A rhamnogalacturonan acetylesterase (RGAE) was purified to homogeneity from the filamentous fungus Aspergillus aculeatus, and the NH₂-terminal amino acid sequence was determined. Full-length cDNAs encoding the enzyme were isolated from an A. aculeatus cDNA library using a polymerase chain reaction-generated product as a probe. The 936-base pair rha1 cDNA encodes a 250-residue precursor protein of 26,350 Da, including a 17-amino acid signal peptide. The rha1 cDNA was overexpressed in Aspergillus oryzae, a filamentous fungus that does not possess RGAE activity, and the recombinant enzyme was purified and characterized. Mass spectrometry of the native and recombinant RGAE revealed that the enzymes are heterogeneously glycosylated. In addition, the observed differences in their molecular masses, lectin binding patterns, and monosaccharide compositions indicate that the glycan moieties on the two enzymes are structurally different. The RGAE was shown to act in synergy with rhamnogalacturonase A as well as rhamnogalacturonase B from A. aculeatus in the degradation of apple pectin rhamnogalacturonan. RNA gel blot analyses indicate that the expression of rhamnogalacturonan degrading enzymes by A. aculeatus is regulated at the level of transcription and is subjected to carbon catabolite repression by glucose.

Pectic polysaccharides are located predominantly in the middle lamella and primary cell wall of dicotyledonous plants (1). The main backbone in pectins can be divided into linear holobifurcation lamella and primary cell wall of dicotyledonous plants (1). Purification of native Rhamnogalacturonan Acetylesterase from A. aculeatus

SYNERGISM BETWEEN RHAMNOGALACTURONAN DEGRADING ENZYMES*

Molecular Cloning and Characterization of a Rhamnogalacturonan Acetylesterase from Aspergillus aculeatus

Sakari Kauppinen, Stephan Christgau, Lene V. Kofod, Torben Halkier, Kurt Dörreich, and Henrik Dalbøge

From Genesearch, Novo Nordisk A/S, Novo Allé, Building 1B1.21, DK-2880, Bagsvaerd, Denmark

The main backbone in pectins can be divided into linear holobifurcation lamella and primary cell wall of dicotyledonous plants (1). Purification of native Rhamnogalacturonan Acetylesterase from A. aculeatus

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Pectic polysaccharides are located predominantly in the middle lamella and primary cell wall of dicotyledonous plants (1). The main backbone in pectins can be divided into linear homogalacturonan (smooth) regions of up to 200 residues of (1,4)-linked α-D-galacturonic acid (GalUA)1 and highly branched rhamnogalacturonan (hairy) regions consisting of repeating α-(1,2)-L-Rha-(1,4)-D-GalUA disaccharide units (1–3). In general, about half of the Rha residues in the hairy regions are substituted with neutral oligosaccharides such as arabinans, galactans, and arabinogalactans. Most pectic substances are further esterified with acetyl or methyl groups at some of the GalUA residues in the backbone (1, 4).

Many saprophytic and plant pathogenic fungi and bacteria possess an array of extracellular enzymes involved in the degradation of plant cell wall polymers (5, 6). In the primary cell wall, the cellulose-xylanoglucon framework is embedded in a matrix of pectic polysaccharides, which thereby control the access of hydrolytic enzymes to the cellulose and hemicellulose substrates (1, 7). Thus, pectinases are often the first cell wall degrading enzymes produced by plant pathogens when cultured on purified plant cell walls or during infection (8, 9). Due to the structural complexity of the pectin matrix a synergistic or sequential action of several different pectinolytic enzymes is required for efficient breakdown (5). For example, the hydrolysis of smooth regions of pectin by polygalacturonases is highly dependent upon demethylation of the homogalacturonan backbone by pectin methyl esterase (5). Similarly, the degradation of rhamnogalacturonan by rhamnogalacturonases (RGAs) depends on the removal of the acetyl esters from the substrate (10, 11). Thus, the presence of a rhamnogalacturonan acetylesterase (RGAE) in the filamentous fungus Aspergillus aculeatus (12) suggests that this enzyme is essential for the action of RGAs in vivo.

