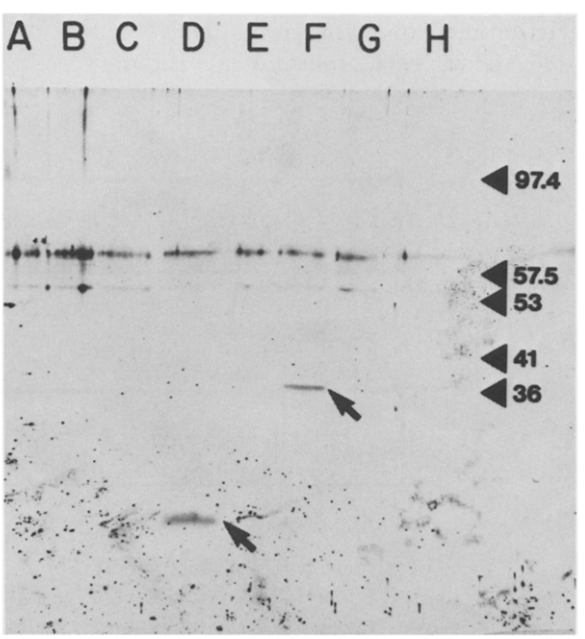

Fig.2. Western blot of non-glycosylated EngA (A–D) and Exg (E–H) after incubation with *C. fimi* protease. The probe was antiserum to synthetic Pro-Thr box. Cellulases, bound to Avicel, were incubated with protease (B,D,F,H) or control buffer (A,C,E,G) for 72 h, at 30°C, then centrifuged to give cellulose-bound (C,D,G,H) and supernatant (A,B,E,F) fractions. Positions of size markers (kDa) are shown in right margin. Arrows indicate immunoreactive products.

could be completely removed by subsequent washing with 50 μM potassium phosphate, pH 7.0. In contrast, the intact enzymes stayed tightly adsorbed to Avicel during incubation and remained so during extensive washing with buffer.

EngA and Exg each contain a highly conserved region of alternating prolyl and threonyl residues termed the Pro-Thr box [11]. Antiserum to synthetic Exg Pro-Thr box was used to probe a Western blot of proteolysis products from non-glycosylated EngA and Exg (fig.2). EngA gave a small (about 15 kDa) immunoreactive fragment (lane D). Exg gave a 39 kDa immunoreactive product (lane F). Corresponding bands were absent from control lanes. Interestingly, EngA and Exg themselves were not immunoreactive. Non-specific bands (55 and 60 kDa) were seen in all lanes.

4. DISCUSSION

Glycosyl substitution has been implicated in the stabilization of glycanases against environmental extremes [10,12] and the adsorption of cellulases to insoluble substrates [13]. These functions are not indicated in EngA or Exg; neither is direct participation in catalysis or binding to cellulose. However, our data point to a key role in protection against proteolysis. Several studies suggest a similar function for sugar groups in other glycoproteins of diverse origin [12].

The action of *C. fimi* protease is quite specific. Non-glycosylated cellulases yield discrete fragments which retain enzyme activity but lose the capacity to bind cellulose. The level of 30 kDa EngA fragment seen in the cellulose pellet following centrifugation of the reaction mix (fig.1, lane K) is greatly exaggerated by deliberate overexposure of the zymogram. This material and corresponding residual 39 kDa Exg fragment were readily washed from the pellet with buffer and therefore represent only soluble entrapped protein.

Endoglucanase activity is expressed from *cen*A missing extensive regions of 5′-proximal coding sequence (Guo, in preparation), but small 3′-deletions result in total loss of activity [5], the 30 kDa EngA fragment must comprise EngA missing about 110 amino acids from its N-terminus. The predicted cleavage site (fig.3), based on product size, agrees with data from the Western blot probed with antiserum to Pro-Thr box (fig.2). The 39 kDa Exg fragment is probably generated by proteolytic removal of a C-terminal region of Exg. Its size and immunoreactivity (fig.2) predict an analogous cleavage site on the carboxyl side of the Pro-Thr box (fig.3). Deletion of 225 base pairs from the 3′-terminus of the *cex* coding sequence permits expression of active enzyme [14].

The N-terminal region of EngA and the C-terminal region of Exg show 50% conservation of sequence [11]. We propose that these regions constitute cellulose-binding domains (fig.3). The 30 and 39 kDa fragments contain catalytic domains. Within the catalytic domains are sequences which

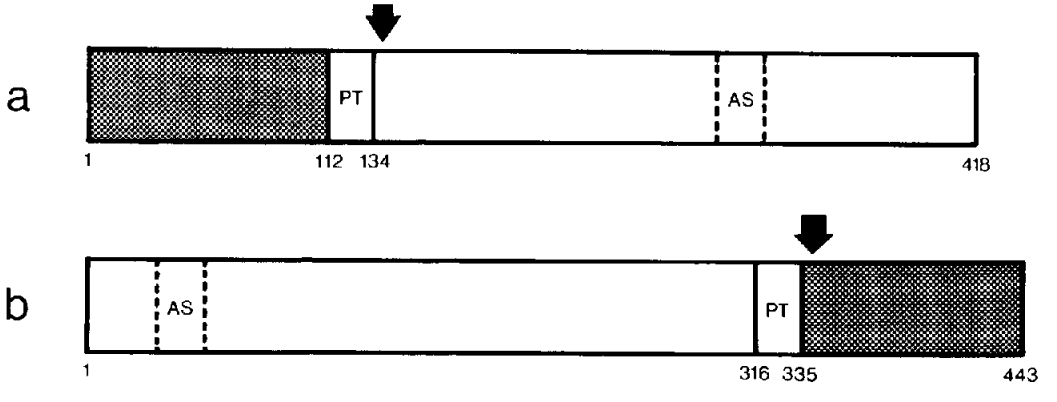

Fig.3. Proposed bifunctional organization of EngA (a) and Exg (b). Cellulose-binding domain, shaded; catalytic domain, unshaded; PT, Pro-Thr box; AS, putative active sites; arrows, deduced *C. fimi* protease cleavage sites of non-glycosylated enzymes. Numbers refer to amino acids, beginning at the mature N-termini [11].

resemble the active site of hen egg white lysozyme [11]. A common mechanism has been suggested for lysozyme and β-1,4-glucanase [15]. We propose that the Pro-Thr box, which joins the two domains, forms a hinge region, like the structurally similar region of immunoglobulins.

The Pro-Thr box may also be the site of glycosylation. *O*-linked sugar moieties frequently occur in clusters in proline-rich sequences and would afford protection against proteolysis to an otherwise vulnerable region. Alternatively, the conserved regions of both proteins contain several potential glycosylation sites for *N*-linked sugars [11].

Our model has several of the essential features of that presented by Van Tilbeurgh et al. [16] to explain the partial proteolysis products of a *Trichoderma reesei* cellobiohydrolase. Papain treatment of cellobiohydrolase I (65 kDa) gave a core protein (56 kDa) with reduced affinity for cellulose and enzyme activity against small soluble substrates but not cellulose. These observations form part of an emerging picture of bifunctional organization for both α- and β-1,4-glucanases, in which interaction with insoluble substrates is regulated by proteolysis (review [17]). Our data suggest that a further level of control may be exerted through glycosyl substitution of exposed regions joining functional domains. It is significant that, while cellulose-bound, glycosylated EngA and Exg are resistant to *C. fimi* protease, the free enzymes are subject to equally specific, though apparently different proteolytic cleavage, both in vitro and in vivo (Langsford, in preparation).

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