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Expression and action pattern of *Botryotinia fuckeliana* (*Botrytis cinerea*) rhamnogalacturonan hydrolase in *Pichia pastoris*

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Abstract

The cDNA sequence coding for the complete rhamnogalacturonan hydrolase (RGase) of *Botryotinia fuckeliana* (*Botrytis cinerea*) was introduced into *Pichia pastoris* and expressed under the control of the alcohol oxidase promoter. The RGase was secreted into the medium of the yeast driven by the α-factor secretion peptide and could be purified using the C-terminal His_6-tag fusion. RGase activity was measured using a traditional reducing end assay with linseed rhamnogalacturonan (RG) as the substrate, or with an assay using a fluorescent RG oligomer as the substrate and detection and identification of hydrolysis products by capillary zone electrophoresis (CZE). Both methods showed the recombinant enzyme to have a specific activity of about ten units per milligram of protein. Since the CZE method allows identification of the hydrolysis products, it was used to show that the RGase lacks a multiple attack mechanism and needs at least five GalA-Rha repeating disaccharides to be active. This finding is contrary to the action pattern of the native RGase of *Aspergillus aculeatus* which has the same substrate length requirement, but exhibits multiple attack, leading to products containing only two and three Rha-GalA repeat units without the appearance of intermediate sized fragments. No plant cell wall degrading enzymes were detected in the culture medium of un-transformed *P. pastoris*, thus the recombinant enzyme, devoid of extraneous activities, can be applied for fine structural studies on cell walls.

Keywords: *Botryotinia fuckeliana*; *Pichia pastoris*

1. Introduction

Pure carbohydrate degrading enzymes are a great help in determining structures of polysaccharides. However, for investigating the structure of plant cell wall polysaccharides, there are few purified enzymes commercially available, and even these retain extraneous activities to a varying extent. Purification of enzymes from culture filtrates of organisms producing them, or from the commercial preparations, can be very time consuming, and extraneous activities often remain. To circumvent these problems, we decided to express cDNAs in a host that does not make cell wall degrading enzymes. Perhaps the widest variety of cell wall degrading enzymes have been characterized from fungi, and fungi often glycosylate the enzymes they secrete. Thus, we chose to use *Pichia pastoris*, a yeast, as the host. Here, we report our results with expression of the rhamnogalact-

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uronan hydrolase of *Botryotinia fuckeliana*.[1] *B. fuckeliana* is more commonly known by the name of its asexual form *Botrytis cinerea*. We have also succeeded in expression of the endoarabinanase of *Bacillus subtillis*[2] in *P. pastoris*.

2. Materials and methods

Microbial strains.—*Escherichia coli* strain DH5α was used as a host for plasmid construction. *P. pastoris* strain X-33 and expression vector pPICZαA were purchased from Invitrogen.[3]

Plasmid construction.—Rhamnogalacturonan hydrolase (RGase) gene from *B. fuckeliana* was obtained from Dr Ken Gross (Horticultural Crops Quality Lab, USDA). PCR primers were designed from the nucleic acid sequence to amplify the coding sequence for 551 amino acids between the signal peptide and the stop codon. *EcoR* I and *Xba* I restriction sites were designed into the 5′ (*N*-terminal) primer and 3′ (C-terminal) primers, respectively. The sequences of the two primers were: 5′-GTAGAATTCGCACAGTTGACTGG-3′ and 5′-CGTCTAGTCTAGACACTCATC-3′. PCR products digested with *EcoR* I and *Xba* I were ligated with the pPICZαA vector and transformed into *E. coli* (DH5α) competent cells on a low salt LB medium with zeocin antibiotics. A clone with a continuous ORF coding for both the α-factor signal and the C-terminal His-tag was identified by DNA sequence analysis (Recombinant DNA/Protein Resource Facility, Oklahoma State University).

Transformation and expression of recombinant RGase in P. pastoris.—The plasmid was integrated into a wildtype strain of *Pichia*, X-33. The transformant phenotype (Mut^+ or Mut^s) for methanol utilization was determined. Mut^+ phenotype colonies (utilizing methanol) were chosen for small-scale expression assays. A single colony was inoculated in 25 mL buffered glycerol-complex medium. After shaking at 30 °C, 300 rpm overnight, the culture was centrifuged at 3000*g* for 5 min. The pellet was resuspended in 250 mL minimal methanol medium and kept in 0.5% methanol by addition of 1.25 mL of 100% methanol every 24 h. Samples of culture (1 mL) were taken every 24 h, centrifuged and supernatants were frozen for further enzyme activity assays.

