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Note

Cloning expression and characterization of a thermostable exopolygalacturonase from *Thermotoga maritima*

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Abstract

A gene encoding for a thermostable exopolygalacturonase (exo-PG) from hyperthermophilic *Thermotoga maritima* has been cloned into a T7 expression vector and expressed in *Escherichia coli*. The gene encoded a polypeptide of 454 residues with a molecular mass of 51,304 Da. The recombinant enzyme was purified to homogeneity by heat treatment and nickel affinity chromatography. The thermostable enzyme had maximum of hydrolytic activity for polygalacturonate at 95 °C, pH 6.0 and retains 90% of activity after heating at 90 °C for 5 h. Study of the catalytic activity of the exopolygalacturonase, investigated by means of ^{1}H NMR spectroscopy revealed an inversion of configuration during hydrolysis of α-(1→4)-galacturonic linkage.

Keywords: Exopolygalacturonase; Thermostable enzyme; *Thermotoga maritima*; Inversion of configuration mechanism

1. Introduction

Poly[(1→4)-α-D-galacturonates] are degradated by two types of enzymes. On one hand lyases (EC 4.2.2.) catalyse a β-4,5-elimination reaction leading to an unsaturated 4,5-glycuronate moiety. For instance, exopolygalacturonate lyases are able to catalyze this elimination at the terminal reducing part of the polygalacturonates either at the first, at the second or at the third galacturonyl bond.[1–5] On the other hand, endopolygalacturonases (endo-PGs, Poly[(1→4)-α-D-galacturonide] glycanhydrolase EC 3.2.1.15) and exo-PGs hydrolyse the (1→4) bond between two α-galacturonic acid (GalA) residues. Exo-PGs EC 3.2.1.67 and EC 3.2.1.82 release GalA and dimeric GalA, respectively. On the basis of their sequence, PGs as well as rhamnogalacturonases have been classified in family 28 of glycosidases.[6] Most of these enzymes have been described as 'inverting' enzymes.[7] Conversely, the mechanism exhibited by glucuronidase seems different since endo β-polyglucuronidase (family 79) or β-glucuronidase (family 2) were recognized as retaining enzymes and thus were able to catalyse regioselective synthesis of glycuronic linkage.[8–10] Nevertheless, PGs are important enzymes since they are commonly used as processing tools in food industries.[11] So far, several exo-PGs have been purified and studied,[12–20] but few have been cloned and overexpressed.[21] The need of new exo-PGs activities is important for food industry applications and particularly from the thermostability point of view. For this reason, we have chosen *Thermotoga maritima* as a source assuming that metabolism of this hyperthermophilic bacterium might reflect the evolutionary adaptation to extreme environmental conditions. Besides, a better solubility of substrates at high temperatures and a longer operational stability of the corresponding enzyme might be a crucial prerequisite for performing the catalytic synthesis with a thermostable biocatalyst. A nucleotidic sequence (Tm0437) coding for a putative exo-PG has been identified in the *T. maritima* genome.[22] Following this indication, we have cloned and overexpressed the corresponding gene in *Escherichia coli* cells. The enzyme thus obtained had an α-exogalactur-

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onase activity. The aim of this paper is to present the biochemical properties of this thermostable hydrolase.

2. Results and discussion

2.1. Cloning of the exopolygalacturonase gene from *T. maritima*

PCR allowed the genesis of a 1.5 kb DNA fragment covering a region with exo-PG gene from the *T. maritima* MSB8 strain and its cloning into a pCR4-TOPO vector (Invitrogen). The coding sequence for exo-PG was subcloned into a pET21d(+) vector and the resulting plasmid pETpgTm was transformed into *E. coli* BL21(DE3) cells.

2.2. Expression and purification of the exopolygalacturonase gene from *T. maritima*

Bacterial cells were grown at 30 °C followed by a 6 h cultivation at the same temperature after addition of isopropyl 1-thio-β-D-galactopyranoside (IPTG) in order to induce the expression of a functionally active enzyme. The overexpressed exo-PG was purified in two steps. Heating the extracts of *E. coli* BL21(DE3)/pETpgTm cells at 70 °C for 1 h allowed to denaturate the majority of proteins of the mesophilic host. A partially purified protein was then subjected to chromatography on a Ni^{2+}/nitriloacetic resin. The purified protein of 51 kDa was detected by SDS-PAGE that corresponds to the deduced molecular mass 51,304 Da of a Tm0437 ORF from *T. maritima* (Fig. 1).

