Inversion of configuration during hydrolysis of α-1,4-galacturonidic linkage by three *Aspergillus* polygalacturonases

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Abstract Endopolygalacturonases I and II (PGI and PGII) of *Aspergillus niger* and an exopolygalacturonase (ExoPG) of *A. tubingensis* were investigated to reveal the stereochemistry of their hydrolytic action. Reduced pentagalacturonic acid (pentaGalU-ol) and reduced trigalacturonic acid (triGalU-ol) were used as non-reducing substrates for the enzymes. The configuration of the reducing ends in the products formed in D_2O reaction mixtures was followed by ^1H-NMR spectroscopy. It has been unambiguously established that primary cleavage of pentaGalU-ol by both PGI and PGII leads to diGalU-ol and the β-anomer of triGalUA. The primary products of hydrolysis of triGalU-ol by ExoPG were diGalU-ol and the β-anomer of GalUA. Thus, all three *Aspergillus* polygalacturonases belong to the so-called inverting glycanases, i.e. they utilize the single displacement mechanism of hydrolysis of the glycosidic linkage.

Key words: Endopolygalacturonase; Exopolygalacturonase; Reaction mechanism; Hydrolysis; NMR; *Aspergillus niger*; *Aspergillus tubingensis*

1. Introduction

It is well established that enzymes hydrolyzing glycosidic linkages of glycosides, oligosaccharides and polysaccharides utilize two different reaction mechanisms that lead to a different configuration of the newly formed reducing end [1–3]. Enzymes catalyzing the hydrolysis in one chemical step, the so-called single displacement mechanism, generate reducing-end products with a configuration of the anomeric carbon reversed to that of the cleaved glycosidic linkage. Such hydrolases are called inverting. Other groups of hydrolases utilize two chemical steps to complete the cleavage of the glycosidic linkage. Such hydrolases are called retaining. Initial studies indicated that, in general, endoglycanases are retaining enzymes and exoglycanases inverting enzymes [4]. More recent data dealing with the stereochemical configuration of enzyme-released products confirmed that most endoglycanases are retaining hydrolases. However, this generalization seems to fail so far for exoglycanases. The two exo-acting α-1,4-glucanases, amyloglucosidase and β-amylase are inverting hydrolases, but a number of maltooligosaccharide-hydrolyzing exo-acting enzymes appear to be retaining glycanases [5]. Of the two cellobiohydrolases of *Trichoderma reesei*, considered as exo-acting enzymes, particularly when acting on cellulose or cellooligosaccharides, one is a retaining and the other is an inverting glycanase [6,7]. However, one valid generalization emerges from the correlation of the mechanism of hydrolysis of glycosidic linkage and the ability of glycanases to catalyze glycosyl transfer reactions at high substrate concentrations. Practically all retaining glycanases tested under conditions of oversaturation with substrate catalyzed the reverse reaction leading to the synthesis of new glycosidic linkages [1,8–10]. There is no report of glycosyl transfer reactions by inverting glycanases although the situation is complicated by the ability of some enzymes, like amyloglucosidase, to catalyze condensation reactions [5].

The mechanism by which endopolygalacturonases (EC 3.2.1.15) and exopolygalacturonases (EC 3.2.1.67) cleave the α-galacturonidic linkage in pectin, pectate or oligogalacturonides is unknown. In review articles on pectic enzymes [11–13] there is no mention of glycosyl transfer reactions by this group of glycanases. The only pectolytic enzyme shown to be capable of catalyzing glycosyl transfer reactions is D-galacturonan digalacturonohydrolase (EC 3.2.1.82) of *Selenomonas ruminantum* [14]. This observation suggests that the *S. ruminantum* digalacturonase is a retaining exoglycanase.

The lack of information on stereochemistry of the catalytic action of polygalacturonases prompted us to examine this property with three *Aspergillus* enzymes.

