Characterization of *Aspergillus niger* Pectate Lyase A[†,‡]

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ABSTRACT: The *Aspergillus niger ply*A gene encoding pectate lyase A (EC 4.2.99.3) was cloned from a chromosomal λ_{EMBL4} library using the *Aspergillus nidulans* pectate lyase encoding gene [Dean, R. A., and Timberlake, W. E. (1989) *Plant Cell 1*, 275−284] as a probe. The *ply*A gene was overexpressed using a promoter fusion with the *A. niger* pyruvate kinase promoter. Purification of the recombinant pectate lyase A resulted in the identification of two enzyme forms of which one appeared to be *N*-glycosylated and the other appeared to be free of *N*-glycosylation. The two enzyme forms showed identical specific activities. The *N*-glycosylation free pectate lyase A was further characterized with respect to product formation on polygalacturonic acid (α-1,4 linked D-galacturonic acid) and mode of action on oligogalacturonides of degree of polymerization 2−8. The bond cleavage frequencies for tetra-, penta-, and hexagalacturonides were studied as a function of [$CaCl_2$]. The bond cleavage frequencies changed in a [$CaCl_2$]-dependent way for penta- and hexagalacturonide. Kinetic studies using tetra- and hexagalacturonide revealed a strong sigmoidal [$CaCl_2$]-dependent relation. The role of Ca^{2+} ions in substrate binding is discussed.

Pectin is one of the major constituents of the primary cell wall in higher plants. It consists of smooth regions of α1,4 linked D-galacturonic acid (Gal*p*A)[1] interspersed with stretches in which α1,2 L-rhamnose alternates with Gal*p*A (*1*). Side chains of arabinan or galactan can be attached to the rhamnose residues (*2*). In naturally occurring pectin, generally 70% of the Gal*p*A residues are methylesterified (*3*).

For the complete decomposition of pectin, saprophytic and phytopathogenic microorganisms have a vast repertoir of pectinolytic enzymes at their disposal (*4*, *5*). These enzymes include those with side chain degrading activities such as arabinanases, galactanases, β-galactosidases, and arabinofuranosidases, methyl- and acetyl esterases, and main-chain depolymerases. The latter group of enzymes consists of two major classes of enzymes: the lyases and the hydrolases. In each of these classes, enzymes specific for the smooth regions, viz., pectate and pectin lyases and polygalacturonases, reviewed in ref *6*, and specific for the hairy regions, viz., rhamnogalacturonan lyase (*7*) and rhamnogalacturonases (*8*), are found. Except for the rhamnogalacturonan lyases, three-dimensional structures are known for all pectin depolymerizing enzymes (*9*−*15*). Despite their different activities, all enzymes share the same unique overall structure, a parallel β helix. The substrate binding cleft, consisting of multiple subsites which accommodate the individual building blocks of the substrate, is formed by loops protruding from the β helical core.

Among the pectin depolymerizing enzymes, the pectate lyases, which act on de-esterified pectin, hold a unique position due to their absolute requirement for Ca^{2+} ions for catalysis (*16*, *17*). A Ca^{2+} ion, coordinated by three Asp residues, has been observed in the three-dimensional structure of *Bacillus subtilis* pectate lyase (*11*). Ca^{2+} ions are also known to complex pectate or polygalacturonic acid (*18*). From a study addressing the role of Ca^{2+} ions in catalysis of *Cephalosporium sp*. pectate lyase (*17*) it was concluded, based on the kinetic data obtained for the pectate lyase acting on (Gal*p*A)$_4$ in the presence of various [$CaCl_2$], that the true substrate was the (Gal*p*A)$_4$−2Ca^{2+} complex and that Ca^{2+} might serve as an activator of the enzyme (*17*).

In this paper, we describe a detailed characterization of the *Aspergillus niger* pectate lyase A. On the basis of the influence of the [$CaCl_2$] on the bond cleavage frequencies for (Gal*p*A)$_5$ and (Gal*p*A)$_6$ and the sigmoidal relationship between the [$CaCl_2$] and the apparent turnover rates of (Gal*p*A)$_4$ and (Gal*p*A)$_6$ the role of Ca^{2+} is discussed.

EXPERIMENTAL PROCEDURES

Strains and Media. *Aspergillus niger* NW188 (*csp*A1, *pyr*A1, *leu*A6, *prt*F28, *gox*C) a derivative of *A. niger* N400 (CBS 120.49) was used for transformation and was grown in minimal medium (*19*), pH 6.0, containing 4% (mass/vol) fructose, 0.1% (mass/vol) yeast extract, 0.02% (mass/vol) leucine, 0.02% (mass/vol) uridine, and a trace element solution according to Vishniac (*20*). *Escherichia coli* DH5αF′-[F′/*end*A1 *hsdR*17 (r_K-m_K+) *supE*44 *thi*-1 *rec*A1 *gyrA* (Nal^r) *rel*A1 Δ(*lacIZYA-argF)U169 deoR* (*ϕ80dlac*Δ*(1acZ)M15*] (*21*) was used for cloning throughout. λ phages were

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[‡] The nucleotide sequence reported for the pectate lyase A gene from *Aspergillus niger* has been deposited in the GenBank database under Genbank Accession Number AJ276331.

