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Essential catalytic role of Glu^{134} in endo-β-1,3-1,4-D-glucan 4-glucanohydrolase from *B. licheniformis* as determined by site-directed mutagenesis

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Site-directed mutagenesis experiments designed to identify the active site of *Bacillus licheniformis* endo-β-1,3-1,4-D-glucan 4-glucanohydrolase (β-glucanase) have been performed. Putative catalytic residues were chosen on the basis of sequence similarity analysis to viral and eukaryotic lysozymes. Four mutant enzymes were expressed and purified from recombinant *E. coli* and their kinetics analysed with barley β-glucan. Replacement of Glu^{134} by Gln produced a mutant (E134Q) that retains less than 0.3% of the wild-type activity. The other mutants, D133N, E160Q and D179N, are active but show different kinetic parameters relative to wild-type indicative of their participation in substrate binding and transition-state complex stabilization. Glu^{134} is essential for activity; it is comprised in a region of high sequence similarity to the active site of T4 lysozyme and matches the position of the general acid catalyst. These results strongly support a lysozyme-like mechanism for this family of *Bacillus* β-glucan hydrolases with Glu^{134} being the essential acid catalyst.

Site-directed mutagenesis; Active site; β-1,3-1,4-Glucanase; *B. licheniformis*

1. INTRODUCTION

Endo-β-1,3-1,4-D-glucan 4-glucanohydrolase (β-glucanase, EC 3.2.1.73) is an enzyme that hydrolyses polymeric natural β-glucans as lichenan and barley β-glucan. The latter is depolymerized by the enzyme up to 3-β-*O*-cellobiosyl-D-glucopyranoside and 3-β-*O*-cellotriosyl-D-glucopyranoside, defining the cleavage specificity at β-1,4 glycosyl bonds adjacent to β-1,3 linkages [1]. β-Glucanase genes from different *Bacillus* species have been cloned and sequenced [2–7]. Nevertheless, studies towards the structural and functional characterization of the enzyme action are still scarce [8]. Although a hybrid β-glucanase has been crystallised, the three-dimensional structure has not been solved yet [9] precluding a straightforward insight into the structural features. A practical approach that allows the direct identification of amino acid residues present at the active site involves the use of mechanism-based inhibitors. Stone et al. [10,11] have reported the use of oligosaccharide epoxides to define the substrate specificity of several glucan hydrolases, EC 3.2.1.73, EC 3.2.1.4 and EC 3.2.1.6. They showed that the epoxides irreversibly inhibit enzyme activity in the same way they inactivate other carbohydrases (i.e. lysozyme [12] or β-galactosidase [13]). However, no further isolation and sequencing of the labeled peptides after protease digestion to locate and identify the active site residues have been reported.

In the absence of structural information, attempts to predict functional catalytic residues are often based on sequence similarities to other carbohydrases for which more detailed information is known. After cloning and sequencing the *Bacillus licheniformis* endo-β-1,3-1,4-D-glucan 4-glucanohydrolase [7], sequence similarity analysis with viral and eukaryotic lysozymes led us to predict two putative active sites. The first is a 25 amino acid segment (residues 132–157) that shows 40% identity and 70% similarity [14] to the T4 lysozyme (T4L) active site, and contains an Asp and Glu residues in the same positions as those assigned to be the general acid (E11) and the oxocarbocation-stabilizing residue or nucleophile (D20) in T4L (Fig. 1). The second putative active site resembles that of hen egg white lysozyme (HEWL) and comprises a fragment of 21 amino acids with 20% similarity to the sequence around the catalytic residues E35 and D52 in HEWL (Fig. 1). While the latter is not

Abbreviations: gluL, gene coding for β-glucanase from *B. licheniformis*; D, aspartic acid; DNS, dinitrosalicilic acid; E, glutamic acid; LB and 2YT, bacterial media as described in Sambrook et al. [28]; N, asparagine; PAGE, polyacrylamide gel electrophoresis; Q, glutamine; SDS, sodium dodecyl sulfate; wt, wild-type.