2. Materials and methods

2.1. Enzymes

Endopolygalacturonases (EC 3.2.1.15) I and II (PGI, PGII) of *A. niger* were products of genetically modified strains overproducing the individual enzymes. The PGs were purified from the culture filtrates using published procedures [15].

Exopolygalacturonase (EC 3.2.1.67) (ExoPG) was a product of *Aspergillus tubingensis* NW756. The strain was grown at 30°C for 55 h on a yeast extract medium containing an enzymic hydrolysate of polygalacturonic acid (a mixture of GalUA and oligogalacturonic acids produced by PGII of *A. niger*) as a carbon source. ExoPG was purified from the medium by using four subsequent chromatographic steps on columns of cross-linked alginate, DEAE-Sepharose, S-Sepharose and MONO Q HR 5/5. Details of the purification procedure will be reported elsewhere [16]. The specific activities of PGI and PGII, determined using polygalacturonic acid as a substrate [15] were 550 and 2760 U/mg protein, respectively. The specific activity of ExPG with diGalUA as a substrate was 12 U/gm [16]. One unit of activity is defined as the amount of enzyme liberating from the corresponding substrate 1 μmol equivalents of reducing sugars per min. For NMR spectroscopy experiments the enzymes were lyophilized three times from D_2O.

2.2. Substrates

GalUA was from Sigma. Oligogalacturonic acids of degree of polymerization 2–5 were prepared by enzymic hydrolysis of citrus pectin followed by chromatographic purification of individual fragments [15,17]. Reduced GalUA (GalU-ol), reduced di-, tri-and pentagalacturonic acid (diGalU-ol, triGalU-ol, pentaGalU-ol) were prepared by $NaBH_4$ reduction of the corresponding aldouronic acids. For NMR spectroscopy all compounds were lyophilized three times from D_2O.

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Abbreviations: PG, endopolygalacturonase; ExoPG, exopolygalacturonase; GalUA, D-galacturonic acid; GalU-ol, reduced D-galacturonic acid (D-galacturonitol)

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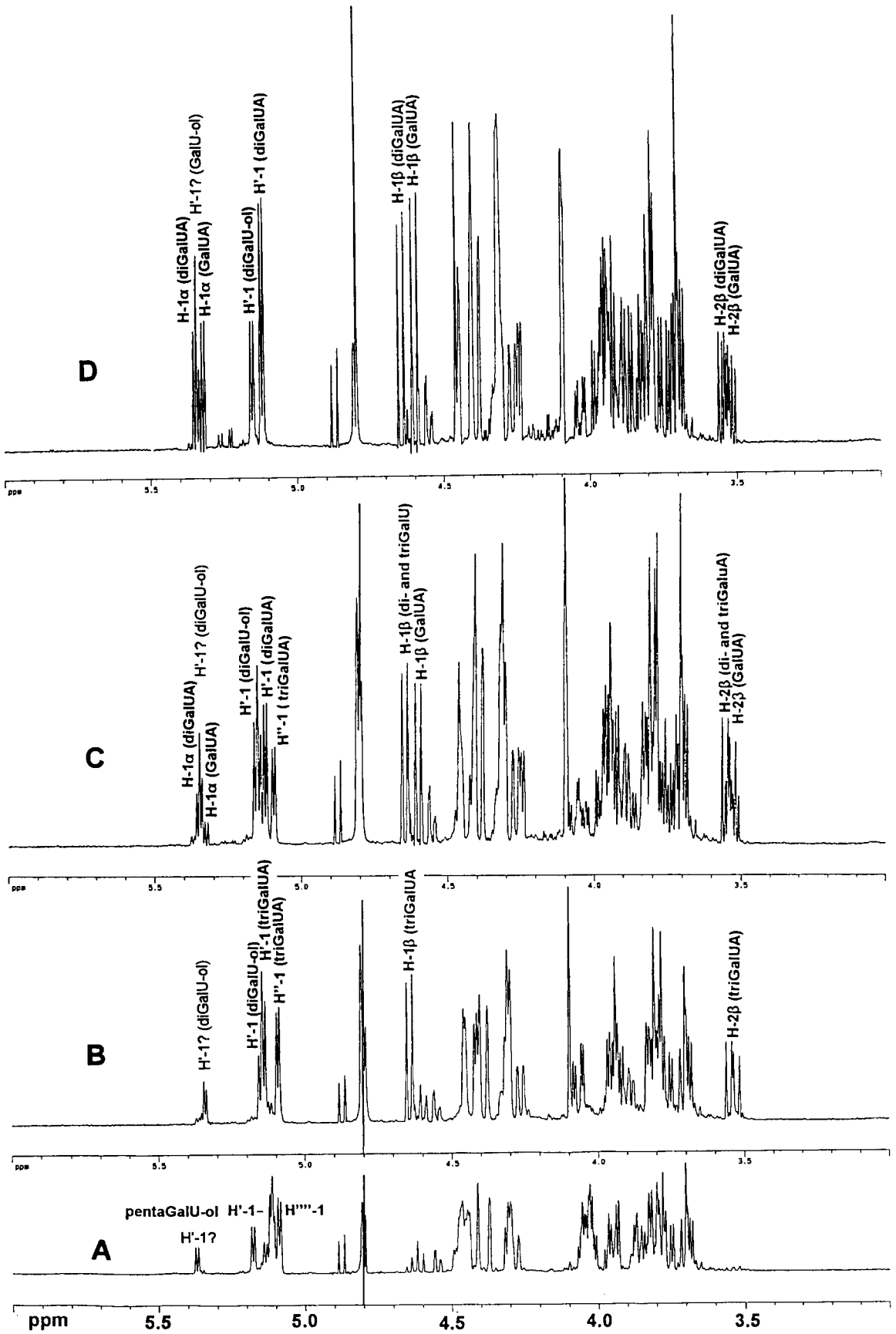