With the goal of elucidating the role of rhamnogalacturonan acetylesterase in the enzymatic degradation of plant cell wall rhamnogalacturonan we have undertaken purification of the RGAE from A. aculeatus and isolation and characterization of full-length rha1 cDNAs encoding the enzyme. In addition, we have overexpressed the rha1 cDNA in Aspergillus oryzae, and characterized the purified, recombinant RGAE (rRGAE). In a previous study we have reported the cloning of two structurally and functionally different rhamnogalacturonases, RGase A and RGase B, from A. aculeatus (11). Here we show that RGAE acts in synergy with RGase A as well as RGase B in the degradation of apple pectin rhamnogalacturonan. As a first step in dissecting the molecular mechanisms that control the regulation of rhamnogalacturonan degrading enzymes in A. aculeatus the expression of their genes is compared at mRNA level.

MATERIALS AND METHODS

Fungal Strains and Growth Conditions—Aspergillus aculeatus strain KSM 510 was cultivated as described previously (11); the mycelium was harvested after 1 to 5 days growth at 30 °C, frozen in liquid N₂, and stored at −80 °C. The amount of glucose remaining in the culture filtrates was determined by using the d-glucose kit according to the manufacturer’s instructions (Boehringer Mannheim, Mannheim, Germany). The A. oryzae A 1560 (13) transformants were grown in YP medium containing (per liter) 35 g of maltodextrin.

Purification of Native Rhamnogalacturonan Acetylesterase from A. aculeatus—A 100-ml aliquot of A. aculeatus supernatant was ultrafiltered in a 200-ml Amicon cell with a 10-kDa Diaflo YM 10 membrane (Amicon). The retentate was diluted 10 times in 0.02 M Tris-HCl, pH 7.0, and applied onto a 250-ml DEAE-Sepharose column (5.0 × 15 cm, Pharmacia, Sweden) in 0.02 M Tris-HCl, pH 7.0, at 4 ml/min. Bound...
proteins were eluted with a linear gradient from 0 to 0.1 M NaCl over 0.25 column volumes and from 0.1 to 0.4 M NaCl over 3.2 column volumes; 10-ml fractions were collected and assayed for activity on para-nitrophenyl (PNP)-acetate and for RGAE activity as described under "Enzyme Characterization." One unit of PNP-acetylesterase activity is defined as 1 μmol of para-nitrophenol released per min. Fractions in which no RGAE activity was detected and pooled, (NH₄)₂SO₄ was added to a 2 M final concentration, and the sample was applied to a 60-ml phenyl-Sepharose column (2.6 × 11 cm, Pharmacia) in 0.05 M Tris-HCl, pH 6.5, 2 mM (NH₄)₂SO₄ at a flow rate of 2 ml/min. Bound proteins were eluted by lowering the concentration of (NH₄)₂SO₄ stepwise from 2 to 1 to 0.5 to 0 M. Each step was eluted in approximately 1 column volume. The enzyme containing fractions were pooled. The pooled fractions were then condensed with ammonium acetate and purity of secreted proteins was evaluated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The RGAE was purified from the culture supernant of an A. oryzae transformant producing RGAE. A 50-ml aliquot of the supernatant was ultrafiltrated into 0.025 M Tris-HCl, pH 8.0, applied on a 50-ml fast Q-Sepharose anion exchange column (Pharmacia), and bound proteins were eluted with a linear gradient from 0 to 0.15 M NaCl over 0.2 column volumes and from 0.15 to 0.5 M NaCl over 12 column volumes. The enzyme was eluted in electrophoretically pure form.

Electrophoresis and Western Blotting—SDS-PAGE was performed according to Laemmli (26), with minor modifications as described (11, 27). Isoelectric focusing was carried out in Ampholine PAG plates, pH 3.5–9.5 (Pharmacia), on a Multiphor electrophoresis unit according to the manufacturer’s instructions (Pharmacia). Goldview (Dionex) was used to detect PNP-acetylesterase activity. SDS-PAGE and Western blotting were performed as described (11). Western blotting was performed with anti-β-tubulin antibodies given as a gift by Dr. R. A. Davis.

"Enzyme Characterization." One unit of PNP-acetylesterase activity is defined as 1 μmol of para-nitrophenol released per min. Fractions containing enzyme activity were pooled. Protein concentrations were determined as described (28). Isoelectric focusing was carried out in Ampholine PAG plates, pH 3.5–9.5 (Pharmacia), on a Multiphor electrophoresis unit according to the manufacturer’s instructions (Pharmacia). Goldview (Dionex) was used to detect PNP-acetylesterase activity. SDS-PAGE and Western blotting were performed as described (11). Western blotting was performed with anti-β-tubulin antibodies given as a gift by Dr. R. A. Davis.