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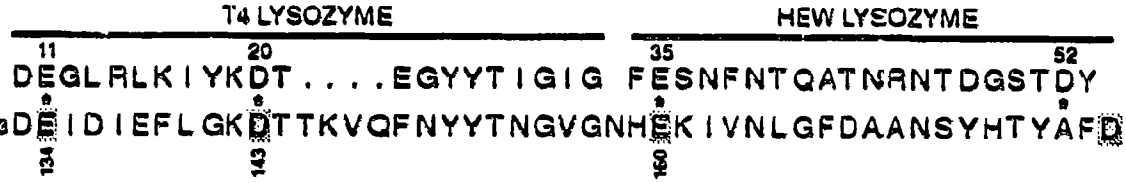

Fig. 1. Alignment of the β-glucanase sequence of *B. licheniformis* with the active site sequences of T4 and hen egg white (HEW) lysozymes. The asterisks indicate the catalytic residues in both lysozymes [23,24].

conserved among the *Bacillus* isozymes, the T4 lysozyme-like active site is completely conserved in the amino acid sequences of other highly homologous *Bacillus* β-glucanases (*B. subtilis* [2], *B. amyloliquefaciens* [3], *B. macerans* [4] and *B. polymyxa* [6]) and almost conserved in the recently reported isozyme from *C. thermocellum* [15]. Therefore, D133, E134 and D143 are candidates to be the catalytic residues in *B. licheniformis* β-glucanase. A similar prediction has been outlined by Borris et al. [16] for the *B. macerans* isozyme and recently discussed by Schimming et al. [15] for the *C. thermocellum* β-glucanase.

We communicate in this report the replacement of single carboxylic amino acid residues from both putative active sites by the isosteric, uncharged Asn and Gln residues by site-directed mutagenesis, as well as the preliminary kinetic characterization of the mutant proteins. This study shows that Glu^{134} is essential for activity.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and culture media*

E. coli TG1 (*supE hsdΔ5 thi Δ(lac-proAB) F'[traD36 proAB⁺ lacIᵠ lacZΔM15]*) was used for plasmid propagation and isolation of ssDNA template for mutagenesis by infection with M13K07 helper phage, and transformed with the mutagenic reaction. *E. coli* HB101 (*supE44 hsdS20($r_B^-m_B^-$) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*) was the host strain for protein expression. For plasmid isolation, bacteria were grown in 2YT medium, while LB medium was used for protein expression. Ampicillin at 100 μg/ml was added when appropriate.

2.2. *Chemicals and enzymes*

Restriction endonucleases and T4 DNA ligase were from Boehringer Mannheim. [^{35}S]α-ATP and the oligonucleotide site-directed mutagenesis kit were purchased from Amersham. DNA sequencing was performed by the T7 Sequencing kit from Pharmacia. Oligonucleotides were synthesized by Clontech. Barley β-glucan was from Sigma.

2.3. *Mutagenesis*

The gene coding for *Bacillus licheniformis* β-glucanase previously cloned from the genomic DNA [7] was subcloned in pUC119 as a 1.2 kb *SacI/SphI* fragment. *E. coli* TG1 was transformed with the ligation product and infected with M13K07 helper phage for ssDNA isolation which was used as template for mutagenesis. The oligonucleotides were 21-mer and contained single mismatches directing the desired mutations: D133N, 5'-GTCGATTTCATTCCAAGGCGT-3'; E134Q, 5'-ATGTCGATTTGATCCCAAGGC-3'; E160Q, 5'-GACGATTTTTTGATGATTTCC-3'; D179N, 5'-AGGCTGCCAG TTGAACGCATA-3'. Oligonucleotide site-directed mutagenesis was performed according to the Eckstein method [17]. Transformants were screened by DNA sequencing using appropriate primers located about 100 bases from the mutation point. Positive clones were confirmed by complete sequencing of the entire gene. *E. coli* HB101 was transformed with the mutant plasmids for protein expression.

