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Stereochemistry of the hydrolysis reaction catalyzed by endoglucanase Z from *Erwinia chrysanthemi*

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Endoglucanase Z from the phytopathogenic bacterium *Erwinia chrysanthemi* (strain 3937) was purified by affinity chromatography on microcrystalline cellulose Avicel PH101. A kinetic characterization using *p*-nitrophenyl β-D-cellobioside and *p*-nitrophenyl β-D-lactoside as substrates was conducted: endoglucanase Z exhibited K_m values of 3 mM and 7.5 mM and V_m values of 129 and 40 nmol·min^{-1}·mg^{-1} towards *p*-nitrophenyl β-D-cellobioside (k_{cat}=0.1 s^{-1}) and *p*-nitrophenyl β-D-lactoside (k_{cat}=0.03 s^{-1}), respectively). The hydrolysis of cellotetraitol by endoglucanase Z was followed by HPLC and ^{1}H NMR. Results show that cellobiitol and β-cellobiose are initially formed, demonstrating that the enzyme is acting by a molecular mechanism retaining the anomeric configuration. This suggests the involvement of a glycosyl-enzyme intermediate.

Cellulase; Endoglucanase; Reaction mechanism; Hydrolysis; *Erwinia chrysanthemi*

1. INTRODUCTION

The degradation of cellulose by micro-organisms is the focus of a wide range of studies from biomass conversion to plant pathology or development [1,2]. The sequences for nearly 80 cellulases and xylanases are known and can be classified into at least 9 different families (A–I) on the basis of amino acid similarities in their catalytic domains [2–5]. As many other cellulases, endoglucanase Z from *Erwinia chrysanthemi* has a bifunctional organization made of (i) a catalytic domain functioning independently of (ii) a cellulose-binding domain [6]. The endoglucanase Z catalytic domain was classified in cellulase family A [3], the largest family since it presently contains over 30 sequences. Conserved glutamate and histidine residues were identified that could be important for the enzymatic activity [2,3] and this was supported by preliminary results of mutants wherein alanine substitutes these residues [6].

The mechanism by which glycosyl hydrolases catalyze glycosidic bond cleavage is formally a nucleophilic substitution at the anomeric center and that can take place with either retention or inversion of the anomeric configuration [7]. The stereochemistry of the reaction product is firmly dictated by the structure of the active site [7]. To test the validity of the sequence similarity-based families of cellulases, it is important to know if members of a given family exhibit the same catalytic mechanism. The mechanism has been determined for cellulases from families B (inversion, [8–10]), C, (retention, [8,10,11]), E (inversion, [12]) and F (retention, [9]) but not for the other families including family A.

In this work we present (i) the use of endoglucanase Z cellulose binding domain for purifying the enzyme, (ii) the kinetic parameters of endoglucanase Z acting on small soluble substrates, and (iii) a ^{1}H NMR study of the stereochemical course of the hydrolysis of cellotetraitol by endoglucanase Z.

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2. MATERIALS AND METHODS

2.1. *Purification of endoglucanase Z*

Escherichia coli TG1 strain was used as a host for pSN1, a pUC18 derivative carrying the *celZ* structural gene [6]. Cells were grown in HR medium which contains: K_2HPO_4 (24 g·l^{-1}), KH_2PO_4 (10 g·l^{-1}), $(NH_4)_2SO_4$ (5 g·l^{-1}), $MgSO_4.7H_2O$ (0.1 g·l^{-1}), vitamin B1 (1 mg·l^{-1}), glycerol (3%), casaminoacids (10 g·l^{-1}), yeast extract (1 g·l^{-1}), ampicillin (50 mg·l^{-1}) and oligoelements (1 ml) as described by Bauchop and Elsden [13]. A 10 l culture was grown in a fermentor (Chemap AG type FZ3000) for 16 h at 37°C where a OD_{600} value of 20 was reached. Cells were harvested by centrifugation (Sharpless AS26YRR, 16,500 × *g*) and the pellet (280 × *g*) resuspended in 1.3 l of KPO_4 (50 mM, pH 7.0) EDTA (3 mM) and broken with a French press. An Avicel column was prepared as follows: 60 g Avicel PH101 (Fluka) were autoclaved in KPO_4 buffer (50 mM) and poured in a 5 cm diameter IBF 50 column giving rise to a 160 ml bed volume. The cell lysate was loaded at 4°C at a rate of 4 ml·min^{-1}. The column was then washed with 1.5 l of a KPO_4 buffer (50 mM, pH 7.0) containing 1 M NaCl and subsequently washed with 0.5 l of KPO_4 (50 mM, pH 7.0); both washings were done at rates of 0.2 ml·min^{-1}. Elution of endoglucanase Z was performed with water at a rate of 4 ml·min^{-1}. Peak fractions were pooled and filtered through a Millipore membrane (0.2 μm) and endoglucanase Z was further purified by anion-exchange chromatography using a FPLC system equipped with a Mono-Q column HR 10/10 (Pharmacia) equilibrated with Tris-HCl 25 mM pH 8.0, EDTA 1 mM and DTT 0.1 mM. Elution of endo-