Fig. 1. ^{1}H-NMR spectra of pentaGalU-ol (A) and of its enzymic digest after incubation with *A. niger* PGI for 3 min (B), 11 min (C) and 3 h (D). The assignment of the crucial signals (several doublets and multiplet of H-2β) is indicated.

2.3. Reaction mixtures and NMR spectroscopy

PentaGalU-ol (20 mM) was used as substrate of PGs and triGalU-ol (20 mM) as a substrate of ExoPG. The reactions were performed in 0.1 M deuterized acetate buffer, pH 4.8 for both PGs and pH 4.0 for ExoPG, at 25°C. The amount of enzyme that would guarantee the substrate hydrolysis rate to be much higher than mutarotation of the newly created reducing end, was established on the basis of TLC examination of different reaction mixtures on silica gel (Kavalier, Czech Republic) in the solvent system 1-butanol-formic acid-water 2 : 3 : 1 (by vol.). Reducing sugars were detected with the aniline hydrogen phthalate reagent. After the appropriate enzyme concentration had been established, 0.5 ml of 20 mM solution of the substrate in 0.1 M D_2O buffer was mixed with 20–100 μl of the enzyme solution in D_2O and ^{1}H-NMR spectra of the reaction mixtures were recorded vs. time on a Bruker ARX 400 spectrometer (Germany) at 25°C. The NMR spectra of reference compunds were obtained under the same conditions. The assignment of important resonances was based on published data [18]. Proton resonances of alditols occurring in the region of H-1α resonances of reducing sugars (5.30–5.40 ppm) (indicated as H′-1 and ? in figures) were not definitively assigned. Because they seem to correspond to the H′-1 signals of some minor forms of oligogalacturonitols, they are referred as to H′-1 resonances.