2.4. *Protein expression and purification of wt and mutant enzymes*

Proteins were expressed from HB101 cells harboring the wt and mutant plasmids grown in 1 liter of LB medium containing 150 μg/ml of ampicillin. β-Glucanases were purified essentially as described [18]; summarizing, the extracellular medium was concentrated by tangential ultrafiltration and the enzyme eluted from a CM-Sepharose CL-6B (Pharmacia) ion exchange column at pH 5.6 with a NaCl gradient. Active fractions were dialysed and lyophilised for storage. Protein concentration was measured by the Bradford procedure (Pierce kit) using BSA as standard. Electrophoretic analyses (SDS-PAGE) were according to Laemmli [19] stained with Coomassie brilliant blue R or by the silver-staining procedure of Blum et al. [20]. The zymogram technique for detection of renatured β-glucanase activity on SDS-PAGE gels (15% acrylamide) was performed according to the protocol of Schwarz et al. [21]. Gel permeation chromatography under native conditions was carried out by FPLC (Pharmacia) on a Spherogel TSK G3000 SW column eluted with 10 mM Bis-Tris propane buffer, pH 6.5.

2.5. *Protein fluorescence*

Fluorescence spectra of wt and E134Q mutant proteins were recorded on a Hitachi-Perkin Elmer MPF-3 spectrofluorimeter at 20°C in 1 × 1 cm cells. Excitation was at 282 nm (2 nm slit) and the emission spectra were recorded from 270 to 440 nm (8 nm slit). Samples were dissolved in 0.1 M HEPES, pH 7.0, at a final concentration of 38 μg/ml (A_{280nm} = 0.5513) for wt and 44 μg/ml (A_{280nm} = 0.6350) for E134Q. Solutions in 8 M urea, 0.1 M HEPES, pH 7.0, at the same final enzyme concentration were prepared and the spectra recorded at different time intervals (20 min, 2 and 4 h).

2.6. *Enzyme kinetics*

Enzyme activity of purified wild-type and mutant proteins was assayed by the net release of reducing sugars from barley β-glucan [18]. The assay mixture containing barley β-glucan (0.2–9 mg/ml) in phosphate-citrate buffer, pH 7.02, was pre-incubated at 55°C for 5 min. The reaction was initiated by adding diluted enzyme to a final concentration of 15–80 ng/ml. Samples were quenched at different time intervals with the DNS reagent [22] and kept 10 min at 100°C. Release of reducing sugars was quantified from absorbance readings at 540 nm using glucose as standard. Kinetic parameters were derived by fitting the data to a rectangular hyperbola by non-linear regression.

3. RESULTS

A phagemid containing the *gluL* gene suitable for mutagenesis and expression was constructed by subcloning the 1.2 kb *SacI/SphI* fragment of pBL339G3 [7] into pUC119. The insert contained the promoter and signal peptide from *Bacillus licheniformis* plus some ad-