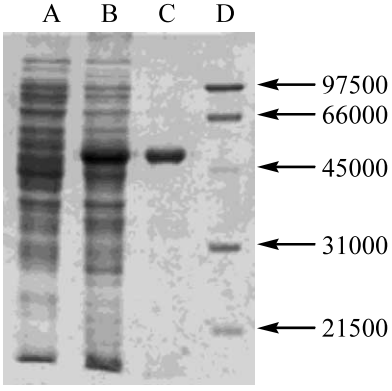


Fig. 1. Purification steps of *T. maritima* exopolygalacturonase from *E. coli* BL21(DE3)/ETpgTm cells. A, crude extracts of *E. coli* BL21(DE3)/ETpgTm cells; B, crude extracts of *E. coli* BL21(DE3)/ETpgTm cells after IPTG induction; C, protein purified on Ni^{2+}/NTA resin; D, reference molecular mass proteins (Da).

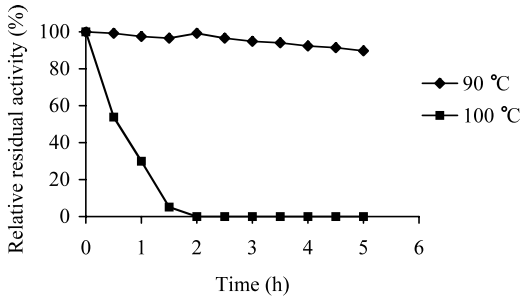


Fig. 2. Thermal stability of exopolygalacturonase from *T. maritima*.

2.3. Biochemical properties of exo-PG

2.3.1. Thermal stability of exo-PG. Fig. 2 shows the variation of the enzymatic activity over a 5 h incubation period at various temperatures. At temperatures up to 90 and below 100 °C, the enzymatic activity remained constant; at $T = 100$ °C, the enzyme lost 50% of its initial activity within 30 min.

The variation versus temperature of the initial rate of the hydrolysis reaction catalyzed by *T. maritima* exo-polygalacturonase was determined under conditions of the initial rate. A maximum was obtained at $T = 95$ °C (Fig. 3). Obviously, the curve shown in Fig. 3 is the sum of two phenomena: the increase of the reaction rate with temperature and the partial inactivation of the enzyme. At $T > 95$ °C, the rate of enzyme inactivation becomes important thus leading to an overall decrease of the activity.

2.3.2. Optimum pH of exo-PG. At pH values below 4.5 or above 7.5, no appreciable activity could be detected. As seen Fig. 4, a maximum activity was obtained at pH 6 in 0.1 M acetate buffer. In 0.1 M phosphate or in 0.1 M citrate/0.2 M phosphate buffers, the maximum activities, obtained for pH 5.8 with the later, were lower than that measured in acetate buffer, suggesting an inhibitory effect by phosphate ions.

2.3.3. Determination of the selectivity and the stereoselectivity of exo-PG. The end products of enzymatic hydrolysis of polygalacturonic acid by exo-PG were

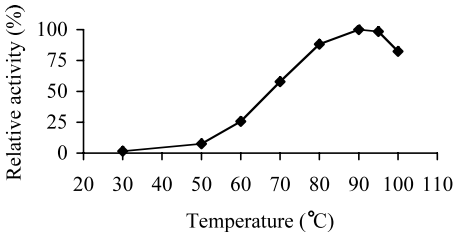


Fig. 3. Variation with temperature of the initial rate of the hydrolysis reaction catalyzed by *T. maritima* exopolygalacturonase.

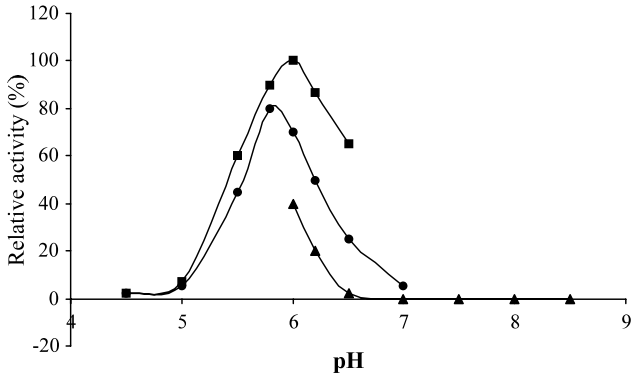


Fig. 4. Variation of the activity of *T. maritima* exopolygalacturonase as a function of pH (■, acetate buffer 0.1 M; ▲, phosphate buffer 0.1 M; ●, citric acid 0.1 M + dibasic sodium phosphate 0.2 M buffer).