3. Results and discussion

3.1. PGI

PentaGalU-ol is hydrolyzed by PGI primarily to triGalUA and diGalU-ol. The first cleavage is followed by slower hydrolysis of triGalUA to diGalUA and GalUA. This mode of attack of the substrates observed by TLC is in full agreement with the ^{1}H-NMR spectra of the enzyme substrate mixture recorded at various time after the enzyme addition (Fig. 1). The signals of pentaGalU-ol (Fig. 1, Table 1) were quickly replaced by signals of the β-anomer of triGalUA (H-1β, 4.64 ppm, $J_{1,2}$ 7.8 Hz) (Table 2). Other important, easily assignable resonance which appears in the spectra immediately after the formation of a new reducing end in β-configuration is that of C-2 proton (H-2β multiplet at 3.53 ppm) (Fig. 1). The signal of the α-anomer of the trimer (H1α, 5.35 ppm, $J_{1,2}$ 3.8 Hz) practically did not appear because the β-anomer of triGalUA was further hydrolyzed to diGalUA and GalUA, and this secondary hydrolysis was faster than mutarotation. As a consequence, the signals of the β-anomer of the primary reducing product (triGalUA) were continuously replaced by signals of the β-anomers of diGalUA and GalUA. There was no triGalUA left in the reaction mixture after 1 h (Fig. 2). H-1α signals of both sugars (5.31 and 5.34 ppm, respectively) appear later as a result of mutarotation. The time course of the appearance of the β-and α-anomers of hydrolysis products of pentaGalU-ol (Fig. 2) demonstrates that PGI hydrolyses the α-1,4-galacturonidic linkage by inversion of its configuration to give the β-anomers of both primary and secondary products of hydrolysis.

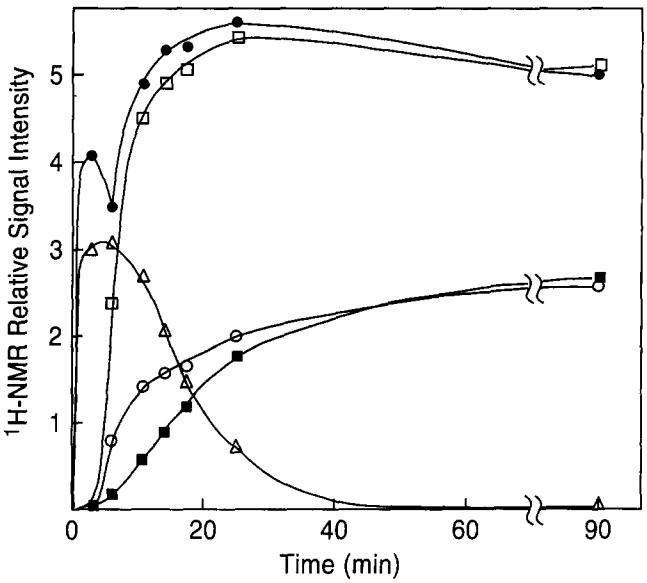

Fig. 2. Changes in relative intensity of anomeric proton resonances during hydrolysis of pentaGalU-ol by PGI of *A. niger*: H-1β of triGalUA and diGalUA (●), H-1α of triGalUA and diGalUA (○), H-1α of GalUA (■), H-1β of GalUA (□), H″-1 of triGalUA (△).

3.2. PGII

The primary attack of pentaGalU-ol by PGII was found to be similar to that of PGI. The substrate is first cleaved to give triGalUA and diGalU-ol. The secondary cleavage of the trimer is much slower than in the case of PGI, but is significant. The trimer is generated exclusively in the β-anomeric form, as only the H1-β resonance (4.64 ppm, $J_{1,2}$ 7.8 Hz), but not that of H-1α, appears in the spectra recorded shortly after enzyme addition (Fig. 3, Table 2). The α-anomer of triGalUA is

Table 1
^{1}H-NMR data for the anomeric and C-1 protons in GalUA, diGalUA, triGalUA, reduced diGalUA (diGalU-ol), reduced triGalUA (triGalU-ol) and reduced pentaGalUA (pentaGalU-ol) (400 MHz, D_2O)