Preparation and purification of recombinant RGase.—Cultures expressing maximal activity (4 days after methanol induction) were harvested by centrifugation at 5000*g* for 5 min. The supernatant was concentrated (from 1000 to 10 mL) using ultrafiltration with a YM10 (10k MW cut off) membrane (Amicom Inc., Beverly, MA) and desalted by washing with water. The concentrated RGase was used directly for activity assays or further purified on poros 20 metal chelate affinity media (Boehringer Mannheim GmbH, Germany) packed in a stainless steel column (8.3 × 1 cm) from Alltech Associates, Inc.

$(GR)_n$ substrates preparation.—Rhamnogalacturonan (RG) was isolated as the ultrafiltration retentate of endopolygalacturonase digested, saponified citrus pectin.[4] The RG was partially hydrolyzed in 2 M TFA at 80 °C for 4 h and products dialyzed in a 1k MW cut off dialysis membrane against water to remove salt and short fragments, including one and two GalA-Rha repeat units. The mixture was separated on a PA1 anion exchange column monitored with a permanganate bleaching detector[5] using the following gradient: 0–20 min, 0.03–0.3 M NH_4OAc; 20–60 min, 0.3–0.5 M NH_4OAc; and 60–80 min, 0.5–1 M NH_4OAc.

Partial acid hydrolysis preferentially cleaves Rha-GalA glycosidic linkages of the repeating Rha-GalA disaccharide backbone of the polysaccharide and cleaves the arabinose- and galactose-containing side chains. Thus, the resulting oligomers have GalA at their non-reducing ends and Rha at the reducing ends. They are designated as $(GR)_n$, where *n* is the number of disaccharide repeats. The $(GR)_{(1-3)}$ oligomers were identified by comparison of electrophoretic mobilities to those of oligomers which had been identified by MALDI-TOF and NMR spectroscopy.[4] Higher oligomers were identified by assuming that the members of the homologous series would produce a characteristic pattern of decreasing migration rates as each oligomer in-

creased in length by the two sugar residues of the repeat unit.

The oligomers produced from RG by RGase have Rha at their non-reducing ends and GalA at the reducing ends. These are designated as the $(RG)_n$ series, and the two and three disaccharide repeat-containing oligomers identified by MS and NMR were used to identify the number of repeats in the homologous series seen by CZE.

Amino-naphthalene trisulfonic acid (ANTS) derivatization.—The purified individual oligomers of $(GR)_5$–$(GR)_{11}$ were derivatized at their reducing ends with ANTS by reductive amination as described previously.[6] Salts and unattached labeling agent were removed from the oligosaccharide by passage through a Toyopearl HW40-S gel-filtration column (15×0.9 cm), the oligosaccharide was eluted with 50 mM NH_4OAc, and detected by a refractive index monitor. The purified substrates were dissolved in water to make a final concentration of 1 nmol/μL.

CZE enzyme activity assay.—Enzyme activity was assayed by adding a 1 μL aliquot of RGase diluted in the appropriate buffer, (~ 2 ng of protein, 2.2×10^{-5} units), to 1 μL of ANTS labeled $(GR)_n$ solution (1 nmol substrate) in a microcentrifuge tube. After the desired incubation period, the enzyme was inactivated by heating at 80 °C for 10 min, and distribution of fluorescence between products and unreacted substrate was determined by CZE with laser induced fluorescence detection.[7] The extent of the reaction was estimated as the fraction of the initial substrate which had been converted to product by dividing the sum of modified peak areas of the product peaks by the sum of all modified peak areas. The modified peak areas were defined as peak areas divided by their retention times to take into account the increased length of time spent in the detection window with decreasing migration rate. We consider all fluorescent oligosaccharides produced from the substrate to have the same molar fluorescence yield as the substrate. Since the initial concentration of substrate (S_0) is known, we calculate the substrate consumption rate as follows:

$$\frac{\text{Sum of modified peak areas of product} \times S_0}{\text{Sum of modified peak areas of product and substrate} \times \text{digestion time}}$$

For kinetic measurements, incubation times were adjusted to ensure less than 10% consumption of substrate.

The pH optimum was found by determining the substrate consumption rate of RGase in 50 mM sodium acetate buffer ranging from pH 3.0 to 6.0. The temperature optimum was derived from the substrate consumption rate of RGase in 50 mM sodium acetate buffer in pH 4.0 ranging from 20 to 50 °C.

