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Exopolygalacturonate lyase from *Thermotoga maritima*: cloning, characterization and organic synthesis application

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Abstract

A new exopolygalacturonate lyase (Pel) gene of the hyperthermophilic bacterium *Thermotoga maritima* was cloned and overexpressed in *Escherichia coli* cells. A 42 kDa monomeric Pel was shown to undergo N-terminal processing by cleavage at a putative site between alanine and serine residues. The enzyme catalyzes selectively a β-4,5 elimination at the third galacturonic unit from the reducing end of polygalacturonic acid by producing (4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-(1→4)-(α-D-galactopyranosyluronic acid)-(1→4)-α-D-galactopyranuronic acid (**3**) with a 60% yield. The optimum activity of the enzyme was detected at pH 9.5 and $T \geq 95$ °C. The highly thermostable enzyme constitutes a useful catalyst for a simplified synthesis of 4,5-unsaturated trigalacturonic acid **3**, a trisaccharide which is extremely difficult to obtain via chemical synthesis.

Keywords: Exopolygalacturonate lyase; Thermostable enzyme; *Thermotoga maritima*; 4,5-Unsaturated trigalacturonic acid; Enzymatic degradation

1. Introduction

Recent understanding of the role of oligosaccharides in biological processes, like their mediator function in molecular recognition, has stimulated the development of several methodological approaches for the synthesis of new molecules for pharmaceutical and biotechnological purposes.[1] The synthesis of di- or trisaccharide can also be achieved via the enzymatic approach using glycosyltransferases and glycosidases.[2–15] Curiously, the potential of enzymes to produce the corresponding uronic di- or trisaccharide has not been widely studied, probably because they are not so easy to handle and only a few enzymatic activities are commercially available for this purpose. Yet, the importance of these carbohydrates, particularly saturated and unsaturated trigalacturonic acids, has been recognized as inducers of proteinase inhibitor genes in plants[16] or as antidotes for heavy metal poisoning.[17]

Considering for instance the α-(1→4)-polyglycuronate series, two types of enzymes: polyglycuronate hydrolases (EC 3.2.1.) and lyases (EC 4.2.2.) participate in the polymeric chain degradation. Some exopolyglycuronate hydrolases act through a double inversion catalytic mechanism and thereby exhibit the transglycuronidasic activity.[18–20] Polyglycuronate lyases catalyze a 4,5-β-elimination reaction leading to an unsaturated 4,5-glycuronate moiety. Unfortunately, these enzymes do not catalyze the reverse addition reaction, but the production of small oligosaccharides containing the α-(1→4)-glycuronidic bond can be approached via a partial degradation of polyglycuronic acid. Thus, some exopectate lyases have been found to be able to perform the elimination reaction on polygalacturonic acids at or near the reducing end releasing 4-deoxy-α-L-*threo*-hex-4-enopyranuronic acid (**1**)—tautomeric form of the "4,5-unsaturated monomer",[21] (4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-(1→4)-α-D-galactopyranuronic acid (**2**) ("4,5-unsaturated digalacturonic acid")[22] or (4-deoxy-α-L-*threo*-hex-4-

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enopyranosyluronic acid)-(1→4)-(α-D-galactopyranosyluronic acid)-(1→4)-α-D-galactopyranuronic acid (**3**) (“4,5-unsaturated trigalacturonic acid”).[23–25]

Searching for a suitable lyase, we have chosen *Thermotoga maritima* as a source, assuming that metabolism of this hyperthermophilic bacterium might reflect the evolutionary adaptation to extreme environmental conditions with unusual carbohydrate substrates. Besides, a better solubility of substrates at high temperatures and a longer operational stability of a corresponding enzyme might be a crucial prerequisite for performing the catalytic synthesis with a thermostable biocatalyst.