Compound	Proton	Chemical shift δ (ppm)[a]	Coupling constants $J_{1,2}$ (Hz)
GalUA	H-1α	5.31	3.8
	H-1β	4.60	7.9
diGalUA	H-1α	5.35	3.8
	H-1β	4.65	7.8
	H′-1	5.13	3.8
diGalU-ol	H′-1	5.16	4.0
triGalUA	H-1α	5.35	3.8
	H-1β	4.65	7.8
	H′-1	5.14	3.7
	H″-1	5.09	3.8
triGalU-ol	H′-1	5.18	4.0
	H″-1	5.10	3.9
pentaGalU-ol	H′-1	5.17	4.0
	H″-1	5.12	4.3
	H‴-1	5.11	4.3
	H⁗-1	5.09	3.8

[a]Values are centered for doublets and are relative to the D_2O resonance (4.80 ppm).

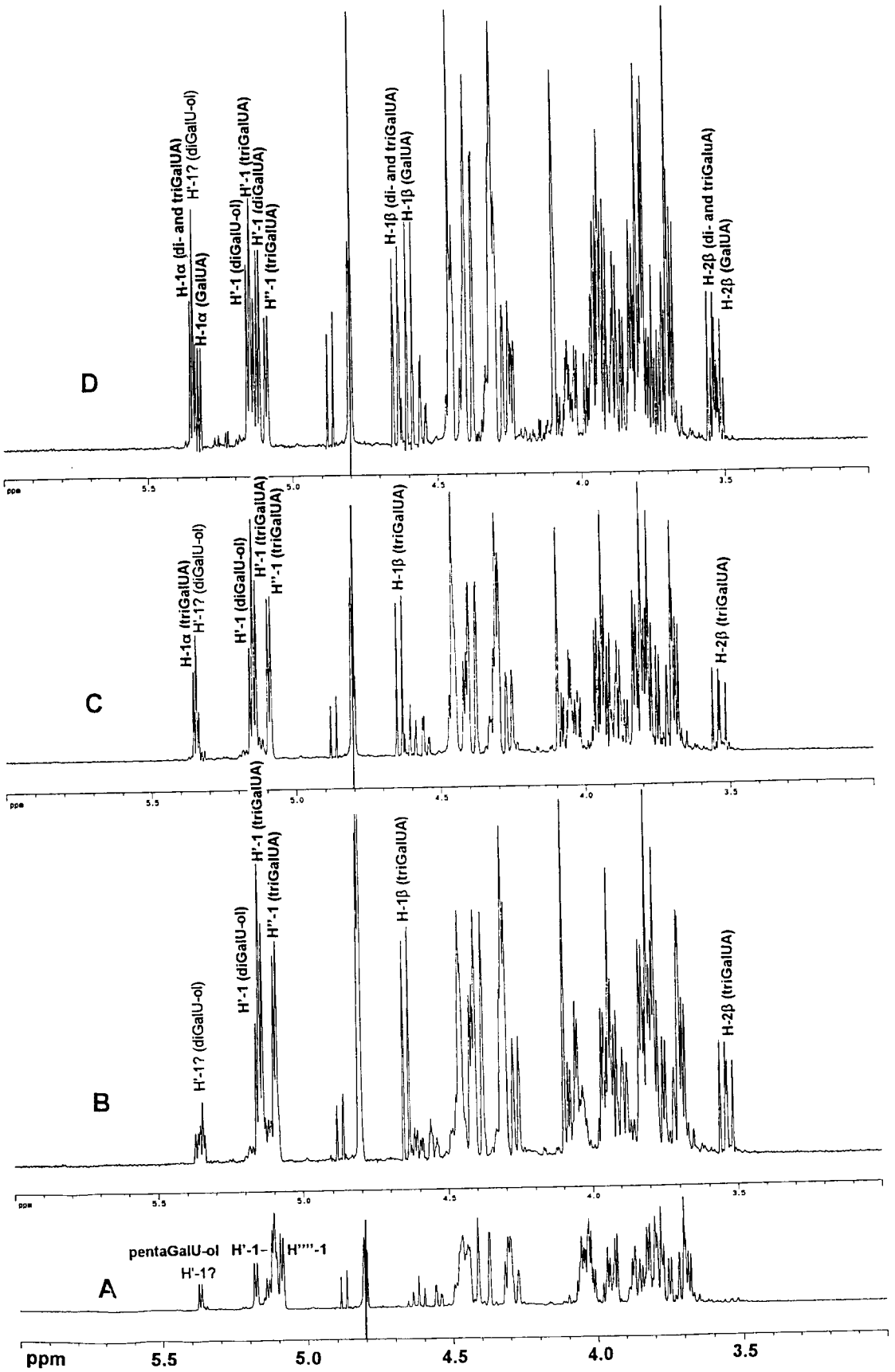

Fig. 3. ^{1}H-NMR spectra of pentaGalU-ol (A) and of its enzymic digest after incubation with *A. niger* PGII for 12 min (B), 2 h (C) and 18 h (D). The assignments of the crucial signals are indicated. The proton resonances of alditols occurring in the region of H-1α resonances (indicated as H'-1 and ?) have not been definitely assigned.

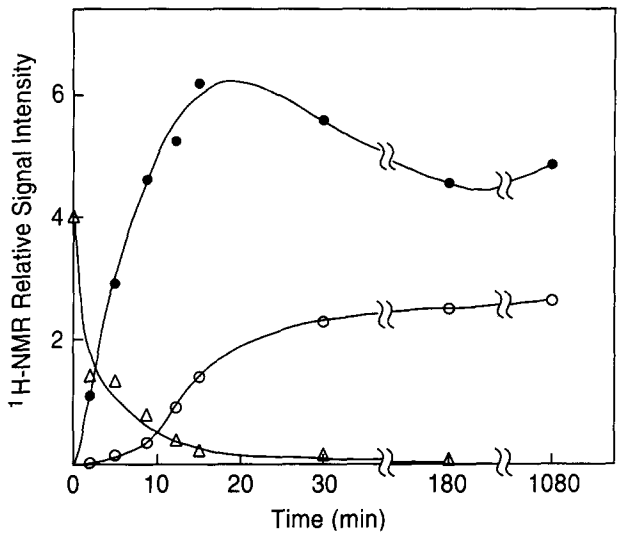

Fig. 4. Changes in relative intensity of anomeric proton resonances during hydrolysis of pentaGalU-ol by PGII of *A. niger*: H-1β of triGalUA (●), H-1α of triGalUA (○), H′-1 of pentaGalU-ol (△).

formed slowly as a result of mutarotation. Hydrolysis of triGalUA by PGII, reflected mainly in the appearance of signals of GalUA, can be observed after an overnight incubation (Fig. 3). The appearance of the H-1 resonances of GalUA is accompanied by a distinct multiplet around 3.53 ppm that corresponds to the H-2β resonance of GalUA. The reducing end proton resonances of diGalUA are identical with those of triGalUA, therefore the formation of diGalUA can be best deduced on the basis of its H′-1 resonance (Table 1, Fig. 3). The time course of appearance of the signals H1-β and H1-α (Fig. 4) represents clear evidence for the inverting character of PGII.