The pH optimum of rRGAE was determined by incubation of 1% (w/v) substrate with 0.01 M Tris-HCl, pH 8.0, 100 μg of enzyme for 15 min at 30°C. The substrate concentration (S) ranged from 0.1 to 1.5% and 43 μg of rRGAE was added to 1.1 ml of substrate. The reaction velocity (V) was calculated and 1 unit is defined as 1 μmol of acetate released per minute.
then \( V/S \) was depicted as a function of \( S \), and \( K_m \) and \( V_{\text{max}} \) were determined by linear regression analysis. The specific activity was calculated as \( V_{\text{max}}/E \), where \( E \) is the amount of enzyme added. To determine the substrate specificity, RGAE and rRGAE were incubated with acetylated xylan and acetylated mannan (kindly provided by Dr. Jürgen Puls) followed by determination of acetate as described.

Degradation of MHR by rRGAE and rRGases—MHR were saponified according to Kofod et al. (11) and 1 ml of 1% MHR or MHR-S in 0.05M sodium acetate buffer was incubated at 30°C in thermomixers with rRGAE (43 μg), rRGase A (10 μg), rRGase B (10 μg), or combinations of rRGAE and rRGases at pH 3.5 and 6.0 for RGase A and RGase B, respectively (11). After 24 h of incubation the enzymes were heat-inactivated at 95°C for 20 min. The degradation products were analyzed by high performance size exclusion chromatography (HPSEC), which included separation on three TSK-gel columns (G4000PWXL, G3000PWXL, and G2500PWXL, TosoHaas, Japan) connected in a row and detection of eluting saccharides by a RID6A refractive index detector (Shimadzu, Kyoto, Japan) (11). The amount of acetic acid released from MHR by saponification or by the prolonged action of rRGAE in citrate/phosphate buffer was determined using the acetic acid kit (Boehringer Mannheim).

RESULTS

Purification of Rhamnogalacturonan Acetylesterase—PNP-acetate was used as substrate in monitoring the purification of acetylesterases, including RGAE, from the culture supernatant of A. aculeatus. An aliquot of the supernatant containing 10 g of protein and 6320 units of PNP-acetylesterase activity was purified on a DEAE-Sepharose anion exchange column. Two peaks active on PNP-acetate were eluted from the column. The last peak contained the RGAE activity, 680 units of PNP-acetylesterase activity, and 432 mg of protein. When the sample was further purified by hydrophobic interaction chromatography, three distinct peaks of PNP-acetate hydrolyzing activity were eluted. The first peak contained 560 units of PNP-acetylesterase activity, the RGAE activity and 16.5 mg of protein. The RGAE was further purified to electrophoretic homogeneity by gel filtration and anion exchange chromatography, resulting in 3 mg of RGAE and 19 units of PNP-acetylesterase activity.

The enzyme was subjected to direct NH2-terminal sequencing (Fig. 1).

Primary Structure of the rha1-encoded Rhamnogalacturonan Acetylesterase from A. aculeatus—To obtain a cDNA probe for rha1, an oligonucleotide corresponding to a part of the NH2-terminal sequence (amino acids 25–33, Fig. 1) was designed. The primer was used pairwise with the forward and reverse pYES primers to amplify the target cDNA from a library pool of approximately 5000 colonies employing the PCR technique (28). Analysis of the PCR products revealed a 0.8-kb product with one primer pair. Sequencing of the subcloned PCR fragment revealed a unique 826-bp cDNA with an open reading frame of 226 amino acids (nucleotide positions 111–936, Fig. 1). In addition to the primer-encoded residues, 12 amino acids coincurred with the NH2 terminus of the purified RGAE, confirming that the PCR had specifically amplified the desired region of the rha1 cDNA. Approximately 10,000 colonies from the A. aculeatus cDNA library in Escherichia coli were screened by colony hybridization using the rha1-specific PCR product as a probe. This yielded 20 positive clones which were further analyzed by sequencing the ends of the cDNAs and determining the nucleotide sequence of the longest cDNA from both strands with synthetic oligonucleotide primers.