Nelson–Somogyi enzyme activity assay.—Nelson–Somogyi reagents[8] were used to measure the reducing groups released by the enzyme from linseed mucilage rhamnogalacturonan. The linseed RG was purified by cetavlon precipitation[9] from flax seed purchased at a local health food store. The substrate stock solution was made as 0.5% (w/v) in 50 mM sodium acetate buffer, pH 4.0. Enzyme assays were initiated by mixing 2 mL of substrate stock solution (10 mg substrate) and 10 μL of concentrated RGase (~ 2 μg) and incubated at 30 °C. Samples (0.5 mL) were removed from the reaction mixture at timed intervals, immediately mixed with 0.5 mL of copper reagent and boiled for 10 min. After the tubes were cooled to rt, 1.0 mL of arsenomolybdate reagent was added and the tubes were incubated at rt for 15–40 min. The assay mixture was centrifuged to remove precipitated material, and the absorbance of the supernatant was read at 500 nm in a spectrophotometer. D-Galacturonic acid was used to prepare a standard curve.

Gel electrophoresis.—Molecular weight of the recombinant RGase was estimated based on a 10% SDS–PAGE gel stained with Coomassie Blue. A broad molecular weight standard (Bio-Rad) from 6.5 to 200 kDa was used for calibration.

GLC sugar composition analysis.—Proteins were electroblotted from SDS–PAGE gels to polyvinylidene difluoride (PVDF) membranes and the membranes stained with Coomassie Blue for localization of the protein bands and estimation of the protein amount. Sugar compositions were determined by gas liquid chro-

matography (GLC) analysis of the trimethylsilyl methyl glycosides. Methanolysis and derivatization of the excised bands were performed using the protocol of Chaplin[10] as modified by Komalavilas and Mort.[11]

3. Results and discussion

RGase optimum expression time, pH and temperature.—Fig. 1 shows the time course of RGase accumulation in methanol-induced recombinant *P. pastoris*. RGase activity was estimated as the consumption rate of 1 nmol of ANTS pre-labeled $(GR)_{10}$ by 1 μL of culture medium. The pH of the culture medium was adjusted to pH 4.0 and the digestion mixture was incubated at rt for 2 h. The highest RGase activity level was reached at 4–5 days after the addition of methanol. Fig. 2 shows the pH and temperature optima of the RGase. RGase activity was measured as the consumption rate of 1 nmol of ANTS pre-labeled $(GR)_{10}$ by 1 ng of concentrated RGase. The optimum pH (Fig. 2(a)) for RGase was determined to be pH 4.0 and the temperature optimum to be 30 °C (Fig. 2(b)).

Properties of the recombinant RGase.—The purified recombinant RGase has an apparent molecular weight, as judged by SDS–PAGE,

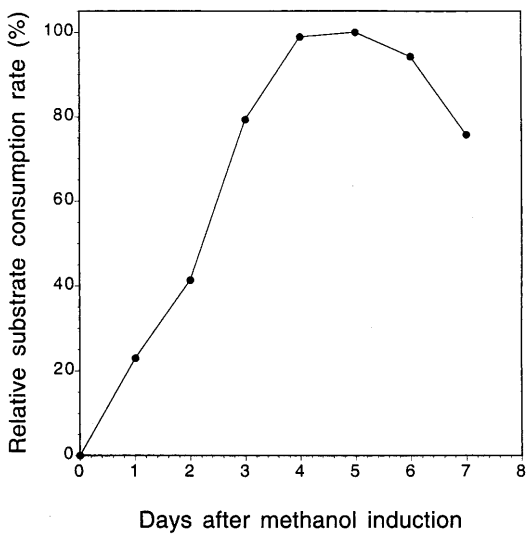

Fig. 1. Time course of RGase accumulation in methanol-induced recombinant *P. pastoris*. RGase activity was estimated as the consumption rate of 1 nmol of ANTS pre-labeled $(GR)_{10}$ by 1 μL of culture medium, normalized to the maximum rate observed.