2. Results and discussion

Cloning and overexpression of the exopolygalacturonate lyase gene from Thermotoga maritima.—A nucleotidic sequence (Tm0433) coding for a putative pectate lyase has been identified in the *T. maritima* genome.[26] We cloned a 2.96 kb DNA fragment covering a region with the putative sugar ABC transporter, pectate lyase and α-glucosidase genes from the *T. maritima* MSB8 strain. The coding sequence for pectate lyase was subcloned into a pET21d(+) vector and the resulting plasmid pETpelTm was transformed into *Escherichia coli* BL21(DE3) cells. In preliminary experiments, we detected that the *pel* gene expression in bacteria at 37 °C leads to the formation of the aggregated protein. Therefore, bacterial cells were grown at 30 °C followed by a 6 h cultivation at the same temperature after the addition of isopropyl 1-thio-β-D-galactopyranoside (IPTG) in order to induce the expression of a functionally active enzyme as described for other cases.[27]

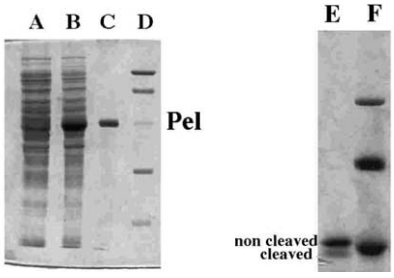


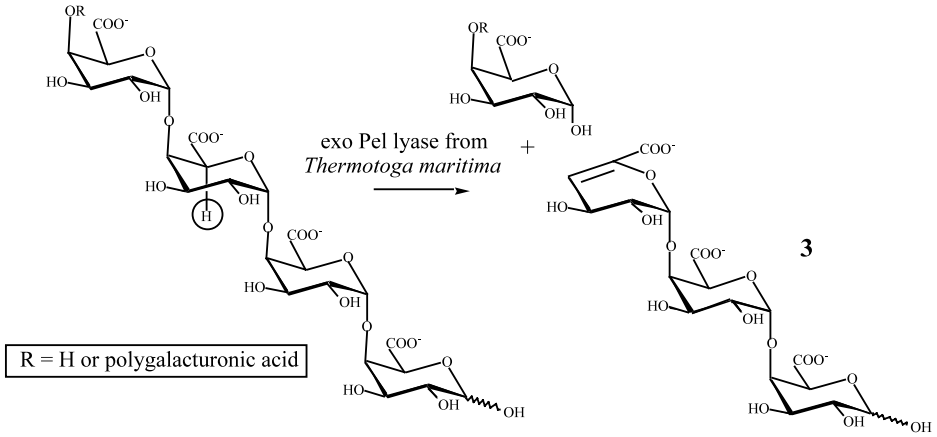
Fig. 1. (a, b) SDS-PAGE protein analysis at various purification steps of *T. maritima* exopolygalacturonate lyase from *E. coli* BL21(DE3)/pPelTm cells. A, crude extracts of *E. coli* BL21(DE3)/pPelTm cells; B, crude extracts of *E. coli* BL21(DE3)/pPelTm cells after IPTG induction; C, protein purified on Ni^{2+}/NTA resin; D, reference molecular mass proteins: 97,500, 66,000, 45,000, 31,000, 21,500 D; E and F are, respectively, the same as C and D apart from the gel composition which is 12% instead of 7%.

Biochemical properties of exopolygalacturonate lyase.—The overexpressed Pel protein was purified in two steps. Heating the extracts of *E. coli* BL21(DE3)/pETpelTm cells at 70 °C for 1 h allowed the denaturation of the major part 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 42 kDa was detected by SDS-PAGE, that corresponds to the deduced molecular mass 41,658 Da of a Tm0433 ORF from *T. maritima* (Fig. 1(a)). Two close bands of different intensity were further identified after a prolonged migration of protein samples (Fig. 1(b)) indicating cleavage of the Pel precursor protein.

Indeed, protein sequence analysis using MacDNAsis V3.6 program (Hitachi Software) showed the presence of a N-terminal hydrophobic region with positively charged arginine and lysine residues, characteristic for bacterial signal peptides. Moreover, two possible and close located cleavage sites comprising alanine and serine residues were detected within this region. Though we did not establish a real cleavage site, nearly 10% of the protein was detected as a faster migrating band, indicating that a small portion of the overexpressed *T. maritima* Pel precursor protein could be processed in heterologous *E. coli* cells.

The enzymatic activity of the *T. maritima* Pel protein was evaluated by monitoring the formation of unsaturated products at OD 235 nm using polygalacturonic acid from orange peels as a substrate. The molar extinction coefficient of 4,5-unsaturated oligogalacturonates was 4482 mol/L/cm (pH 9.5). The specific enzymatic activity was expressed in enzymatic units, EU (number of μmoles of unsaturated saccharides synthesized per minute) per mg of protein using polygalacturonic acid as a substrate. The addition of EGTA totally inhibited the enzymatic activity but could be restored by the addition of $CaCl_2$ indicating that the *T. maritima* Pel mediated catalysis requires Ca^{2+}.