The 936-bp cDNA clone pC1RGAE1 contains a 750-bp open reading frame initiating at nucleotide position 39 and terminating with a TGA stop codon at nucleotide position 789, thus predicting a 250-residue polypeptide of 26,350 Da (Fig. 1). The open reading frame is preceded by a 38-bp 5'-noncoding region and followed by a 116-bp 3'-noncoding region and a poly(A) tail. The deduced primary structure of RGAE matches the NH2-terminal sequence determined from the purified enzyme (Fig. 1). In addition, the rha1 cDNA encodes an apparent signal peptide of 17 amino acids (29), revealed by comparison with the mature RGAE and 19 units of PNP-acetylesterase activity. The enzyme was subjected to direct NH2-terminal sequencing (Fig. 1).
search of the Swiss-Prot protein sequence data base with the deduced RGAE amino acid sequence revealed no significant similarities, implying that RGAE from *A. aculeatus* is a novel enzyme representing a new family of esterases.

The copy number of the rha1 gene in the *A. aculeatus* genome was determined by Southern blot hybridization. Total DNA isolated from *A. aculeatus* was digested to completion with BamHI, BglII, EcoRI, or HindIII and hybridized with the rha1 cDNA. The rha1 probe detects only single strongly hybridizing fragments in each digest, indicating that the rha1 gene is present as a single copy in the *A. aculeatus* genome (Fig. 2).

Regulation of rha1, rhgA, and rhgB Transcript Levels in *A. aculeatus*—To study the effect of glucose on the expression of rhamnogalacturonan degrading enzymes, *A. aculeatus* was cultivated in a medium containing both glucose and soybean meal as carbon sources (11), and the culture supernatants were assayed for glucose concentration after 1–5 days of growth. During the first 3 days, the concentration of glucose in the culture filtrates decreased from 178 to 0.14 mg/mL, while no glucose was detected in the 4- and 5-day-old cultures, respectively. Furthermore, *A. aculeatus* produced RGAE and total RGase activity (11) in culture only after glucose had been expended from the medium.

The rha1 cDNA probe hybridized readily to a single 0.9-kb mRNA species in the 5-day-old mycelium, while the rha1 message did not accumulate to detectable levels in the fungus when glucose was present in the growth medium (Fig. 3). This is consistent with the RGAE activity observed in the culture filtrate from day 5. Reprobing the same filter with the rhgA and rhgB cDNAs encoding RGase A and RGase B (11), respectively, revealed a similar expression pattern for the 1.6-kb rhgA and 1.8-kb rhgB mRNAs, except that the message levels appeared to be lower compared with rha1 (Fig. 3). In contrast, the 1.5-kb benA encoded β-tubulin mRNA accumulated to comparable levels in both samples irrespective of the presence of glucose in the medium (Fig. 3).

Heterologous Expression of the rha1 cDNA in *A. oryzae*—To obtain high level production of RGAE in *A. oryzae*, the rha1 cDNA was subcloned behind the *A. oryzae* α-amylase promoter, and the resulting construct was introduced into the *A. oryzae* wild type strain A 1560 by co-transformation with the amdS plasmid as described (13). Ten transformants were purified twice through conidial spores and screened for RGAE secretion by the activity assay and SDS-PAGE. The highest yielding transformant was grown in 1-liter fermentor, and the culture supernatant was harvested after 5 days of growth for purification of rRGAE by anion exchange chromatography.