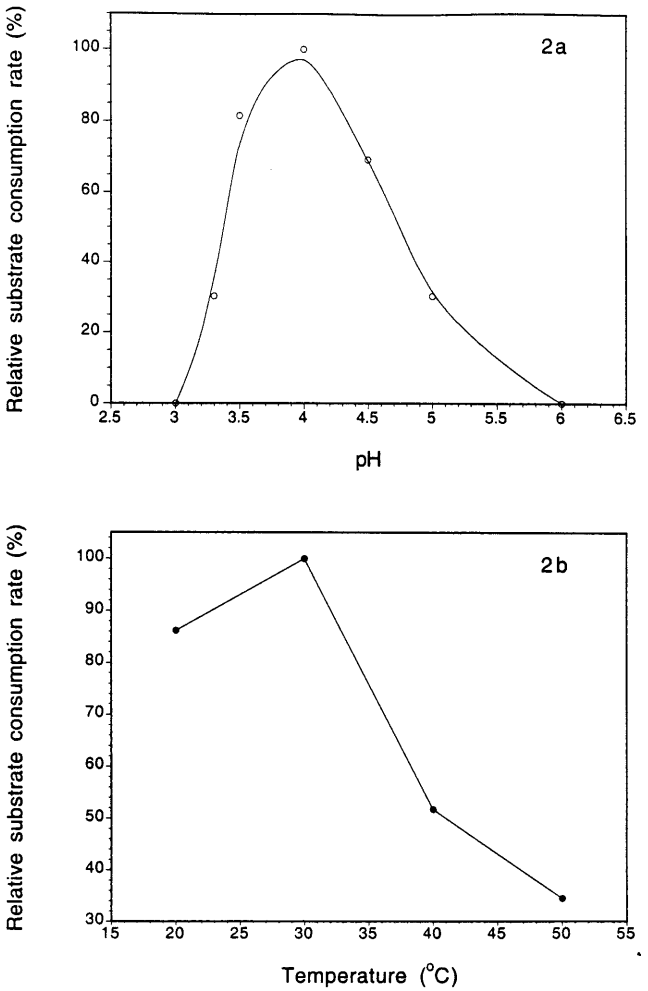

Fig. 2. pH and temperature optima for recombinant RGase. RGase activity was estimated as the consumption rate of 1 nmol of ANTS pre-labeled $(GR)_{10}$ by 1 ng of concentrated RGase, normalized to the maximum rate observed.

of 100 kDa. The expression level of the RGase from the 4-day culture was estimated at about 1 μg/mL by comparison of the staining intensity of the enzyme band on the gel to that of known amounts of standard proteins. The molecular weight of the recombinant RGase deduced from its amino acid sequence is 60 kDa. The difference of 40 kDa from the predicted mass could be a result of 'hyperglycosylation' of recombinant proteins expressed in Pichia.[12] The native RGases of *Aspergillus niger* and *Aspergillus aculeatus* contain approximately 10–15 kDa sugars contributing to their weight.[13,14] Sugar composition analysis of the recombinant RGase protein band blotted onto PVDF membrane revealed approximately 1 μg of mannose per 1 μg of the RGase peptide with

only traces of other sugars. The predicted amino acid sequence homologies for RGase from *B. fuckeliana* and RGases from *A. aculeatus*[14] and *A. niger* (*rhg*A and *rhg*B)[13] are compared in Fig. 3. All four RGases show a similar (average 60% identity) 440 amino acid region in which, domains A, B, D, F and G are RGase specific and domain C and E are pectinase specific. In addition, RGase of *B. fuckeliana* and RGase of *A. niger* (*rhg*B) have a C-terminal extension of 105–110 amino acids (domain H). This domain neither shows significant homology to any related pectin degrading enzymes nor between the two RGases. Visser and colleagues suggested that this C-terminal extension may modify the specificity of the enzyme and substrate binding or localization.[13]

Recombinant RGase activity generated fragments.—The specific activity of the recombinant RGase was determined using two different methods (Table 1). Previous assays for RGase have mainly used the so called modified 'hairy' region from apple pectin[15] or a partial acid hydrolysate of sugar beet pectin[16] as a substrate with quantitation of the enzyme reaction products by HPAEC in combination with pulsed amperometric detection. Gross et al.[17] devised an assay involving release of rhamnose from RGase-generated oligomers by rhamnosidase followed by quantitation of the rhamnose by GLC. No rhamnogalacturonan pure enough for assaying RGase activity is commercially available. However, linseed mucilage has been known for many years to contain a rhamnogalacturonan with galactose- and fucose-containing