3.3. ExoPG

Reduced triGalUA (triGalU-ol) was used as a substrate for the enzyme releasing GalUA from the non-reducing end of oligogalacturonides. GalUA and diGalU-ol were the products of the primary attack. Monitoring of the reaction by ^{1}H-NMR spectroscopy revealed that the released monomer has the configuration of the β-anomer (H-1β, 4.60 ppm, $J_{1,2}$ 7.9 Hz) (Fig. 5, Table 2). Its slow mutarotation, reflected in the appearance of the H-1α signal at 5.32 ppm ($J_{1,2}$ 3.8 Hz), was surprisingly accompanied by hydrolysis of the primary product of hydrolysis, diGalU-ol, to give GalU-ol and an additional molecule of GalUA. The fact that identical reducing product (GalUA) is formed on the first and second cleavage and that no glycosyl transfer reactions take place is documented by the presence of only one H-1β and one H-2β signal through the whole experiment. diGalU-ol has not so far been reported to serve as a substrate of exopolygalacturonases. This reaction can obviously be observed only at very high enzyme concentrations like those used here. The data in Fig. 6, showing the time course of appearance of the resonances of C-1 protons during hydrolysis of triGalU-ol, confirm that *A. tubingensis* ExoPG liberates the β-anomer of GalUA from oligogalacturonides. This means that ExoPG is also an inverting glycanase.