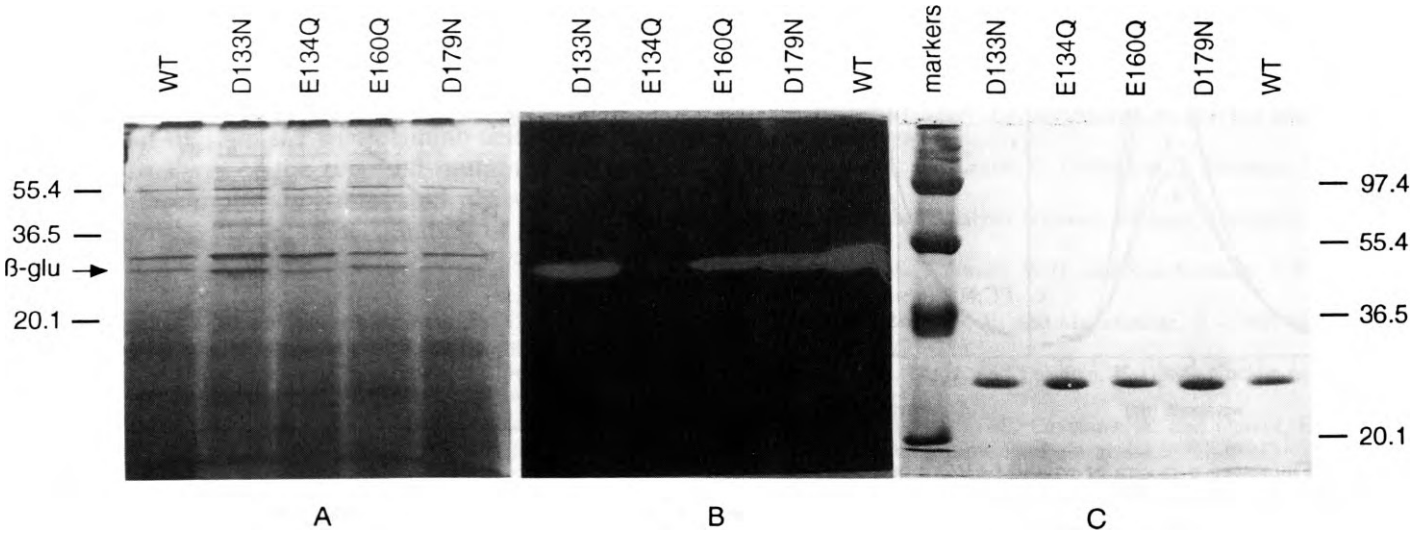

Fig. 2. Electrophoretic analysis of wt and mutant β-glucanases. (A) SDS-PAGE (15% acrylamide, silver stained) of the extracellular medium of *E. coli* HB101 cells expressing β-glucanase (D133N mutant is 3× concentrated). (B) zimogram (SDS-PAGE, 15% acrylamide, 0.5% barley β-glucan, Congo red activity staining after renaturation) of 3× concentrated extracellular extracts. (C) SDS-PAGE (10% acrylamide, Coomasie brilliant blue R stained) of purified enzymes. Molecular weight markers (Boehringer Mannheim): 97.4 kDa, phosphorylase *b*; 55.4 kDa, glutamate dehydrogenase; 36.5 kDa, lactate dehydrogenase; 20.1 kDa, trypsin inhibitor.

ditional bases upstream of the promoter. Expression of β-glucanase in *E. coli* is constitutive and 60–70% of the total enzyme is secreted into the extracellular medium, thus showing that the signal peptide is operative in *E. coli*. Four mutant enzymes designed to identify the active site and test the predictive analysis outlined above were prepared by oligonucleotide site-directed mutagenesis and expressed in *E. coli* HB101. SDS-PAGE analysis of the extracellular extracts (Fig. 2A) showed that all the mutant proteins are secreted and that the expression levels are similar to that of wt enzyme. A preliminary activity assay on the extracellular medium by the zymogram technique stained with Congo red (Fig. 2B) indicates that E134Q is nearly inactive while D179N, D133N and E160Q have reduced activity compared to wt. Likewise, enzyme activity from supernatants of overnight cultures grown to stationary phase were assayed by the DNS method [22] upon incubation with 0.5% barley β-glucan as substrate. Percentages of activity relative to wt are shown in Table I. These figures are a composite of specific activity and expression level for each mutant and are consistent with the results from the zymogram analysis which, in addition, involves protein renaturation.

Table I

Expression and kinetic parameters of β-glucanase mutants

Mutant	Extracellular medium		Purified enzymes	
	Activity[a]	Expression[b]	% V_{max}	K_m (mg/ml)[c]
wt	100%	1	100	1.30 ± 0.15
D133N	25%	0.8–0.95	20 ± 5	1.75 ± 0.25
E134Q	<0.5%	~1	<0.3	-
E160Q	20%	0.8–0.95	30 ± 7	7.0 ± 1.2
D179N	90%	0.8–0.85	100 ± 10	4.2 ± 1.0

[a] Relative activity at 5 mg/ml barley β-glucan, 55°C, pH 7.0.
[b] Relative expression level to wt enzyme calculated from the extracellular activity using the kinetic parameters of the purified enzymes.
[c] Errors in K_m determinations are large mainly due to the limited solubility of barley β-glucan. Substrate concentration ranged from 0.2 to 9 mg/ml, and consistently, no saturation was achieved for those mutants with high K_m.