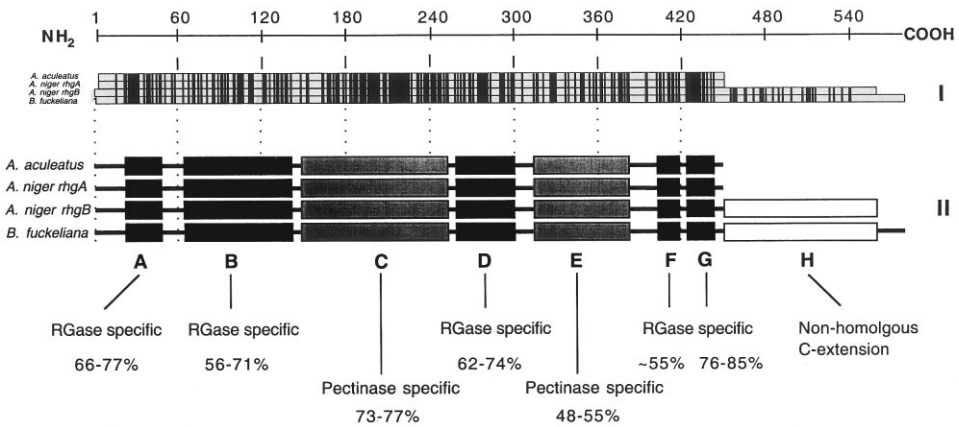

Fig. 3. Amino acid sequence homology comparison among four fungal rhamnogalacturonan hydrolases: *A. aculeatus*, accession no. X83525; *A. niger rhg*A, accession no. X94220; *A. niger rhg*B, accession no. X94221; and *B. fuckeliana*, accession no. U62397. **I** Homology scanning (identity) of aligned amino acid sequences. Black boxes indicate amino acids identical to all four RGases. **II** Discrete homology domains are indicated by the boxed regions as RGase-specific (dark boxes), pectinase-specific (light boxes) and non-homologous (white boxes). Domains A, B, D, F and G are highly conserved among RGases and do not appear in other pectin degrading enzymes. Domains C and E are highly conserved in a large number of pectin degrading (RGases, *endo*- and *exo*-polygalacturonase) enzymes. Domain H is rich in serine and threonine and is both non-homologous and has no similarity with any entry in Genbank.

Table 1
Comparison of RGase assay methods and calculated specific activities [a]

Method	Nelson–Somogyi	CZE	CZE
Substrate	linseed RG	ANTS labeled $(GR)_{10}$	$(GR)_{10}$
Substrate amount	2.5 mg	1 nmol	1 nmol
RGase amount (ng)	500	1	1
Assay volume (μL)	500	1	1
Specific activity (unit/mg)	10	11	11

[a] One unit of enzyme activity is defined as the amount of enzyme required to release one micromole of reducing-sugar equivalents per minute under the defined assay conditions.

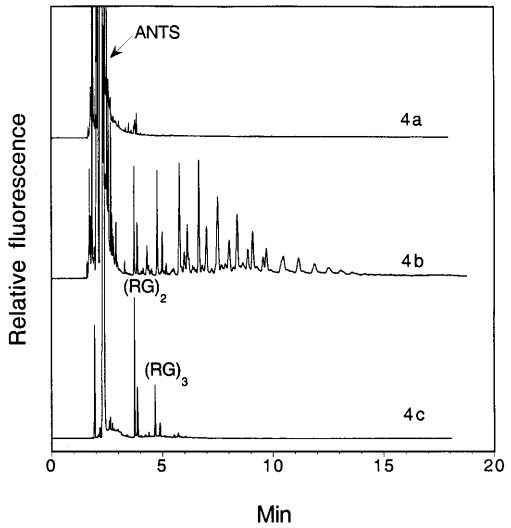

Fig. 4. Electropherograms of ANTS labeled linseed RG (a), linseed RG partially digested by RGase (b), and linseed RG completely digested by RGase (c). 1 μg of RGase was added to 2.5 mg of linseed RG in 0.5 mL of 50 mM sodium acetate buffer, pH 4.0.

side-chains. An enzyme activity which caused a rapid reduction of viscosity of linseed mucilage and which must have been RGase, was isolated from *A. niger*.[18] Fig. 4 shows electropherograms of the ANTS labeled linseed mucilage and products of a partial hydrolysis by RGase. The linseed mucilage does not contain small fragments (Fig. 4(a)). After incubation with RGase, a series of fragments accounting for about 40% of the total polymer was generated (Fig. 4(b)), and most of them could be degraded into $(RG)_2$ and $(RG)_3$ after extended digestion (Fig. 4(c)). The remainder of the polymer appeared to be resistant to digestion and eluted at about 100–200 kDa molecular weight on a Toyopearl HW-55S gel-filtration column. Thus, linseed mucilage is a suitable substrate for RGase. From experiments using 0.5% (w/v) linseed RG, an incubation temperature of 30 °C, a pH of 4.0, and the Nelson–Somogyi assay for determination of reducing groups, we calculated that one mg of enzyme would produce 10 μmol of product per minute (Table 1).

An alternative way to assay the enzyme activity is to use fluorescent labeled substrate and CZE. With this method we used much less substrate and enzyme because the assay can be carried out in as little as 1 μL, although similar concentrations were used for both assay methods. The concentration of the linseed RG used for the Nelson–Somogyi assay is 0.5% (5 mg/mL) which is equivalent to approximately 1.5 mM of $(GR)_{10}$. The concentration of ANTS pre-labeled $(GR)_{10}$ substrate for CZE assay was 1 mM. The same RGase concentration (1 ng/μL) was used for both CZE and Nelson–Somogyi assays. Electropherograms at several time points during the course of degradation of ANTS pre-labeled $(GR)_{10}$ are shown in Fig. 5. Because only the ANTS labeled substrate and products are detected, the non-labeled products do not ap-