Molecular Characterization of Rhamnogalacturonan Acetylesterase—The molecular masses were determined by SDS-PAGE to 32–34 kDa for native RGAE and to 32–35 kDa for rRGAE (Fig. 4). The discrepancy of 8–11 kDa compared with the calculated molecular weight of 24,605 implies that a substantial amount of glycan structures is attached to the enzyme. The validity of this conclusion was assessed by mass spectrometry of RGAE and rRGAE. As shown in Fig. 5, both native and recombinant RGAE are glycosylated as the determined molecular masses exceed the predicted molecular weight of the rha1-encoded polypeptide. The masses of native RGAE range from 25.5 to 28 kDa, with an average of 26.7 kDa, while the average mass for rRGAE is 28.3 kDa, ranging from 25 to 31 kDa, suggesting that the glycosylation of the recombinant enzyme is slightly different from that of the native RGAE. In addition, the glycosylation of both enzymes is clearly heterogeneous. The recombinant, but not native, RGAE binds Galanthus nivalis lectin specific for a terminal mannose residue in glycan moieties (Fig. 4). A similar pattern was observed with peanut lectin specific for Galβ1–3GalNAc in some N-linked glycans, supporting the notion that the glycans attached to the enzymes are different. The polyclonal anti-RGAE antiserum recognized a number of polypeptides in the *A. aculeatus* supernatant, suggesting that they contain identical or similar antigenic epitopes, such as glycan structures. This is supported by the fact that both G. nivalis and peanut lectins reacted with several extracellular proteins from *A. aculeatus* (Figs. 4, C and D). The differences seen in the amount of mannose (6 pmol/pmol of RGAE, 11 pmol/pmol of rRGAE) and galactose (0 pmol/pmol of RGAE, 1 pmol/pmol of rRGAE) in the glycan moieties of RGAE and rRGAE confirmed that the two enzymes are differentially glycosylated. Furthermore, the presence of glucosamine (2 pmol/pmol of protein) in both RGAE and rRGAE indicates that at least some of the carbohydrate is N-linked, in accordance with two potential N-glycosylation sites in the primary structure of RGAE (Fig. 1).

Characterization of Enzymatic Properties—The enzyme characteristics of the purified rRGAE are shown in Table I. The temperature optimum of 40 °C, the pH optimum of 6.0, and the
specific activity of about 1 unit/mg are consistent with the results obtained by Searle-van Leeuwen et al. (12). The rRGAE released about 60% of the total amount of saponifiable acetyl esters (Table I) present in the substrate. This is slightly lower than the previously reported 70% (12) and could be a result of a lower degree of acetylation in the MHR preparation used in this study (results not shown). Although the pH optimum indicates that the rate of hydrolysis is slower under acidic conditions, it was shown that upon prolonged incubation rRGAE hydrolyses acetyl groups from MHR to the same extent at pH 3.5 as at pH 6.0 (Table I). Neither RGAE nor rRGAE released acetate from acetylated xylan or mannan, in accordance with previous reports (12).

Synergistic Degradation of MHR by rRGAE and rRGases—The HPSEC analysis of MHR incubated with rRGAE showed that the enzyme had no inherent cleaving activity within the backbone of MHR (Fig. 6). MHR incubated with rRGase A or rRGase B alone was only slightly depolymerized, whereas the combination of rRGase A or rRGase B with rRGAE resulted in extensive depolymerization of the substrate (Fig. 6). The concerted action of rRGAE and rRGases on the MHR substrate was comparable with the degradation of saponified MHR by rRGases alone (Fig. 6).

**DISCUSSION**

Several independent lines of evidence indicate that the cDNA clone pC1RGAE1 encodes the rhamnogalacturonan acetylesterase from *A. aculeatus*. First, the deduced primary structure of the cDNA encoded protein contains an amino acid sequence with 100% identity to the NH2-terminal sequence determined from the purified, native RGAE from *A. aculeatus*. Second, heterologous expression of the rha1 cDNA in *A. oryzae*, a filamentous fungus that does not possess a rhamnogalacturonan acetylesterase activity, resulted in a RGAE activity in the culture supernatant, similar to that found in *A. aculeatus*. Both recombinant and native RGAE were shown to be active on apple pectin rhamnogalacturonan and PNP-acetate, but not on acetylated mannan or xylan. Furthermore, the enzyme characteristics of the purified rRGAE correspond well with the values reported previously for the native RGAE from *A. aculeatus* (12). Third, a polyclonal rabbit antiseraum raised against authentic RGAE. The molecular ions carrying one, two, and three positive charges are indicated as (M + H)+, (M + H)2+, and (M + H)3+.

