Site-directed mutagenesis of the putative catalytic residues of *Trichoderma reesei* cellobiohydrolase I and endoglucanase I

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Received 8 October 1990

Site directed mutagenesis has been performed to test hypotheses concerning the putative active sites of *Trichoderma reesei* cellobiohydrolase I and endoglucanase I. It is shown that mutagenesis of the residue E126, previously proposed to be the proton donor in CBHI, did not totally inactivate the enzyme while mutagenesis of the residue E127 in the homologous enzyme EGI resulted in complete loss of activity. These results are compared with those obtained in similar studies of other glucanases and the effects on enzymatic activity of hyperglycosylation of the yeast produced cellulases are discussed.

Site-directed mutagenesis; Active site; Cellulase, Hyperglycosylation

1. INTRODUCTION

Two cellobiohydrolases (CBH) and two or more endoglucanases (EG) of *Trichoderma reesei* act synergistically to bring about efficient hydrolysis of native cellulose. The key enzymes in the degradation of highly ordered crystalline substrates are the two cellobiohydrolases, CBHI and CBHII [1-3]. The active sites of no cellulases have so far been characterized in detail but chemical modification of carboxyl groups of *T. reesei* CBHI and EGI suggests that the glutamic acid, E126 in CBHI and the corresponding glutamic acid, E127 in EGI are essential for catalysis [4,5]. We have used site-directed mutagenesis to test this hypothesis. In this report we show that after expression in yeast and partial purification of the mutant proteins all of the CBHI mutants made have retained at least a third of their original activity on small soluble substrates. Somewhat surprisingly the E127Q mutant of EGI is, however, totally inactivated. These results are compared with those obtained in similar studies of other glucanases.

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Abbreviations: A, alanine; CBH, cellobiohydrolase; *cbh*, gene coding for CBH; D, aspartic acid; E, glutamic acid; EG, endoglucanase; *egl*, gene coding for EG; N, asparagine; PAGE, polyacrylamide gel electrophoresis; Q, glutamine; SDS, sodium dodecyl sulfate; wt, wild-type

2. MATERIALS AND METHODS

2.1. *Strains and vectors*

The *cbh*I and *egl*I cDNAs were derived from the plasmids pTTe9 and pTTe11 [6]. Bluescribe M13+ vector (Vector Cloning Systems, San Diego, USA) was used for in vitro mutagenesis *E. coli* JM101 [7]. The cellulase genes were expressed in *Saccaromyces cerevisiae* AH 22 (*leu2*-3 *leu2*-112 *his4*-519 *can1 gal2 cir*$^+$) [8] as described by Penttilä et al. [9,10].

2.2. *DNA techniques*

Site-directed mutagenesis was performed using the in vitro mutagenesis kit of Amersham UK, and the mutations were confirmed by DNA sequencing.

2.3. *Yeast transformation and cultivation*

Yeast transformation was carried out according to Keszenman-Pereyra and Hieda [11]. The transformants were grown in shake flasks in 50 ml of YEP-D [12]. Aliquots of 2 ml were withdrawn at appropriate intervals and used to measure cell density (A_{600nm}) and enzyme activity.

2.4. *Enzyme activity determination and quantification*

Enzyme activities were determined using 4-methylumbelliferyl lactoside as substrate as described by van Tilbeurgh et al. [13] with the exception of reaction temperature which was 50°C (*n*kat = activity U/min).

Quantification of the different forms of CBHI and EGI in the yeast growth media was performed as follows; 4 μl of culture medium was spotted on a nitrocellulose filter which was blocked as for Western blotting, treated with polyclonal rabbit antiserum against CBHI [14] or a monoclonal antibody against the EGI C-terminal part [15], and finally with ^{35}S-labelled protein A. The membrane was cut into pieces and the radioactivity of each dot was counted in a scintillation counter. The specific radioactivity was estimated from a standard curve (cpm/protein concentration) obtained by the same procedure using purified native CBHI or EGI from *T. reesei*. To visualize the CBHI protein on the membranes alkaline phosphatase-linked goat anti-rabbit IgG was used instead of protein A.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

2.5. *Gel filtration*

Gel filtration chromatography (Sephacryl S-300) was used to determine the molecular weight distributions of the different forms of CBHI and EGI by comparison to the elution positions of the activities of the proteins included in the Pharmacia high molecular weight protein standard kit.