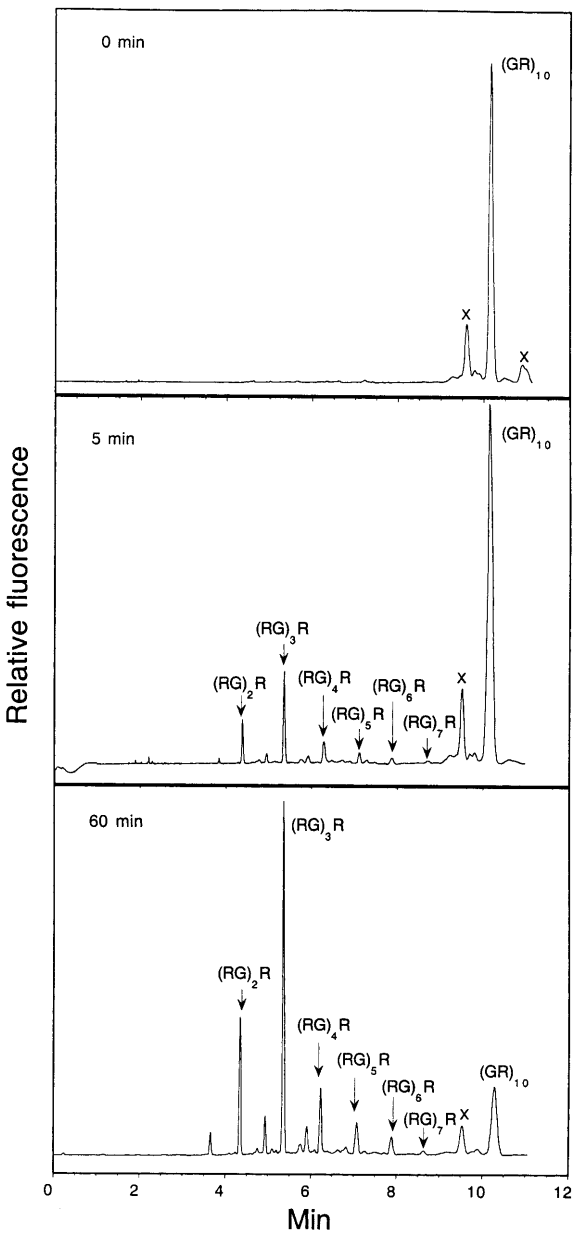

Fig. 5. Time course of RGase digestion of ANTS pre-labeled $(GR)_{10}$. Peaks under 'X' are unidentified.

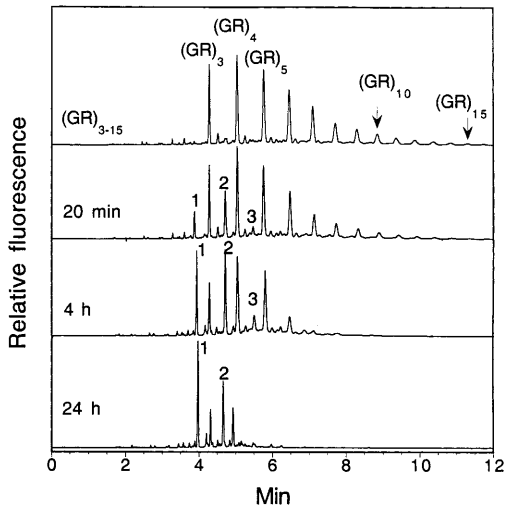

Fig. 6. Time course of RGase digestion of ANTS pre-labeled $(GR)_{3-15}$ mixture. (1) $(RG)_2R$; (2) $(RG)_3R$; (3) $(RG)_4R$.

pear in the electropherogram. If the enzyme cleaves the oligomer only once, the amount of ANTS labeled products is equal to that of the non-labeled products, and the substrate consumption rate, if it were expressed in μmol/min, is equivalent to the number of units of enzyme added. However, if the enzyme cleaves the substrate '*n*' times, the actual activity of the enzyme would be '*n*' times that seen with the fluorescence assay.

To determine if the ANTS label itself has an effect on the enzyme digestion, non-labeled $(GR)_{10}$ was digested by RGase. The digestion mixture was then labeled with ANTS and the substrate consumption rate was calculated (counting only those fragments from the original reducing end of the oligomer, i.e. $(RG)_nR$) (data not shown). The result showed that the substrate consumption rate of the non-labeled $(GR)_{10}$ is similar to the ANTS pre-labeled $(GR)_{10}$. Both specific activities are approximately 11 unit/mg (Table 1). This indicates that labeling of the substrate does not affect the RGase digestion.