Determination of the selectivity of Pel. In initial experiments, we found that the exopolygalacturonate lyase was able to produce high amounts of the unsaturated trisaccharide **3** (see Scheme 1) starting from polygalacturonic acid when working at pH 9.5, $T = 70$ °C. Since this trisaccharide was easily purified by anion-exchange chromatography from the reaction mixture, we were not sure that this compound was the only unsaturated saccharide synthesized. In order to clarify the matter, we incubated each oligomer of polygalacturonic acid with the enzyme in order to achieve a degree of polymerization from 2 to 6. These compounds were prepared from the acid hydrolysis at 100 °C of polygalacturonic acid (see Section 3). Neither the digalacturonic acid, nor the corresponding trimer, were found to be substrates for the enzyme. Conversely, tetragalacturonic acid and higher oligomers produced **3** as a



Scheme 1. Selectivity of the elimination reaction catalyzed by Pel from *T. maritima*.

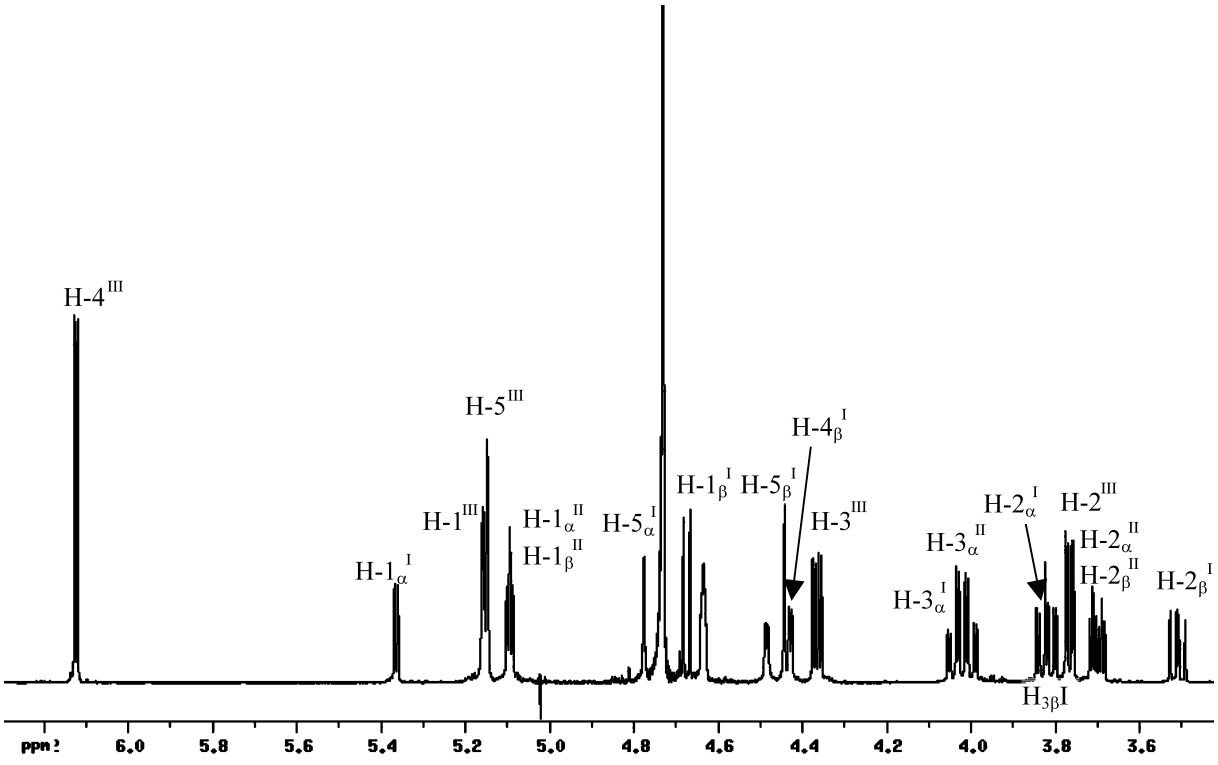


Fig. 2. Proton NMR spectra (500 MHz, solvent D_2O, pH 1.0, 25 °C) of the unsaturated trigalacturonic acid (mixture of α and β anomers) **3**. The chemical shifts are quoted from the ^1H trimethylsilyl resonance of DSS.

single unsaturated compound. The initial rate determined for each oligomer decreased from the hexamer to the tetramer.