The mutant enzymes were purified up to >95% purity as judged by SDS-PAGE (Fig. 2C) following the protocol developed for the wt enzyme [18]. To assess that the inactive mutant E134Q is properly folded, its chromatographic behaviour and fluorescence properties were analysed: (i) both E134Q and wt eluted at identical retention time under native conditions on gel permeation chromatography (TSK G3000 SW column) at pH 6.5; (ii) E134Q and the active enzymes came out from an ionic exchange column (CM-Sepharose CL-6B or TSK CM-3SW on FPLC at pH 5.6) at approximately the same ionic strength; and (iii) the fluorescence spectra of wt and E134Q at pH 7.0 showed an emission maximum at 340 nm with similar intensities for both proteins (Fig. 3). Under unfolding conditions in 8 M urea, the fluorescence is partially quenched and the emission maximum shifts to higher wavelength. The unfolding is rather slow as seen by the decrease in the fluorescence up to 4 h. Taken together with the fact that E134Q is also secreted into the extracellular medium and not degraded intracellularly, these results strongly support a folded structure for the inactive E134Q mu-

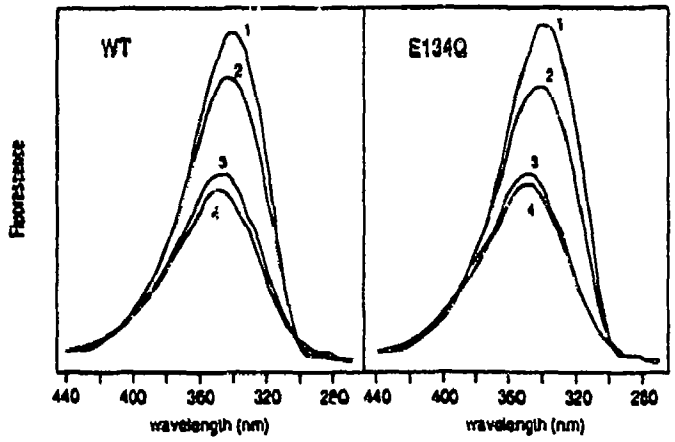

Fig. 3. Fluorescence spectra of wt and E134Q mutant β-glucanases. Curves in both plots are: (1) native enzyme in 0.1 M HEPES pH 7.0, (2) in 8 M urea, 0.1 M HEPES, pH 7.0, after 20 min, (3) same as (2) after 2 h, and (4) same as (2) after 4 h. Enzyme concentration was 38 μg/ml for wt and 44 μg/ml for E134Q.