3. RESULTS

3.1. *Mutant design*

Recently Tomme and Claeyssens [4] using Woodward reagent (WRK) were able to modify carboxylgroups in native and core proteins of CBHI with concomittant loss of activity. Peptides of the modified CBHI were obtained after denaturation, reduction and subsequent proteolysis of the enzyme. A specifically labelled peptide was sequenced and shown to contain three potentially catalytic residues, E126, D130 and D132. Only one of these was specificallly protected by a substrate analogue, but could not be unambiguously identified. Through sequence comparisons with lysozymes and β-galactosidases, however, E126 was proposed as putative proton donor [4,5]. In order to confirm these results we have now made the mutations E126Q, D130N and D132A in CBHI and E127N in EGI; the latter shares 45% amino acid sequence similarity with CBHI.

3.2. *Growth and enzyme production of the recombinant yeast*

The yeast transformants producing mutated CBHI and EGI proteins were grown in shake flasks and their growth and enzyme production were determined. The growth of all strains producing mutated proteins were identical with those of the strains producing the corresponding wt enzymes but the production in the culture medium of each of the mutant proteins was reduced to 15-60% of that of the corresponding wt enzyme (Fig. 1, Table I). A similar phenomenon has been previously observed in the production of mutants of human lysozyme in yeast [16].

3.3. *Specific activiti of the mutant enzymes*

All *Trichoderma* cellulases so far expressed in yeast are more extensively glucosylated than the native enzymes [9,10]. The heterogeneity of glycosylation prevents the purification of these yeast produced cellulases to homogeneity. Therefore, the amounts of specific protein in the culture supernatants were estimated by the immunological method described in section 2.

As calculated from the data presented in Table I, the specific activities of the secreted CBHI mutants were 29% (E126Q), 36% (D130N) and 60% (D132A) of the wt CBHI activity. Thus none of the CBHI mutant proteins is totally inactivated. On the other hand, the EGI mutant E127Q was apparently totally inactive.

3.4. *Molecular weight determination of the mutant enzymes*

Again, due to the hyperglycosylation of the fungal cellulases produced in yeast, the size distribution of im-