Thus, it is likely that at least four galacturonic units are necessary to stabilize the transition state. These experiments demonstrated that the enzyme catalyzes selectively the elimination reaction at the third galacturonic unit from the reducing end of polygalacturonic acid. The Δ4,5-uronic acid **3** was produced from polygalacturonic acid at more than 60% yield of the isolated substance. The structure of **3** has been elucidated by means of standard one- and two-dimensional NMR spectroscopy and by mass spectrometry comparing the obtained results with available data on related polyuronates (Fig. 3).[28,29] Compound **3**, although described by several authors,[21–23] has not yet been fully characterized. The complete analysis of proton and carbon spectra is given in Figs. 2 and 3 and the values for chemical shifts are given in Table 1. The presence of an ethylenic proton H-4^{III} ($\delta = 6.12$ ppm) and of two ethylenic carbons ($\delta = 115.0$ ppm, C-4^{III} and $\delta = 143.4$ ppm, C-5^{III}) indicates the unsaturation of the molecule.

High variations in chemical shifts on pH are observed for H-5, H-4 and the corresponding carbons. A systematic study of the dependence of chemical shifts on pH is currently under investigation in this laboratory with the aim of obtaining the pK_a values of each carboxylic function.

Thus, we found that the *T. maritima* Pel is a rather efficient catalyst to produce the valuable trisaccharide **3**. Currently, we investigate the reactivity of the double bond of this compound on its capacity to provide, through addition reactions, new rare oligosaccharides like L-altrosuronic acid derivatives.