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glucanase Z was done at 4°C by an increasing gradient of KCl (0–0.5 M). Fractions (3 ml) were collected and analysed for activity on *p*-nitrophenyl β-D-cellobioside. Endoglucanase Z was eluted at KCl 100 mM in a single 3 ml fraction and was subsequently aliquoted in 0.5 ml fractions for storage at −80°C. For the NMR analysis, endoglucanase Z was desalted by passing through a PD10 column (Pharmacia) and lyophilized. No loss in enzymatic activity of endoglucanase Z on *p*-nitrophenyl β-D-cellobioside was observed after lyophilization.

2.2. *Enzyme assays*

Enzyme assays were performed in HEPES buffer (100 mM, pH 7.4) at 37°C and using 8.75 μg pure enzyme. A range of concentrations of *p*-nitrophenyl β-D-cellobioside (0.15–20 mM) and *p*-nitrophenyl β-D-lactoside (0.25–10 mM) was used for determining K_m and V_m values. Enzymatic reactions were conducted in 96-well ELISA plates. Release of *p*-nitrophenol was followed in a Titertrek Multiskan apparatus by monitoring OD at 405 nm. A molar extinction coefficient of 0.035 was found for *p*-nitrophenol in the above-mentioned experimental conditions.

2.3. *Preparation of cellotetraitol and HPLC conditions*

Cellotetraose was obtained by acetolysis of cellulose [14] and fractionation of the oligosaccharides by preparative steric exclusion chromatography on Bio-Gel P2 [15]. Cellotetraose (20 mg) was reduced with $NaBH_4$ (10 mg) in water (10 ml) and purified by HPLC on a C-18 Sperisorb ODS2 10 μm column (S.F.C.C.) eluted with H_2O/methanol (95:5). HPLC Analyses of the enzymatic hydrolyzates were done with a Nucleosil C18 5 μm column (S.F.C.C.) and confirmed with a CHO-682 column (Interchim). In both cases the eluent was water. Detection of the products was achieved with a refractometric detector (Waters Associates).

2.4. *Proton NMR*

The enzyme and substrate were lyophylized twice from 99.8% D_2O and once from 99.95% D_2O to exchange labile ^1H atoms for ^2H. Cellotetraitol (2.5 mg) was then redissolved in 0.3 ml 99.95% D_2O and placed in a dry 5 mm NMR tube. Proton NMR spectra were recorded at 45°C with a Bruker AC300 spectrometer operating at 300 MHz, equipped with a 5 mm $^1H/^{13}C$ probe, a temperature controlling unit and an Aspec3000 computer. Each experiment was run with 8 dummy scans followed by 64 impulsions of 90° pulses using 4000 data points and a spectral width of 3000 Hz resulting in a acquisition time of 0.68 s per scan and roughly 60 s per spectrum. After the spectrum of the substrate alone was recorded, 120 μl of endoglucanase Z (10 mg · ml^{-1} in 99.95% D_2O) were added to the tube which was immediately placed back in the spectrometer. Spectra were then recorded every 2.5 min during the first hour and one was recorded 3 hours after the addition of enzyme.

3. RESULTS AND DISCUSSION

3.1. *Endoglucanase Z purification*

Small amounts of enzyme had been previously prepared by batch adsorption of endoglucanase Z on Avicel and desorption by urea treatment [6]. Such a protocol being unpractical for culture volumes larger than 1 l, a two-step purification procedure was developped involving (i) affinity chromatography on cellulose followed by (ii) anion-exchange chromatography. This procedure allowed to obtain 104 mg pure endoglucanase Z from 10 l culture medium (Table I; Fig. 1)