analysed using thin layer chromatography (TLC). Galacturonic acid was the only product detected during hydrolysis, demonstrating that this enzyme is an EC 3.2.1.67 exopolygalacturonase (Scheme 1). A complete absence of digalacturonic acid and of DP < 6 was noticed on the TLC plates. Exo-PG also catalyses hydrolysis of polygalacturonic acid of $2 < DP < 6$. These results indicate that D-galacturonic acid was released from the non-reducing end of polygalacturonate. Other possible substrates like *p*NP-α-D-galacturonic acid and *o*NP-α-D-galacturonic acid were not substrates for this enzyme. This result suggests that exo-PG may have at least two residues-binding subsites and thus requires at least two carboxylates for substrate recognition.

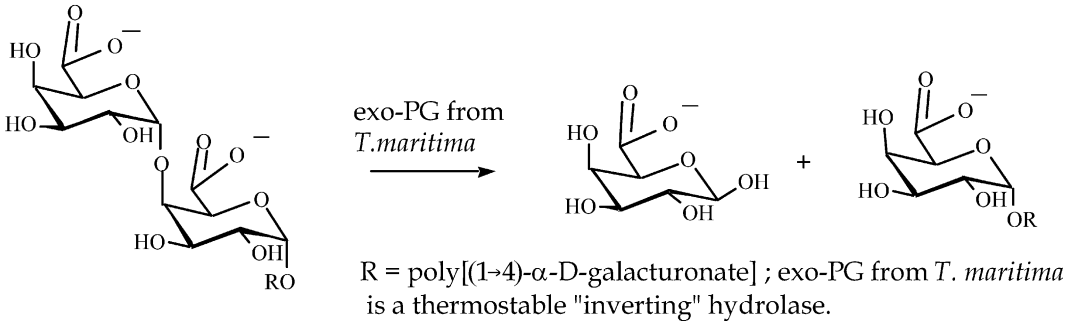
In order to determine the mode of action of the exo-PG, we have followed hydrolysis of digalacturonate by means of ^{1}H NMR in situ. Monitoring of the reaction revealed that the released monomer II had the β-configuration [δ(H-1II) = 4.56 ppm, $J_{1,2} = 7.9$ Hz) (see Fig. 5). The slow apparition of a H-1 resonance for α-galacturonic acid at 5.28 ppm is due to the mutarotation of the β-anomer of galacturonic acid II and to monomer I (Scheme 1). This experiment shows that exo-PG induces the synthesis of the β-anomer of galacturonic acid from the hydrolysis of oligogalacturonates and consequently is an 'inverting' glycanase. This result confirms many earlier observations that the molecular mechanism is conserved in the sequence-based families of glycoside hydrolases.[23]

In conclusion, the putative 'inverting' exo-PG from *T. maritima* was cloned and overexpressed in *E. coli*. This very thermostable hydrolase which present a maximum activity at pH 6, constitutes a powerfull tool for food industry applications.

3. Experimental

3.1. DNA isolation and cloning

Chromosomal DNA of *T. maritima* MSB8 was isolated as described by Dimova with minor modifications.[24] The *T. maritima* DNA region containing a putative PG gene was amplified by PCR with oligonucleotide primers 5′-CTGCACTAGCTAGTACC and 5′-TTCTCCAGA-GGCATGTC. Thirty cycles of PCR amplification with Goldstar *Taq* DNA polymerase (2.5 U) were carried out in a 50 μL solution containing 400 μM dNTPS each, 20 pmol each primers and 200 ng genomic DNA template. The PCR product (1.5 kb) was cloned using the TOPO TA Cloning kit (Invitrogen). Each cycle consisted of heating at 94 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min 30 s. Next, the PG coding sequence was amplified using two flanking primers 5′-ATGACCATGGAAGAACTGGCAAAAAAG-3′ and 5′-CCGCTCGAGTTTCAGCAGGCTTACCTTC-3′, carrying additionally *Nco*I or *Xho*I restriction sites. PCR Amplification 30 cycles with Goldstar *Taq* DNA polymerase (2.5 U) was carried out in a 50 μL reaction containing 400 μM dNTPS each, 20 pmol each primers and 10 ng plasmidic DNA template. Each cycle consisted of heating at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min 30 s. The PCR product was purified using the QIAquick PCR purification kit (Qiagen) and digested with *Nco*I and *Xho*I restriction enzymes. A band of the correct size predicted for the digested PG gene was purified by electrophoresis and ligated into the T7 expression vector pET21d(+) (Novagen) that allowed the creation of a His-tag at the C-terminal extremity of the protein.