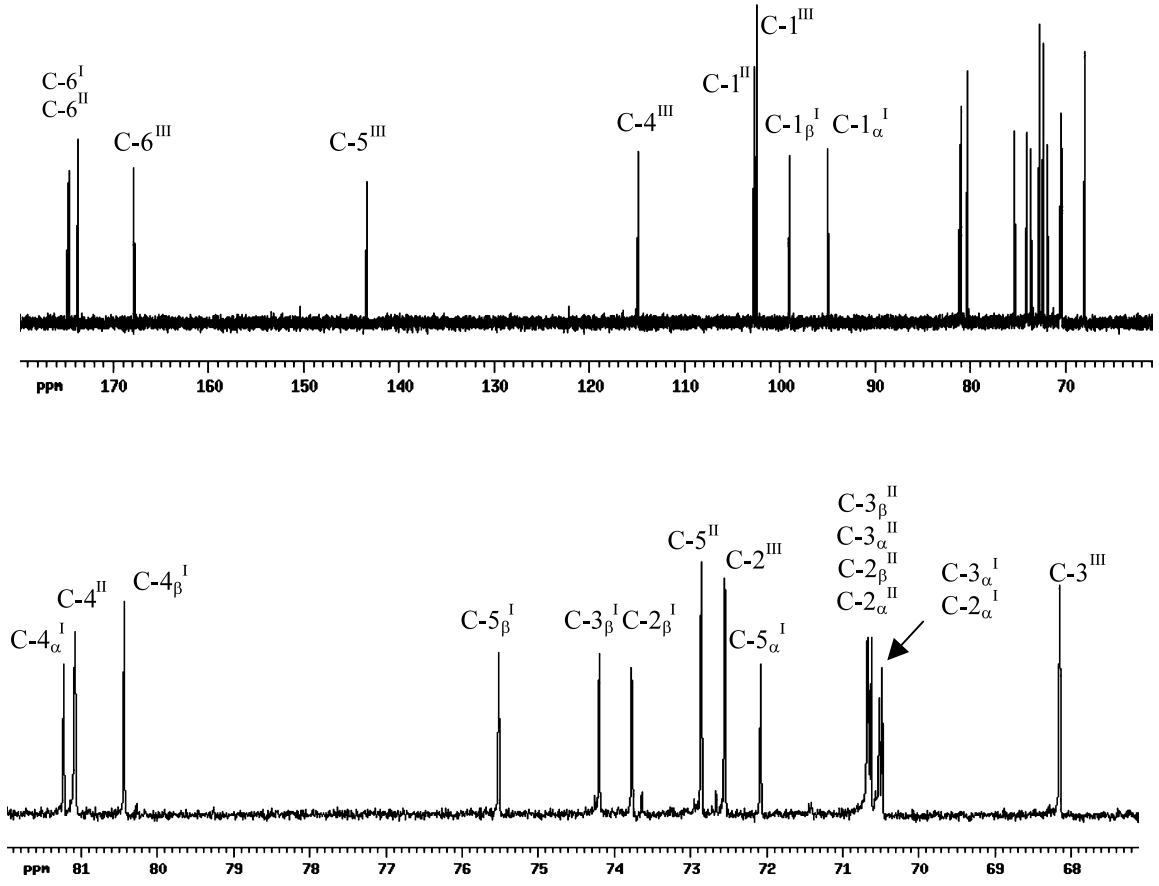


Fig. 3. Carbon NMR spectra (126 MHz, solvent D_2O, pH 4.5, 25 °C) of the unsaturated trigalacturonic acid (mixture of α and β anomers) **3**. The spectra below are an expansion of the 67–82 ppm window. Chemical shifts are quoted from the ^{13}C trimethylsilyl resonance of DSS.

Table 1
Chemical shifts in ppm for the unsaturated trisaccharide **3** [solvent D_2O, pH 1, chemical shifts quoted from the trimethyl resonance of sodium 3-(trimethylsilyl)-1-propanesulfonate]

^{1}H	1	2	3	4	5	
I	5.37 (α)	3.83 (α)	4.04 (α)	4.49 (α)	4.78 (α)	
	4.68 (β)	3.51 (β)	3.81 (β)	4.43 (β)	4.45 (β)	
II	5.09 (α)[a]	3.69 (α)	4.00 (α)	4.64 (α, β)	5.15 (α, β)	
	5.10 (β)[a]	3.70 (β)	4.03 (β)			
III	5.16	3.77	4.37	6.12		
^{13}C	1	2	3	4	5	6
I	95.1 (α)	70.5 (α)	70.5 (α)	81.2 (α)	72.1 (α)	173.8 (α)[a]
	99.1 (β)	73.8 (β)	74.2 (β)	80.4 (β)	75.5 (β)	174.7 (β)[a]
II	102.8	70.7 (α, β)	70.7 (α, β)	81.1	72.9	174.8 (α, β)
III	102.6	72.6	68.2	115.0	143.4	167.9

[a] May be reversed.

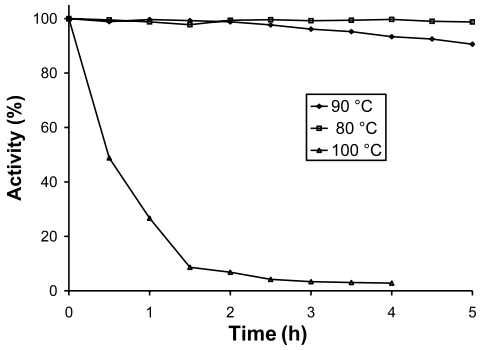


Fig. 4. Thermal stability of exopolygalacturonate lyase from *T. maritima*.

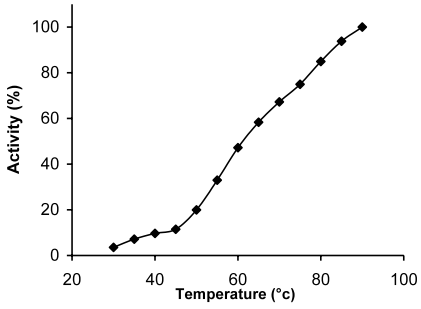


Fig. 5. Optimum temperature for exopolygalacturonate lyase activity from *T. maritima*.

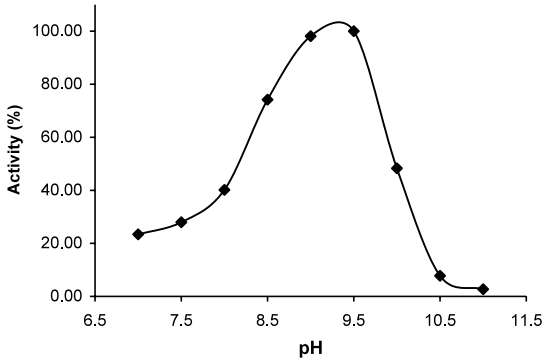


Fig. 6. Optimum activity as a function of pH of exopolygalacturonate lyase from *T. maritima*.