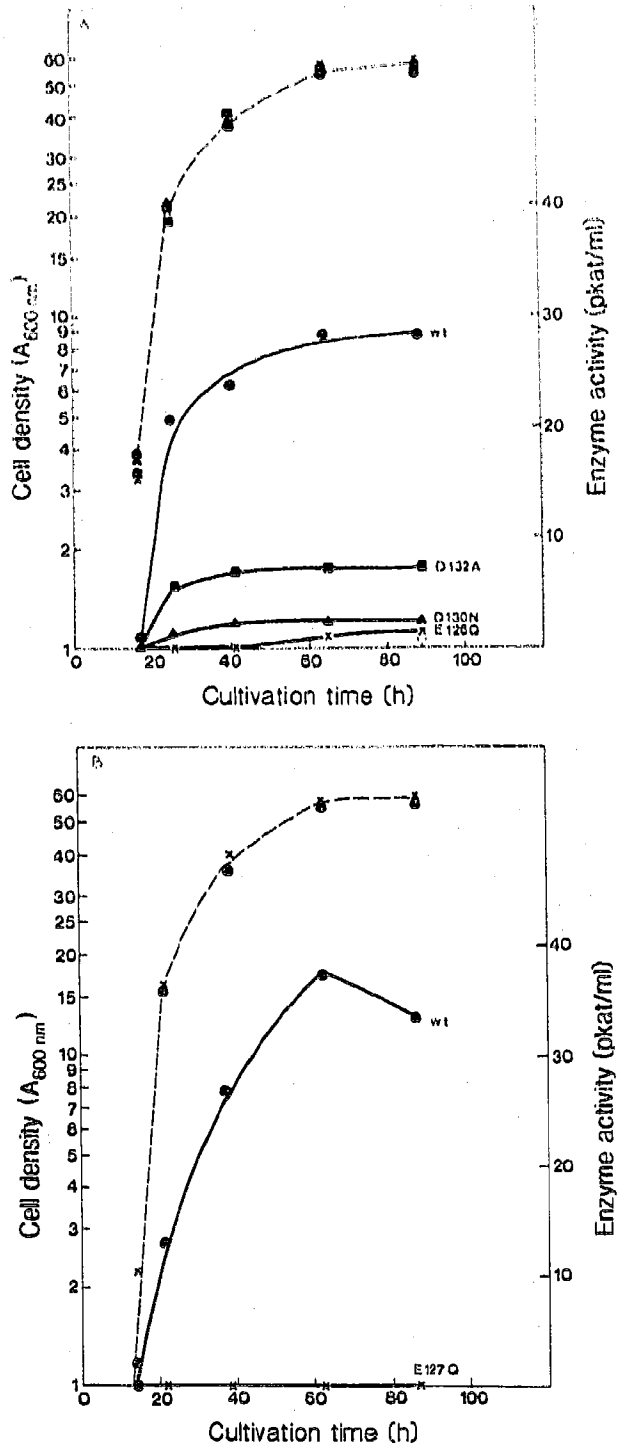

Fig. 1. Growth (dashed lines) and enzyme production (solid lines) of the recombinant yeast producing weight or mutant proteins of CBHI (A) and EGI (B).

Table I

Production levels and specific activities on 4-methylumbelliferyl lactoside of the wt and mutant enzymes of CBHI and EGI produced in yeast

Protein	Enzyme activity in medium (nkat/ml)	Specific protein in medium (μg/ml)	Spec. act. (nkat/μg)
CBHI wt	1.74	1.79	0.97
E126Q	0.075	0.27	0.285
D130N	0.17	0.47	0.35
D132A	0.51	0.86	0.585
EGI wt	2.2	1.25	1.76
E127Q	0	0.75	0

All values given are an average of two independent experiments.

munologically reactive and enzymatically active proteins was determined by gel filtration instead of SDS-PAGE. As seen in Fig. 2A the molecular weight/activity distribution of each of the CBHI mutants was essentially unchanged from that of the wild-type enzyme.

In the case of the EGI mutant, an interesting phenomenon was observed. In gel filtration immunologically reactive wt EGI was distributed over a large number of fractions while the enzymatic activity was only seen in the lower molecular weight fractions (Fig. 2B, fractions 24–38). It seems as if the extensive overglycosylation apparently present in the large molecular weight EGI renders the protein enzymatically inactive. In the case of the EGI mutant, E127Q, the lower molecular weight immunologically reactive fraction was not detected and the high molecular weight fraction was enzymatically inactive, similar to the wt EGI. Therefore, as it may be possible that the mutation introduced in EGI renders it more susceptible to hyperglycosylation, which as such may inactivate the enzyme, no conclusions can be drawn on the catalytic role of E127.

4. DISCUSSION

Since none of the mutations introduced in CBHI inactivated the enzyme completely, it seems highly unlikely that any of the affected residues could act as proton donor. The proton donors of e.g. lysozymes and glucoamylases have been mutagenized before and in all cases published the resulting mutant enzyme has been totally inactivated [17–19]. On the other hand, mutant enzymes in which residues involved in substrate binding or in stabilizing the reaction intermediates, have been changed may retain part of their activity. For example, in chicken lysozyme the mutation D52N in the residue stabilizing a reaction intermediate results in a mutant enzyme with 5% residual activity [18]. The glucoamylase of *Aspergillus awamori* carrying the mutation E179Q in the putative general base of the enzyme retains 5–10% of the wt activity depending on the