Scheme 1. Stereoselectivity of the hydrolysis reaction catalyzed by exo-PG from *T. maritima*.

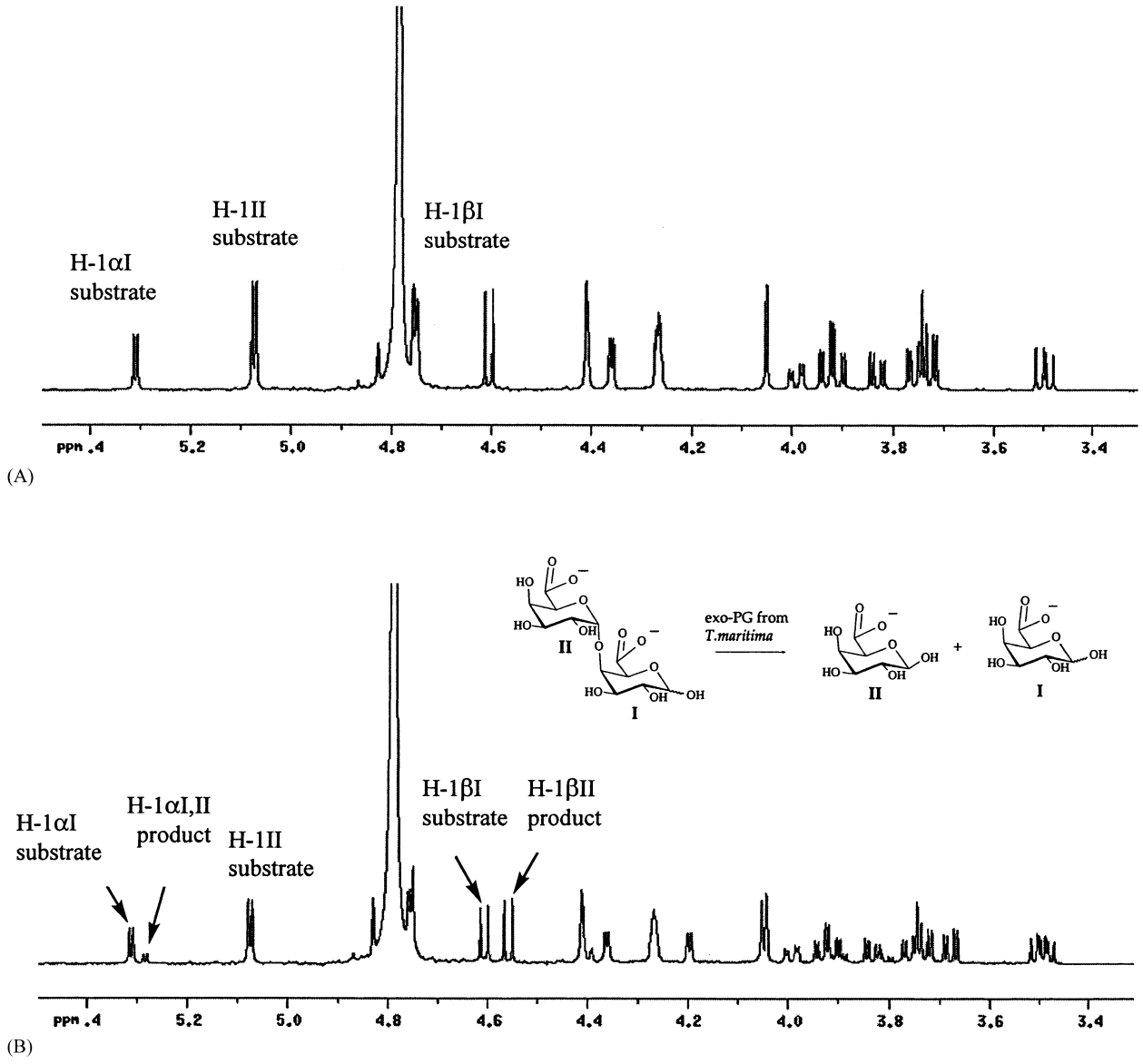


Fig. 5. Proton NMR spectra (500 MHz, solvent D_2O, pH 6.0, 25 °C) of pure digalacturonate (A) and after incubation with *T. maritima* exo-PG for 5 min (B). The chemical shifts are quoted from the ^1H trimethysilyl resonance of DSS.