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[1] Abbreviations: Gal*p*A, pyranose form of galacturonic acid; (Gal*p*A)$_n$, Gal*p*A with degree of polymerization *n*.

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propagated in *E. coli* LE392 [F` e14$^-$ (*Mcr*A$^-$) *hsdR*514 (r_K-m_K+) *supE*44 *supF*58 Δ(*lac*IZY)6 *galK*2 *galT*22 *metB*1 *trpR*55] (*22*). *E. coli* DH5α was grown in Luria-Bertani medium supplemented with 80 μg/mL ampicillin. *E. coli* LE392 was grown in NZYCM medium. Media were solidified using 1.5% (mass/vol) bactoagar. Top agar was prepared using NZYCM medium solidified with 0.7% agarose (electrophoresis grade).

Manipulation of DNA and RNA. Isolation of phage and plasmid DNA and other molecular manipulations were carried out essentially as described (*22*). Total RNA was isolated by disrupting frozen mycelium in liquid nitrogen using a micro dismembrator (Braun Melsungen AG, Melsungen, FRG). The mycelium powder was extracted with TRIzol Reagent according to the supplier (GibcoBRL, Life Technologies Inc., Gaithersburg, MD). Restriction enzymes were used as recommended by the supplier (GibcoBRL).

For construction of the *pki-ply*A promoter fusion, two primers were designed: primer 1, a 33-residue-long oligonucleotide 5′GTTACAATGCATAACTTCAAGTGGATCGTTGCC 3′ located in position −6 to 27 of the *ply*A gene which allows introduction of a *Nsi*I site at the start codon, and primer 2, a 17-residue-long oligonucleotide 5′CAGCAAGGACCTTGGAG 3′ that anneals at position 492 to 475, downstream of the *Xho*I site at position 366. The PCR-generated fragment was checked for undesired mutations. This fragment was then digested with *Nsi*I and *Xho*I and ligated together with the 1235 bp *Xho*I/*Nsi*I fragment containing the remainder of the gene into *Nsi*I-digested plasmid pPROMH, which contains the *pki*A promoter (*23*), yielding plasmid pIM3642.

cDNA was generated essentially according to Gilliland et al. (*24*). In the first step, a reverse transcriptase reaction using the reverse transcriptase MoMuLV and reaction buffers as described by the supplier (GibcoBRL) was performed on 5 μg of total RNA isolated from an *A. niger pki-ply*A transformant (752.1−235) using a specific 3′ end primer. This primer 3 is a 19-residue long oligonucleotide 5′AGCCTTCTTGGAGTCGACG 3′ which anneals in the 3′ coding region (position 1005 to 987 of the coding region). In the second step, the reverse transcriptase reaction was used for PCR employing the second, 5′-end-specific primer (primer 1).

Nucleotide sequences were determined using either the Cy5 AutoCycle Sequencing Kit (Pharmacia Biotech., Uppsala, Sweden) or the Cy5-dATP Labeling Mix (Pharmacia Biotech). The reactions were analyzed with an ALFexpress DNA Sequencer. Computer analysis was done using the program DNASTAR (DNASTAR Inc., Madison, WI). Calculations of molecular mass and iso-electric point were performed using the program Gene Runner from Hastings Software, Hastings, NY.

Screening of the Genomic Library. A ^{32}P-dATP labeled (*25*) 1.1 kb *Eco*R1 fragment from plasmid pRD091, containing the *A. nidulans* pectate lyase gene (*26*), was used as a probe. Plasmid pRD091 was kindly provided by Dr. R. Dean. The probe was used to screen approximately 1×10^5 plaque forming units from an *A. niger* N400 library in λ_{EMBL4} (*27*). Hybridization was carried out at 56 °C for 18 h in 6 × SSC, 5 × Denhardt's solution, 0.5% (mass/vol) SDS. Two subsequent washing steps were carried out for 30 min at 56 °C in 4 × SSC, 0.5% (mass/vol). For secondary screening, identical conditions were used with the exception that phage dilutions yielding 200−300 plaques/plate were used, allowing the selection of individual plaques. Phages were propagated in *E. coli* LE392, and phage DNA was isolated as described (*22*).