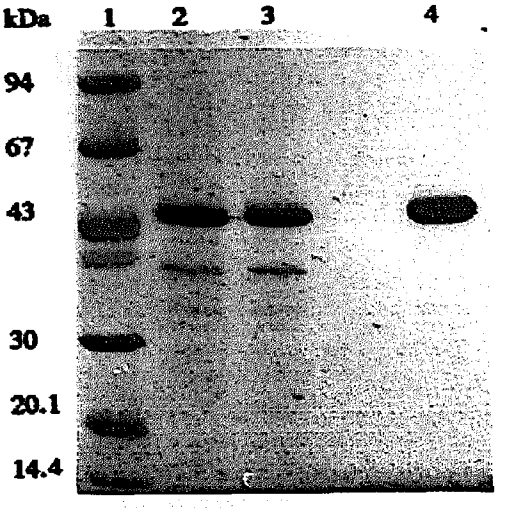

Fig. 1. SDS-PAGE analysis of samples taken during purification. Lane 1, size markers; lane 2, sample after affinity chromatography on Avicel; lane 3, sample after filtration on a 0.2 μm Millipore membrane; lane 4, mono-Q eluted sample.

3.2. *Substrate specificity and catalytic parameters*

In a previous report *p*-nitrophenyl β-D-cellobioside was used as the substrate for endoglucanase Z [6]. Other compounds have been tested, and no activity could be found with *o*-nitrophenyl β-D-glucoside, *p*-nitrophenyl 1-thio-β-D-glucoside, arbutin and esculin. In contrast, endoglucanase Z was found to be active against *p*-nitrophenyl β-D-lactoside. Kinetic constants were determined with *p*-nitrophenyl β-D-cellobioside (K_m = 3 mM, V_m = 129 nmol · min^{-1} · mg^{-1}, k_{cat} = 0.1 s^{-1}) and *p*-nitrophenyl β-D-lactoside (K_m = 7.5 mM, V_m = 40 nmol · min^{-1}, k_{cat} = 0.03 s^{-1}).

3.3. *Stereochemical anylysis*

Characterization of a highly related cellulase produced from another *Erwinia chrysanthemi* (strain 3665) have shown that it hydrolyzed MN300 cellulose to yield cellobiose and cellotriose as products [16]. Therefore, in order to study the stereochemical course of the hydrolysis, a G-4 oligomer seemed appropriate. Cellotetraitol was chosen as the substrate for this experiment because (i) HPLC analysis shows that it is degraded at only one site, releasing cellobiose and cellobiitol, and (ii) it has no reducing ^1H which could prevent the detection of the reaction product by ^1H NMR spectroscopy. Preliminary experiments having

Table I

Purification of endoglucanase Z

Purification step	Total activity[a] (U)	Total protein (g)	Specific activity[a] (U/g)	Recovery of activity (%)	Purif. factor (x-fold)
Crude Extr.	1141	66.3	17	100	1
Avicel	325	0.232	1403	28.5	82.5
Mono-Q	277	0.104	2666	24.3	157

[a] Units are defined as μmol of *p*-nitrophenol liberated per hour. *p*-nitrophenyl β-D-cellobioside was used at a concentration of 2.15 mM.