Thermal stability of Pel. Fig. 4 shows the variation of the enzymatic activity over a 5 h incubation period at various temperatures. At temperatures up to 80 °C and below 90 °C, the enzymatic activity remained constant; at $T = 100$ °C, the enzyme lost 50% of its initial activity within 30 min.

The variation of the enzymatic activity versus temperature was determined under conditions of the initial rate and the maximum activity was detected at $T \geq 95$ °C (Fig. 5).

Optimum pH of Pel. At pH values below 7 or above 11, no appreciable activity could be detected. As seen in Fig. 6, a maximum activity was obtained at pH 9.5. Such a behaviour of a *T. maritima* Pel and other pectate lyases[21–25] is not surprising as a basic amino acid residue in the active site is necessary to remove H-5 of the galacturonic ring.

3. Experimental

DNA isolation and cloning.—Chromosomal DNA of *T. maritima* MSB8 was isolated as described by Dimova[30] with minor modifications. The *T. maritima* DNA region containing a putative *pel* gene was amplified by PCR with oligonucleotide primers 5′-CTGCACTAGCTAGTACC and 5′-TTCTCCAGA-GGCATGTC and the obtained 2.96 kb DNA fragment was cloned into a pCR4-TOPO vector (Invitrogen). Next, the *pel* coding sequence was amplified using two flanking primers, carrying additionally *Nco*I or *Xho*I restriction sites and inserted into the expression vector pET21d(+) (Novagen) that allowed the creation of a His-tag at the C-terminal extremity of the protein. pET-21d(+) provides overexpression of a cloned gene from a strong T7 bacteriophage promoter in *E. coli* BL21(DE3) cells after the IPTG induction of a T7 RNA polymerase.

Biochemical analysis of T. maritima *exopolygalacturonate lyase*

Protein overexpression and purification. The *E. coli* BL21(DE3) strain harbouring the cloned *T. maritima pel* gene in pET21d(+) was grown in LB with 100 μg/mL ampicillin at 30 °C to OD600 0.8 and incubated further with isopropyl β-D-thiogalactopyranoside (IPTG, 1 mM) for 6 h. 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 using a 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 concn of imidazole 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 Pel protein was analyzed by SDS-PAGE (7% or 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).

Determination of the protein concentration. The Bradford method was used by adding 50 μL of the reactant to 200 μL of the sample on microplates. The optical density was measured at 620 nm after 10 min incuba-

tion at rt. The protein concentration was calculated from a calibration curve obtained with BSA (Sigma).

Measurement of the exopolygalacturonate lyase activity. In standard conditions, the lyase activity was measured at 70 °C and pH 9.5. The polygalacturonic acid (1 mg) was dissolved in 0.5 mL of 100 mM Tris buffer containing 100 mM glycine, 100 mM ethylamine and 1 mM $CaCl_2$ (pH 9.5). This solution was warmed for 5 min at 70 °C and 1 μL (about 200 ng of protein) of the lyase preparation was added. The degradation of the PGA was followed by means of a UV spectrophotometer (Kontron Uvikon 860) at 235 nm (absorption for the double bond). The UV cell used as a reference contained the same soln as above except for the enzyme. The activity was calculated by means of a standard curve determined using the pure unsaturated trisaccharide **3** at different concentrations in standard conditions (pH 9.5, $T = 70$ °C).

Thermal inactivation. The standard conditions described for the measurement of the lyase activity were used to determine the residual enzymatic activity after partial thermal denaturation. Thus, 20 μL aliquots of the lyase were incubated at different temperatures (80, 90, 100 °C) for 5 h. The residual activity was measured every 30 min.

Determination of the optimal temperature and pH. The activity of the lyase was measured in the standard conditions given above at temperatures from 25 to 95 °C using polygalacturonic acid from orange peels as a substrate. Similarly, the optimal pH was determined in the standard conditions by varying pH from 7 to 11.