Transformation of A. niger and Analysis of Pectate Lyase A Production. The *A. niger* strain NW188 was used for cotransformation with 1 μg of plasmid pGW635, containing the *pyr*A gene (*28*) and 20 μg of plasmid pIM3642 as described previously (*29*). In short, protoplasts were prepared from freshly grown mycelium using NovoZyme 234 (Novo Nordisk, Denmark) in isotonic buffer (STC: 1.33 M sorbitol, 10 mM Tris/HCl, pH 7.5, 50 mM $CaCl_2$). To 10^6 STC washed protoplasts in STC (200 μL), transforming DNA was added. DNA uptake occurred by the addition of 50 μL of poly(ethylene glycol) 6000 in 10 mM Tris/HCl, pH 7.5, 50 mM $CaCl_2$, and subsequent incubation on ice for 20 min. After addition of 2 mL of poly(ethylene glycol) 6000 in 10 mM Tris/HCl, pH 7.5, 50 mM $CaCl_2$ solution, the mixture was left at room temperature for 5 min, 4 mL of STC was added, and aliquots plated in topagar on selective medium [minimal medium with 0.95 M sucrose and 0.02% (mass/vol) leucine]. Individual transformants were selected and grown in 50 mL cultures of minimal medium (see above) without uridine added, and the culture fluid was analyzed for pectate lyase A expression by a standard assay and by SDS−PAGE.

*Production and Purification of Pectate Lyase A. pki-ply*A transformant 752.1−235, which produced the highest amount of pectate lyase A (see Results), was used for large-scale production and purification of the enzyme as described previously for endopolygalacturonase E (*23*). In short, mycelia were separated from the culture medium by filtration and following adjustment of the growth medium to pH 6.0, and proteins were absorbed batchwise to DEAE-sephadex A50 and pulse-eluted using 1.0 M NaCl. Following extensive dialysis against 20 mM piperazine/HCl, pH 6.0, proteins were loaded onto Source Q preequilibrated with 20 mM piperazine/HCl, pH 6.0, and eluted with a linear gradient of 0 to 0.5 M NaCl in 20 mM piperazine/HCl, pH 6.0. The two pectate lyase activity peaks were partially overlapping, and therefore, the enzymes were rechromatographed under identical conditions. Finally, the two pectate lyase pools were dialyzed against 20 mM piperazine/HCl, pH 6.0 and stored at 4 °C with 0.01% NaN_3 added as an antimicrobial agent.

Enzyme Assay and Kinetic Analysis. Pectate lyase activity was assayed spectrophotometrically by following the formation of Δ4,5 unsaturated bonds at 235 nm [$\epsilon_{235nm} = 4800$ M^{-1} cm^{-1} (*30*)] at 30 °C in 1.0 mL of 45 mM Tris/HCl, 45 mM glycine, 1.0 mM $CaCl_2$, pH 8.45, using 0.25% (mass/vol) polygalacturonic acid (United States Biochemical Corp, Cleveland, OH) as a substrate. The determination of the pH optimum for pectate lyase A was done using polygalacturonic acid as a substrate in 45 mM Tris/HCl/glycine and 50 mM 2 amino-2 methyl-1 propanol/HCl. Determination of bond cleavage frequencies was done in 0.6 mL assays using 0.50 mM oligogalacturonides with different degrees of polymerization ($n = 2-8$) at 30 °C in 20 mM Tris/HCl, 1.0 mM $CaCl_2$, pH 8.0. One-hundred microliter samples were taken at timed intervals, and reactions were stopped by lowering the pH to 3−4 by the addition of 10 μL of 1% (vol/vol) acetic acid. Analysis of the reaction products was done by

high-performance anion-exchange chromatography with pulsed amperometric detection and UV detection (see below). To assess the kinetic behavior of pectate lyase, tetra- and hexagalacturonide reaction rates were determined spectrophotometrically using a thermostated Shimadzu UV-2501PC spectrophotometer (Shimadzu Seisakusho LTD, Kyoto, Japan) interfaced to a computer. Kinetic traces were recorded, and corresponding rates, corrected for spontaneous chemical β-elimination of the substrate, were determined using the kinetics module obtained from Shimadzu. Reactions were started by the addition of 10 μL of diluted enzyme and were carried out at 30 °C in buffer, pH 8.45 (45 mM Tris/HCl, 45 mM glycine, 10 mM NaCl), in 0.6 mL of total reaction volume using various concentrations of substrate and $CaCl_2$. The enzyme was diluted in 20 mM piperazine/HCl, pH 6.0, 0.01 mM $CaCl_2$. For (Gal*p*A)$_6$, the concentrations (mM) used were: 0.05, 0.075, 0.10, 0.15, 0.25, 0.40, 0.60, 0,80, 1.0, and 1.3. For (Gal*p*A)$_4$, concentrations (mM) used were 0.05, 0.075, 0.10, 0.15, 0.25, 0.40, 0.60, 0.80, and 0.95. The $CaCl_2$ concentrations (mM) used were 0.05, 0.75, 0.10, 0.20, 0.40, 0.60, 0.80, 1.0, 2.0, 5.0, and 10.