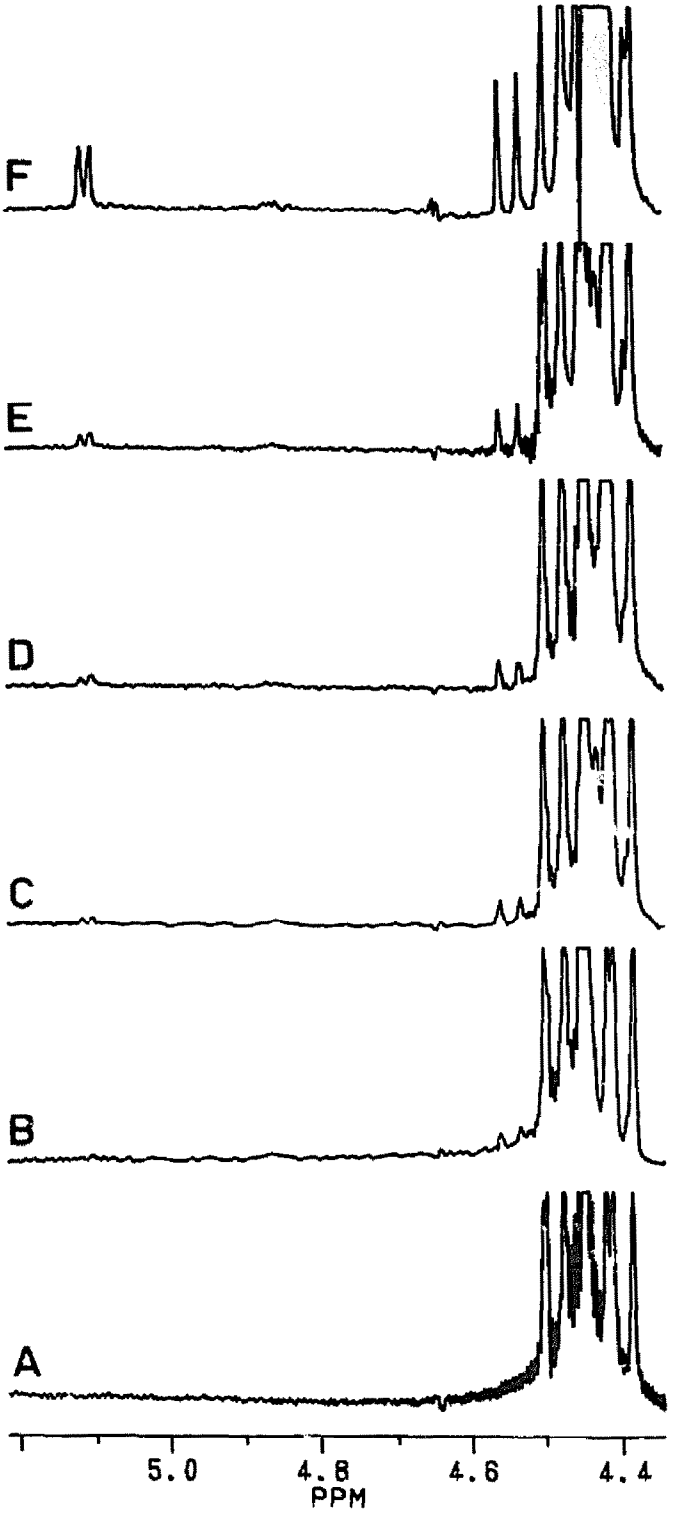

Fig. 2. ^{1}H NMR spectra of cellotetraitol during the hydrolysis by endoglucanase Z in the region of the free hemiacetal H-1 resonance. (A) Reference spectrum of cellotetraitol. (B–F) Same as (A) but 5, 15, 25, 35 and 180 min after the addition of endoglucanase Z.

shown that the enzyme was active in distilled water, the NMR experiment was conducted in unbuffered D_2O. Unambiguous determination of the anomeric configuration of the reaction product was obtained by selecting an enzyme/substrate ratio such as to obtain a hydrolysis rate of cellotetraitol by endoglucanase Z faster than mutarotation.

Fig. 2A shows the partial ^{1}H NMR spectrum of the starting cellotetraitol in the 4.4–5.2 ppm region. No signal referable to a free hemiacetal H-1 resonance is observed, confirming that the starting cellotetraitol was free from contamination by unreacted cellotetraose. Spectra recorded 5, 15, 25, 35 and 180 min after the addition of endoglucanase Z and illustrating the stereochemical course of the reaction are shown in Fig. 2B–F. The spectrum corresponding to 5 min of hydrolysis (Fig. 2B) shows a new signal centered at 4.55 ppm ($J_{1,2} \sim 8$ Hz) assignable to the H-1 of β-cellobiose produced in the early times of the reaction. This demonstrates that the enzymatic hydrolysis occurs with retention of the anomeric configuration. Fifteen minutes after the addition of endoglucanase Z (Fig. 2C), the doublet at 4.55 ppm keeps growing while a very small signal, almost hidden in the background noise, develops at 5.12 ppm ($J_{1,2} \sim 3$ Hz). This corresponds to a small amount of α-cellobiose originating from mutarotation of β-cellobiose produced by enzymatic hydrolysis. The α/β ratio (calculated after integration of the peaks at 4.55 and 5.12 ppm) of 0.26, far from the 0.66 value expected from the mutarotation equilibrium, further demonstrates that β-cellobiose is indeed initially formed. Fig. 2D and E show the spectra recorded 25 and 35 min. after the addition of endoglucanase Z, respectively. The peaks assignable to β- and α-cellobiose (at 4.55 and 5.12 ppm, respectively) continue to grow with α/β ratios of 0.41 and 0.52. This indicates that, although β-cellobiose is the primary product of the reaction, mutarotation now progressively becomes dominant. The spectrum recorded 3 h after the addition of endoglucanase Z shows a α/β ratio of 0.66 which corresponds to the value of the mutarotation equilibrium (Fig. 2F).