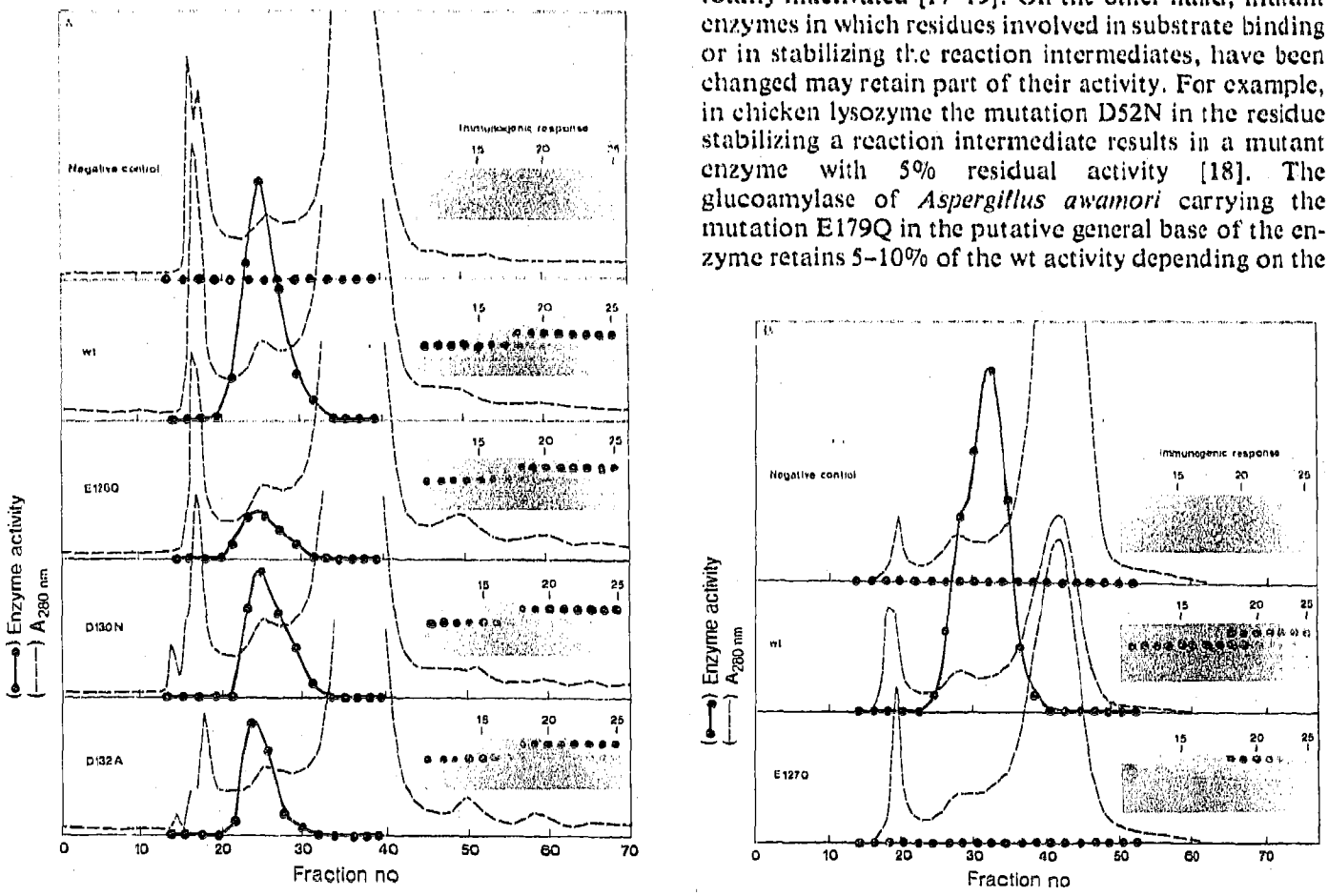

Fig. 2. Gel filtration chromatography (Sephacryl S-30) of the wt and mutant proteins of CBHI (A) and EGI (B). The enzymatic activity on 4-methylumbelliferyl lactoside and the immunogenic reactivity to specific antibodies in each fraction are indicated by the solid line and the dot blot (respectively).

substrate. Similarly another form of the same enzyme, carrying the mutation E180Q in a residue with a stabilizing function, is 11–38% active as compared with the wt enzyme [19]. Therefore, by analogy to the lysozyme and glucoamylase results and based on our results of the chemical modification and site-directed mutagenesis it is reasonable to assume that the residue E126 and possibly D130 are located in the active center of CBHI and participate in substrate binding or stabilizing events.

It is possible that the EGI mutant E127Q was inactive because of overglycosylation. However, evidence of fundamental differences in the catalytic mechanisms of CBHI and EGI has recently been obtained [20]. Therefore, the possibility remains that the catalytic residues are not strictly conserved between CBHI and EGI.

Acknowledgements: Financial support from the Technology Development Center of Finland (Y.M.), the Ministry of Finnish Foreign Affairs (S.N.) and Neste Foundation (M.S.) is gratefully acknowledged. Dr S. Aho is thanked for providing the monoclonal antibodies.

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