As seen from Table 1, the activities determined from CZE assay and Nelson–Somogyi assay are comparable. However, the Nelson–Somogyi assay requires a minimum of 50 nmol of reducing sugars for detection, while the CZE assay needs 1 nmol or less. Furthermore, the CZE method using ANTS labeled substrate is more convenient, more specific and allows identification of the products of the reaction.

Recombinant RGase cleavage pattern.—The RGase of *A. aculeatus* was reported to hydrolyze the GalA linkages in the alternating Rha-GalA backbone of RG, yielding $(RG)_2$ and $(RG)_3$ oligomers.[19] Mutter et al.[16] reported that RGase from *A. aculeatus* is able to cleave oligomers of five Rha-GalA units or more and cleaves at four or six residues from the non-reducing end Rha. The crystal structure of RGase (*A. aculeatus*) suggests that the catalytic groove can accommodate 12 sugar residues.[20] The $(GR)_n$ oligomers prepared from TFA hydrolysis of the RG from citrus pectin have Rha at the reducing end and GalA at the non-reducing end because the glycosidic bond between Rha and GalA is more acid labile. A time course of the recombinant RGase digestion of ANTS pre-labeled $(GR)_{3-15}$ mixture is shown in Fig. 6. After a short time of incubation, new peaks corresponding to $(RG)_nR$ products appeared between the $(GR)_n$ substrate peaks. This indicates that RGase of *B. fuckeliana* cleaves at the glycosidic bond between GalA and Rha. Substrates longer than $(GR)_5$ were degraded very quickly and $(GR)_5$ was degraded relatively slowly. However, $(GR)_3$ could not be degraded and $(GR)_4$ was degraded only very slowly.

According to the *A. aculeatus* RGase cleavage pattern,[16] it is clear that three types of final products would be generated from the ANTS pre-labeled $(GR)_n$ substrates: ANTS labeled reducing-end fragments, $(RG)_2R$ or $(RG)_3R$; non-reducing end products, $G(RG)_2$ or $G(RG)_3$; and internal fragments from double digestion, $(RG)_2$ or $(RG)_3$.

For a more detailed understanding of the cleavage pattern of the recombinant RGase from *B. fuckeliana*, individual GR oligomers pre-labeled with ANTS were used as substrate. Fig. 5 shows an example of a time course of RGase digestion of ANTS pre-labeled $(GR)_{10}$. Initially, a series of reducing end products was formed with elution order of $(RG)_2R$, $(RG)_3R$, $(RG)_4R$, $(RG)_5R$, $(RG)_6R$ and $(RG)_7R$. The highest peak was $(RG)_3R$ and the second highest was $(RG)_2R$. The ratio between the whole series of fragments remained about the same during the digestion until the majority of the $(GR)_{10}$ had been

consumed. At this time the larger fragments were degraded into the final products of $(RG)_2R$ and $(RG)_3R$. Thus, it appears that the initial $(GR)_{10}$ oligomer was a much better substrate than the intermediate digestion products. Fig. 7 summarizes the observed RGase cleavage patterns for ANTS pre-labeled $(GR)_n$ ($n = 5$–11) oligomers during the initial phase of the reaction. The relative cleavage rates between different positions were

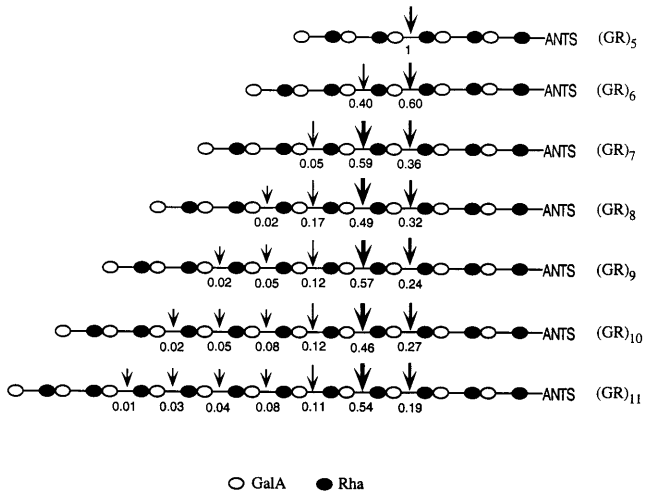

Fig. 7. Observed RGase cleavage pattern of ANTS pre-labeled $(GR)_5$–$(GR)_{11}$ oligomers during the initial phase of the reaction. Arrow indicates the cleavage position and the number under the arrow represents the fractional cleavage at the site. Solid oval represents Rha and empty oval represents GalA.