4. CONCLUSION

Enzymatic hydrolysis of cellotetraitol by endoglucanase Z proceeds with overall retention of the anomeric configuration in the reaction products. This is consistent with a double-displacement mechanism involving two catalytic residues promoting (i) the protonation of the glycosidic oxygen and (ii) a nucleophilic assistance leading to a glycosyl–enzyme intermediate [7]. Glu and/or Asp residues are frequently found as the residues responsible for this type of catalysis [7]. Only two Glu residues (and no Asp residues) are totally invariant in cellulase family A [2] immediately suggesting that one of these could act as the nucleophile mediating the formation of the glycosyl–enzyme intermediate while the other would be involved in the protonation of the glycosidic bond. Preliminary

analysis of the behaviour of endoglucanase Z derivatives in which one of these Glu residues (Glu-133) has been changed to Ala support their involvement in catalysis [6]. As a working hypothesis, one might suppose that the nucleophilic Glu residue might be under stronger geometrical constraint as compared with that involved in glycosidic bond protonation. This is currently under investigation by analysis of mutants of endoglucanase Z containing Asp residues in place of Glu-133 and Glu-220.

Grouping of cellulases based on amino acid sequence similarity was meant for predicting that all cellulases from a given family share the same folded conformation [3]. A corrolary to such a prediction is that all members of a family should act with the same molecular mechanism. To investigate this issue is especially important in the case of cellulases since the level of amino acid sequence conservation is so weak that sensitive comparison methods such as Hydrophobic Cluster Analysis [17] had to be used to achieve the grouping [3]. The establishment of the stereochemistry of the hydrolysis reaction catalyzed by endoglucanase Z is, to our knowledge, the first reported for a cellulase from family A. Assessment of the homogeneity of this family will require analysis of the catalytic mechanism prevailing in other family A members.

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REFERENCES

[1] Blume, J.E. and Ennis, H.L. (1991) J. Biol. Chem. 266, 15432–15437.
[2] Béguin, P. (1990) Annu. Rev. Microbiol. 44, 219–248.
[3] Henrissat, B., Claeyssens, M., Tomme, P., Lemesle, L. and Mornon, J.-P. (1989) Gene 81, 83–95.
[4] Gilkes, N.R., Henrissat, B., Kilburn, D.G., Miller, R.C. and Warren, R.A.J. (1991) Microbiol. Rev. 55, 303–315.
[5] Henrissat, B. (1991) Biochem. J. 280, 309–316.
[6] Py, B., Bortoli-German, I., Haiech, J., Chippaux, M. and Barras, F. (1991) Prot. Eng. 4, 325–333.
[7] Sinnott, M.L. (1990) Chem. Rev. 90, 1171–1202.
[8] Knowles, J., Lehtovaara, P., Murray, M. and Sinnott, M. (1988) J. Chem. Soc. Chem. Commun. 1401–1402.
[9] Withers, S.G., Dombroski, D., Berven, L.A., Kilburn, D.G., Miller, R.C., Warren, R.A.J. and Gilkes, N.R. (1988) Biochem. Biophys. Res. Commun. 139, 487–494.
[10] Claeyssens, M., Tomme, P., Brewer, C.F. and Hehre, E.J. (1990) FEBS Lett. 263, 89–92.
[11] Claeyssens, M., van Tilbeurgh, H., Kamerling, J.P., Berg, J., Vrsanska, M. and Biely, P. (1990) Biochem. J. 270, 251–256.
[12] Meinke, A., Braun, C., Gilkes, N.R., Kilburn, D.G., Miller, R.C. and Warren, R.A.J. (1991) J. Bacteriol. 173, 308–314.
[13] Bauchop, T. and Elsden, S.R. (1960) J. Gen. Microbiol. 23, 457–469.
[14] Hess, K. and Dziengel, K. (1935) Ber. 68B, 1594–1605.
[15] Heyraud, A. and Rinaudo, M. (1981) J. Liq. Chromatogr. 4, suppl., 175–293.
[16] Boyer, M.H., Chambost, J.P., Magnan, M. and Cattaneo, J. (1984) J. Biotechnol. 1, 241–252.
[17] Gaboriaud, C., Bissery, V., Benchetrit, T. and Mornon, J.P. (1987) FEBS Lett. 224, 149–155.