Preparation of the oligomers of polygalacturonic acid (DP from 2 to 6).—The oligomers were prepared from the partial acidic hydrolysis at 100 °C of orange peels polygalacturonic acid (Aldrich). Typically, 5.45 g of the latter were suspended in 500 mL of deionized water and warmed for 48 h at 100 °C. After lyophilization of the resulting soln, 57 mg of solid was dissolved in 0.6 mL of 0.1 mM acetate buffer (pH 3.6). The separation of the oligomers was performed by means of gel steric exclusion chromatography using two successive columns. The first one (86 cm long, 3.2 cm o.d.) contained P6 Biogel (Bio-Rad, 106 g of dry matter, 691 mL) and the second (86.4 cm long, 3.2 cm o.d.) contained P4 Biogel (Bio-Rad, 173 g of dry matter, 694.5 mL). The oligomer mixture was eluted with 0.1 M acetate buffer (pH 3.6) and fractions of 4.5 mL were collected while the evolution of the separation was monitored by means of a refractometer.

The separation of the salts from each oligomer was done over a Sephadex G10 column (78 cm long, 4.4 cm o.d. containing 1.18 mL of gel). The rate of elution was 91 mL/h of deionized water. After lyophilization of the solns, about 20 mg of each oligomer (DP from 2 to 6) was obtained. The purity of the compounds checked by HPAEC was shown to be satisfactory.

Synthesis of the unsaturated trisaccharide **3**.—Polygalacturonic acid from orange peels (6 g from Sigma) was suspended in 600 mL of 0.1 M ethylamine buffer (pH 9.5) containing 1.8 mg (30 units) of exopolygalacturonate lyase from *T. maritima*. The mixture was incubated at 25 °C for 140 h. Although the lyase is a very stable enzyme, rt was used for the reaction considering the low stability of the unsaturated trisaccharide **3**. Then, the water and ethylamine were removed under reduced pressure. The resulting powder was partially dissolved in 100 mL of water. The non-soluble material consisted of unreacted starting material and other high molecular weight polymers. The resulting solution was lyophilized and the solid obtained was purified via ion-exchange chromatography using the resin Dowex 1X2-400. Thus 250 mg of lyophilisate dissolved in 4 mL of distilled water was introduced into a column (height 3.5 cm, o.d. 1.5 cm) containing the above resin. The column was washed with 25 mL of deionized water and 25 mL of a 150 mM ammonium bicarbonate soln. The elution of the unsaturated trigalacturonic acid was carried out with 100 mL of a 250 mM ammonium bicarbonate soln. Each fraction (5 mL) was analyzed by means of thin layer chromatography plates (precoated silica gel F254 E. Merck) using a mixture of 4:3:2 butan-1-ol–water–AcOH as eluent (in such conditions, the R_f for polygalacturonic acid was 0 while R_f for **3** was 0.2). The fractions containing **3** (as tris(ammonium) salt) were collected and submitted to lyophilization. The tris(acid) form of **3** was obtained using the resin Amberlite IR 120. Thus, 1 g of lyophilisate dissolved in 5 mL of deionized water was introduced into a column (height 85 cm, o.d. 1.4 cm). The elution of the unsaturated trigalacturonic acid was carried out with 200 mL of deionized water. The resulting solution was submitted to lyophilization. The structure of **3**, established unambiguously by NMR spectroscopy, was confirmed by means of ESMS spectra (M − H 527.4). The compound **3** was produced with a yield of 60%. The specific optical rotation for **3** is $[\alpha]_D$ +190.1° (*c* 1, H_2O), lit. no data. $C_{18}H_{24}O_{18}$ (528.4) Calcd: C, 40.92; H, 4.58. Found: C, 40.83; H, 4.62.

*NMR spectroscopy of (4-deoxy-α-*L*-threo-hex-4-enopyranosyluronic acid)-(1→4)-(α-*D*-galacto-pyranosyluronic acid)-(1→4)-α-*D*-galactopyranuronic acid* (**3**).—Complete analysis of the NMR ^1H and ^{13}C resonances and the subsequent structure assignment were made using standard 2D sequences (COSY HH and HCOOR correlations). The spectra were recorded with a Bruker AX500 spectrometer operating at 500 MHz for ^1H and 126 MHz for ^{13}C. The chemical shifts are reported in Table 1 [solvent D_2O, pH 1, chemical shifts in ppm quoted from the trimethylsilyl resonance of sodium 3-(trimethylsilyl)-1-propanesulfonate].

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