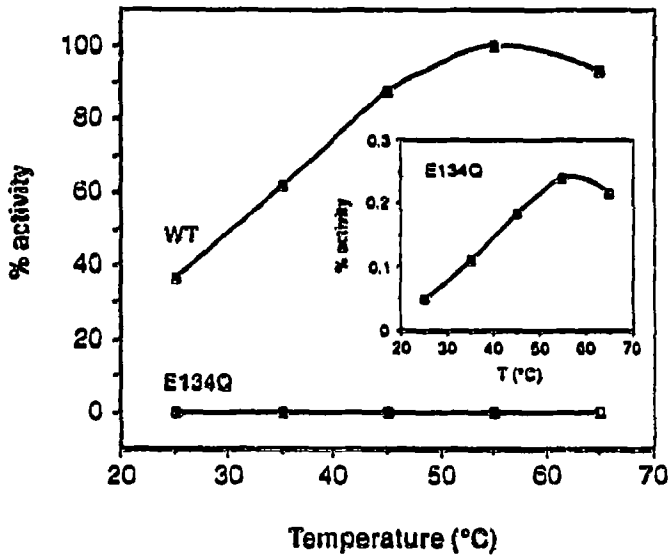

Fig. 4. Specific activity of wild-type and E134Q mutant β-glucanases at different temperatures. (Inset) Magnification of the E134Q activity-temperature profile. Conditions: citrate-phosphate buffer, pH 7.0; 6 mg/ml barley β-glucan; enzyme concentration, 23 ng/ml for wt, 4.8 μg/ml for E134Q.

tant. Therefore, the large decrease in catalytic activity can be assigned to the replacement of an essential functional residue.

Kinetic constants for the enzyme-catalysed hydrolysis of barley β-glucan at the wt optimum temperature (55°C) and pH (7.0) were determined. Results are summarized in Table I. The question of a possible thermal inactivation of the E134Q mutant under the assay conditions as a reason of its low activity was addressed by performing the assay at lower temperatures as shown in Fig. 4. A curve that parallels the profile of wt was observed down to 25°C. Thus, the residual activity of <0.3% is ascribable to the functional effect of the mutation.

Asn and Gln residues are susceptible to hydrolysis to the corresponding Asp and Glu residus if they are solvent exposed at acidic or neutral pH. Therefore, mutations as those described here have to be handled with care. At least for the D179N mutant it has been observed a slow hydrolysis reverting to wt enzyme. After a month of storage at 4°C in aqueous solution, kinetic parameters became identical to those of wt. This result indicates that the side chain of residue 179 is solvent exposed as it would correspond to an active site residue interacting with the substrate. The mutant proved to be stable as a lyophilizate for several months.

4. DISCUSSION

Provided that a right-folded structure for the E134Q mutant occurs as argued above, Glu^{134} is essential for activity and participates in glucosidic bond hydrolysis. This result is similar to those obtained upon mutation of the general acid catalyst of other carbohydrases [23–26]. Moreover, E134 matches the position of the general acid catalyst in T4 lysozyme (E11, Fig. 1) within a region of high sequence identity.

The mutants at position 133, 160 and 179 show diverse effects on the kinetic parameters suggesting different roles of each residue in the enzyme mechanism along the reaction coordinate. It can be pointed out as a preliminary conclusion that D179 is likely involved in substrate binding since the asparagine mutant possesses a 3-fold higher K_m value (that only implies a decrease of 0.75 kcal · mol^{-1} in the energy of interaction) while no effect on k_{cat} is observed. On the other hand, D133 may assist in catalysis as a neighbour group to the essential E134 (5-fold decrease in k_{cat} for D133N), but it does not significantly contribute to substrate binding as seen by no change in K_m upon mutation. The fourth mutant analysed, E160Q, has an effect on both k_{cat} and K_m indicative of a significant contribution of E160 to transition state complex stabilization.

The amino acid sequence alignment of *B. licheniformis* β-glucanase to T4 and hen egg white lysozymes suggested E134 and E160 as candidates to be the general acid catalyst in a lysozyme-type mechanism. It is clear from the results reported here that the active site sequence of β-glucanase resembles that of T4 lysozyme, and E134 is proposed as the general acid. The role of E160 is not so clear. However, since both E160Q and D179N mutations show an effect on kinetic parameters, they are somehow involved in substrate binding and/or substrate transition-state complex stabilization. For an extended active site cleft with multiple subsites as discussed by Stone et al. [10] and commonly accepted for endo-glycosyl hydrolases [27], a number of residues will

interact with the substrate. The region from residues 133 to 195, proposed as the functional core, is rich in acidic amino acids containing 9 Asp and Glu (all of them conserved in the other highly homologous *Bacillus* enzymes) out of 17 in the whole amino acid sequence.

Further analysis of the reported mutants with synthetic oligosaccharide substrates and pH activity profiles will allow a deeper understanding of the enzyme action.

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