3.4. Conclusion

The fact that both endo-and exo-acting polygalacturonases of the *Aspergillus* species utilize the single displacement mechanism of the hydrolysis of glycosidic linkage is in agreement with the lack of glycosyl transfer reactions within this class of enzymes. The results also pose the question of whether this mechanism may not be a consequence of the presence of the charged groups in the sugar residues of the substrate molecule. Therefore, it will be of interest to examine the mechanism of cleavage of glycosidic linkage for other types of polygalacturonic acid and pectin depolymerizing enzymes, such as digalacturonohydrolase, pectin-and pectate-lyase, as well as glycanases attacking similarly charged polysaccharides, e.g. alginates.

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Table 2
^{1}H-NMR data for the anomeric and C-1 protons in early products liberated: (i) by exopolygalacturonase from reduced triGalUA (triGalU-ol); (ii) by endopolygalacturonase I and II from reduced pentaGalUA (pentaGalU-ol) (400 MHz, D_2O)

Enzyme	Chemical shift δ (ppm)	Coupling constants $J_{1,2}$ (Hz)	Assignment
EPG I	4.64	7.8	H-1β in triGalUA
	5.09	3.9	H″-1 in triGalUA
	5.14	4.2	H′-1 in triGalUA
	5.15	4.9	H′-1 in diGalU-ol
EPG II	4.64	7.7	H-1β in triGalUA
	5.09	4.8	H″-1 in triGalUA
	5.14	4.3	H′-1 in triGalUA
	5.15	4.0	H′-1 in diGalU-ol
ExoPG	4.60	7.9	H-1β in GalUA
	5.16	3.5	H′-1 in diGalU-ol
	5.32	3.8	H-1α in GalUA