**TABLE I**

| Characteristic                          | Values          |
|-----------------------------------------|-----------------|
| pH optimum                              | 6.0             |
| Temperature optimum                     | 40 °C           |
| Specific activity, pH 6.0, 30 °C        | 0.3–1.0% MHR    |
| Acetate concentration in a 1% solution  | 0.8–1.3 units/mg|
| saponification                          | 8.5 mm          |
| RGAE, pH 3.5, 24 h                      | 4.9 mm          |
| RGAE, pH 6.0, 24 h                      | 4.9 mm          |
olite repression of the polygalacturonase expression by glucose has been reported to operate at the transcriptional level (30, 33, 34). The rha1 mRNA accumulates in substantial amounts in A. aculeatus grown on the mixed carbon source after glucose has been depleted from the medium coinciding with the extracellular RGAE activity, whereas the rha1 message is absent in the fungus grown on glucose-containing medium. The comparable expression patterns observed for the rhgA and rhgB mRNAs imply that they are coordinately regulated. Furthermore, the message levels correlate well with the high total RGase activity detected in the 5-day-old culture of A. aculeatus (11). Taken together, these results indicate that the expression of rhamnogalacturonan degrading enzymes by A. aculeatus is primarily regulated at the level of transcription and is subjected to carbon catabolite repression by glucose.

The heterogeneous size in SDS-PAGE together with the difference of 8–11 kDa in apparent mobility and molecular mass compared with the predicted molecular weight of RGAE imply that the enzyme is modified by glycosylation. By comparison, mass spectrometric analyses resulted in average molecular masses of 26.7 and 28.3 kDa for RGAE and rRGAE, respectively. While the values are significantly lower than those determined by SDS-PAGE, they are consistent with substantial glycosylation of the predicted 24,605-Da RGAE polypeptide. The deduced amino acid sequence of RGAE contains two potential sites for N-linked glycosylation, in good agreement with the presence of glucosamine in both RGAE and rRGAE. Yet, this is probably not sufficient to explain the discrepancy between the observed and calculated molecular weights, suggesting that additional, O-linked glycans could be attached to the enzyme. In addition, the observed differences in the molecular masses, lectin binding patterns, and monosaccharide compositions indicate that the glycan moieties on the native and recombinant RGAE are structurally different.

The HPSEC chromatograms showed only limited depolymerization of unsaponified MHR when the rRGases were used alone (Fig. 6), although the degradation of MHR by rRGase A was more pronounced than that obtained by Schols et al. (35). In contrast, the combination of rRGAE with rRGase A and rRGase B revealed a marked synergism between the enzymes, resulting in extensive breakdown of the MHR substrate. Although only 60% of the saponifiable acetyl esters in MHR were hydrolyzed by rRGAE, it enhanced depolymerization of MHR almost to the level seen with MHR-S, implying that acetyl esters are a major hindrance for the action of RGases on harnogalacturonan. This is in accordance with previous reports for RGase A (10, 11) and is here shown to be valid for RGase B as well. The remaining acetyl esters seem to inhibit the rRGases mainly in MHR fragments of molecular weight exceeding 30,000 (Fig. 6), indicating that RGAE is sterically hindered by the side chains in heavily substituted regions of the rhamnogalacturonan backbone.

An expanding inventory of purified microbial pectinolytic enzymes, active within the smooth regions of pectin, along with a number of cloned enzymes, has significantly improved our understanding of the structure and degradation of plant cell wall pectin (5, 31, 36–39). In contrast, the enzymatic breakdown of the rhamnogalacturonan backbone in pectic hairy regions has received only limited attention. The recently reported rhamnogalacturonan α-L-rhamnopyranohydrolase from A. aculeatus (40) together with the previously described RGase A and RGase B (10, 11), and the rhamnogalacturonan acetyleraser (12) cloned and characterized in this study, imply that analogous to the degradation of the smooth regions, a family of rhamnogalacturonan degrading enzymes can be found in nature. Furthermore, the data presented here demonstrate that the deduced primary structure of RGAE is unique, representing a novel family of esterases and that the enzyme acts in synergy together with RGase B as well as RGase A in the degradation of plant cell wall rhamnogalacturonan.

Acknowledgments—We thank Maria Holm, Jan Juel de Jong, Margit T. Kjaer, Ina Nørgaard, Marcel Mischler, and Susanne Jacobsen for skillful technical assistance. We also thank Dr. M. T. Hansen for the bamA clone.

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FIG. 6. Synergistic degradation of MHR by RGAE and RGases. The incubations of MHR and MHR-S were performed for 24 h in acetate buffers at optimal pH for RGase A (A) and RGase B (B), and the degradation products were analyzed by HPSEC. The estimated molecular weight (Mw) and degree of polymerization (DP) are indicated on the horizontal axis.