3.2. Protein overexpression and purification

The *E. coli* BL21(DE3) strain harbouring the cloned *T. maritima* PG gene in pET21d(+) was grown in LB with 100 μg/mL ampicillin at 30 °C to OD_{600} 0.8 and incubated further with isopropyl β-D-thiogalactopyranoside (IPTG, 1 mM) for 6 h. The cell culture (500 mL) was centrifuged (2500*g*, 15 min) and resuspended in 10 mL of 50 mM sodium phosphate buffer (pH 8.0) containing NaCl (300 mM) and imidazole (10 mM). The cells were lysed by ultra-sound Raytheon 250 W apparatus, centrifuged (18,000*g*, 15 min) and the clear lysate was incubated at 70 °C for 1 h in order to denature *E. coli* proteins. After centrifugation at 18,000 g for 15 min at 4 °C, the debris were discarded and the supernatant was loaded on a column containing 3 mL Ni^{2+}/NTA resin (Quiagen). The column was first washed twice with 10 mL of the described buffer except the concentration of imidazole which was 50 mM. The protein attached to the resin was eluted with the same buffer containing 250 mM imidazole and the collected protein was dialysed against 10 mM Tris–HCl buffer, pH 8.0. The purified PG protein was analyzed by SDS-PAGE (12% polyacrylamide gel, 0.1% SDS, 375 mM Tris–HCl buffer pH 8.8) at a constant voltage (120 V). The gel was coloured with Coomassie blue G-250 (Bio-Rad). Protein concentrations were determined using Bradford method. The reactant (50 μL) was added to 200 μL of the sample on microplates. The optical density was measured at 620 nm after 10 min incubation at

room temperature. The protein concentration was calculated from a calibration curve obtained with bovine serum albumin (Sigma).

3.3. Biochemical analysis of *T. maritima* exopolygalacturonase

3.3.1. Measurement of the exopolygalacturonase activity. Enzymatic activities were calculated from increase of reducing saccharides using Nelson method[25] by comparison with standard calibration curves obtained in the presence of known concentrations of galacturonic acid. In standard conditions, reaction mixtures containing 0.5 mL polygalacturonate solution (2 mg/mL in 100 mM acetate buffer pH 6) were incubated 25 min with 15 μL (1.08 μg) of the exo-PG preparation at 70 °C.

3.3.2. Thermal inactivation. The standard conditions described for the measurement of the enzyme activity were used to determine the residual enzymatic activity after partial thermal denaturation. Thus, 200 μL aliquots of the exo-PG were incubated at different temperatures (90, 100 °C) for 5 h. The residual activity was measured every 30 min.

3.3.3. Determination of the optimal temperature and pH. The activity of the exo-PG was measured in the standard conditions given above at temperatures from 30 to 100 °C using polygalacturonic acid from orange peels as a substrate. Similarly, the optimal pH was determined in the standard conditions by varying pH from 4.5 to 8.5.

3.4. Determination of the selectivity and catalytic mechanism of exo-PG

The analysis of hydrolysis products was followed by means of TLC plates. Thus, 0.5 mL of polygalacturonate solution (2 mg/mL in 100 mM acetate buffer pH 6) was incubated 25 min with 15 μL (1.08 μg) of exo-PG preparation at 70 °C. Aliquots of 1 μL were analyzed by means of TLC using 4:3:2 *n*-butanol–water–acetic acid (v/v) as eluent. D-galacturonic acid was used for comparison.

The catalytic mechanism of the enzyme was investigated by ^{1}H NMR spectroscopy using digalacturonic acid (Sigma) as substrate. Thus, 600 μL of 40 mM of the substrate in D_2O phosphate buffer pH 6.0 was mixed with 70 μL (2.88 mg) of enzyme preparation and ^{1}H NMR spectra of the reaction mixtures were recorded on a Brucker AX500 apparatus at 500 MHz every 5 min at 25 °C.

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