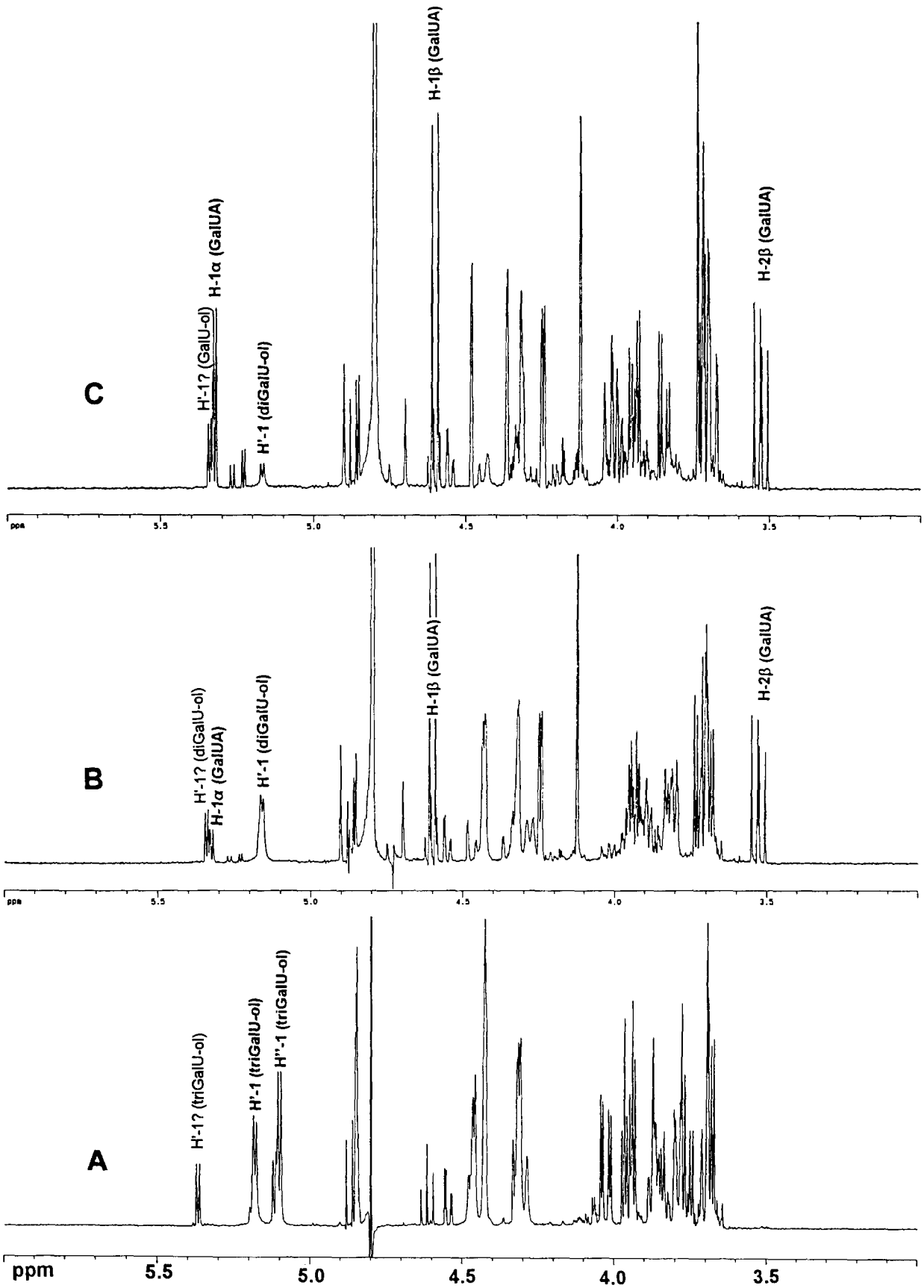

Fig. 5. ^{1}H-NMR spectra of triGalU-ol (A) and of its enzymic digest after incubation with *A. tubingensis* ExoPG for 4 min (B), 1.5 h (C). The assignment of the crucial signals is indicated. The proton resonances of alditols occurring in the region of H-1α resonances (marked as H'-1 and ?) have not been definitely assigned.

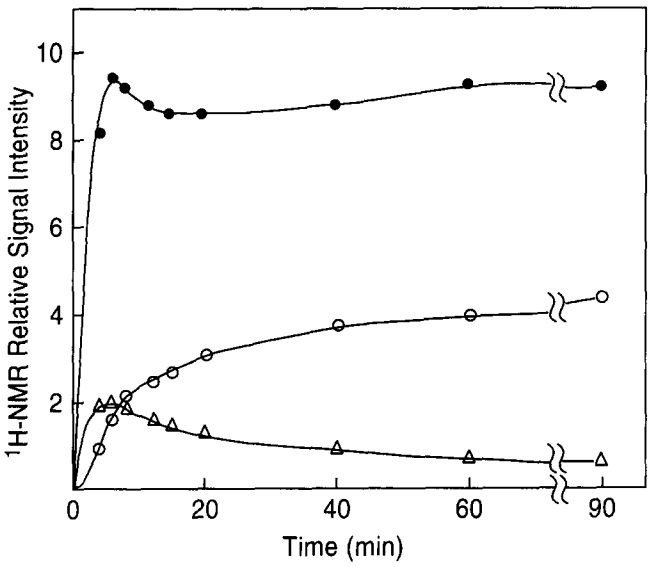

Fig. 6. Changes in relative intensity of anomeric proton resonances during hydrolysis of triGalU-ol by ExoPG of *A. tubingensis*: H-1β of GalUA (●), H-1α of GalUA (○), H′-1 of diGalU-ol (△).

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