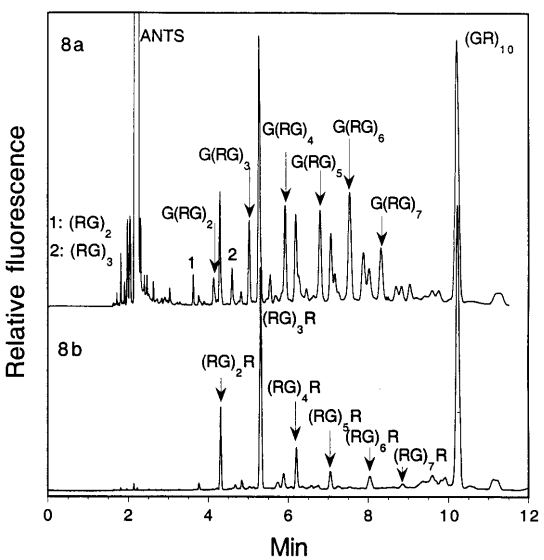

Fig. 8. Electropherograms of partially digested pre-labeled $(GR)_{10}$ by RGase after (a) and before the second ANTS labeling (b).

calculated based on the ratios of modified peak areas (peak area/retention time) obtained from CZE assays. The result reveals that the recombinant RGase preferentially cleaves three or two repeating units of RG from the reducing end, disregarding the ANTS label.

To check if the RGase cleaves once or multiple times, progressing along the substrate prior to release, we needed to identify the products formed from the non-reducing end of the substrate. A second ANTS labeling was used to detect those fragments. The second ANTS labeling (Fig. 8(a)) showed all products including the originally labeled reducing-end fragments, non-reducing end fragments and the internal fragments cleaved from the substrate. Fig. 8(b) shows the electropherogram of the singly labeled, digested oligomer, in order to indicate the migration time of the original reducing-end fragments. In the doubly labeled electropherogram there was an additional series of fragments migrating faster than the corresponding $(RG)_nR$, which we assumed to be the non-reducing end $G(RG)_n$ series. The faster migration rate of the $G(RG)_n$ oligomers is quite reasonable because of the higher proportion of GalA. The migration rate of the oligomer is dependent on both its charge and effective radius of gyration. The ANTS label itself provides three negative charges to any oligomer to which it is attached. At the pH of the running buffer, 2.5, there is a small amount of charge on the GalA residues, and no charge on the Rha residues. The $GalA_n$ series of oligomers migrates considerably faster than the RG oligomers of the same number of sugar residues.[4] The presence of the $G(RG)_7$ and $G(RG)_6$ fragments (the non-reducing end products corresponding to the preferential cuts of the $(GR)_{10}$ substrate) indicates that when RGase cleaves the oligomer it releases the products before it makes the next cut. The products formed by two cuts, i.e., $(RG)_2$ and $(RG)_3$ were not detected at the beginning stages of the digestion (data not shown). Thus, the recombinant RGase does not show multiple attack as does the RGase from *A. aculeatus*.[16] This is also indicated by the formation of many different lengths of oligomers during partial digestion of linseed RG (see Fig. 4).

Tests for extraneous activities.—The recombinant RGase preparation without purification on an affinity column was tested with several other fluorescent oligosaccharides for the presence of extraneous activities. These included labeled arabinoheptaose, $GalA_{18}$, cellopentaose and xylohexaose. No hydrolytic activity against these substrates was detected. In our investigations of crosslinking between rhamnogalacturonan and xyloglucan of cotton cell walls,[21] we need to degrade RG without degrading xyloglucan (XG). A mixture of oligomers containing two repeat units of XG was prepared by partial digestion of tamarind XG with endoglucanase followed by size fractionation on a Toyopearl HW-40S column. The ANTS labeled XG dimer was used as a substrate to test for the presence of xyloglucan degrading activity. No endoglucanase activity was found in the concentrated recombinant RGase. Thus, the concentrated and desalted recombinant RGase can be directly applied for cell wall structure study without the necessity of His-tag column purification.

4. Conclusions

The recombinant RGase of *B. fuckeliana* expressed in *P. pastoris* does not contain extraneous cell wall degrading enzyme activities. The enzyme activity was simply detected and estimated using fluorescent labeled oligosaccharide CZE assay. The CZE-determined enzyme specific activity is comparable to that calculated from the Nelson–Somogyi reducing-sugar assay. The recombinant RGase preferentially cleaves two and three RG repeating units from the reducing end of the $(GR)_{n>5}$ substrate and a multiple attack mechanism is absent. The extra C-terminal extension region of the RGase from *B. fuckeliana* may be responsible for the cleavage pattern differing from that of the RGase from *A. aculeatus*.

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