Analytical Methods. The protein content of purified pectate lyase was estimated spectrophotometrically at 280 nm. The molar extinction coefficient, 38 950 M^{-1} cm^{-1}, was calculated by the method based on the Trp, Tyr, and Cys content (*31*) taken from the deduced primary sequence. It should be noted here that the enzyme was not unfolded in 6 M guanidinium chloride prior to absorbance determination which may result in underestimation of the actual protein content. The purity of the enzyme preparation was monitored by SDS−PAGE. The gel was stained with Coomassie brilliant blue R250. The protein molecular mass was estimated by SDS−PAGE calibrated with protein test mixture 4 (Serva, Boehringer Ingelheim, GFR).

Oligogalacturonides were isolated as described (*32*). Quantitation of the oligomers was performed with the *m*-hydroxydiphenyl colorimetric assay for uronic acids (*33*). Reaction products were analyzed on a Dionex BioLC high-performance chromatography system (Dionex Corporation, Sunnyvale, CA), using a CarboPac PA-100 anion-exchange column (25 cm × 4 mm) equilibrated with 0.1 M NaOH, 0.15 M sodium acetate, using pulsed amperometric detection with settings for potential and pulse sequence for carbohydrate detection as recommended by the manufacturer, and by UV detection at 235 nm using a Pye Unicam LC−UV detector. The samples loaded were eluted isocratically for 5 min followed by a linear gradient of 0.15 to 0.75 M sodium acetate in 0.1 M NaOH at 1 mL/min in 20 min. Unsaturated products were quantitated from the integrated signal from the UV detector using $\epsilon_{235nm} = 4800$ M^{-1} cm^{-1}.

RESULTS

Cloning and Nucleotide Sequencing of the ply*A Gene from A. niger*. Screening of the *A. niger* N400 library with the *A. nidulans* pectate lyase cDNA, as isolated from plasmid pRD091 (*26*), resulted in four individual λ-clones. From Southern analysis, it was concluded that the entire gene should be located on a 2.3 kb *Eco*R1 fragment. This fragment was subcloned into pUC18 (*34*), and its nucleotide sequence was determined over both strands by further subcloning of restriction fragments and using specific oligonucleotides. Four introns were identified by determination of the nucleotide sequence of the cDNA obtained via RT-PCR using mRNA isolated from *pki-ply*A promoter fusion transformant *A. niger* NW188 752.1−235. The putative mature pectate lyase has a calculated M_r of 32.2 kDa and a calculated pI of 4.4.

Overproduction and Purification of Pectate Lyase A from A. niger. To produce the pectate lyase A, a promoter−gene fusion was made with the pyruvate kinase promoter and the *ply*A gene, and the resulting plasmid pIM3642 was used to transform *A. niger* NW188 as described in the Experimental Procedures. This allowed the production of pectate lyase A by cultivating the transformants on media with a glycolytic carbon source which represses expression of other pectinolytic genes (*35*) as demonstrated recently for the overproduction of endopolygalacturonase E from *A. niger* (*23*).

Of the transformants obtained for the promoter-gene fusion, 20 individual colonies were grown in 50 mL cultures. After 22 h of cultivation, 30 μL culture fluid samples were taken and analyzed for production of pectate lyase in a standard assay and by SDS−PAGE (not shown). No pectate lyase activity was detected, and no bands were visible in the lane which contained a control sample from the host strain itself, *A. niger* NW188. Several transformants which produced pectate lyase activity also showed two bands, a minor band at 47 kDa and a major band at 43 kDa, which is larger than the expected size, viz., 32.2 kDa.

*A. niger pki-ply*A transformant 752.1−235 produced the highest amount of the 47 and 43 kDa bands and was used for purification of pectate lyase A. Since there was no further increase in the amount of pectate lyase produced after 22 h of growth under the conditions chosen (see Experimental Procedures), large-scale cultivation was performed for 22 h. Approximately, 35 mg of the 47 kDa band and 80 mg of the 43 kDa band were obtained from 5 L of culture fluid. Analysis of the two proteins by mass spectrometry revealed that the 43 kDa band represents non-*N*-glycosylated pectate lyase of 32.3 kDa, whereas the 47 kDa band appeared to be *N*-glycosylated pectate lyase of 33.5 kDa. The glycon was of the high mannose type ($Man_5GlcNAc_2$)*(36)*.

Characterization of Pectate Lyase A from A. niger. In a standard assay using polygalacturonic acid as a substrate the specific activity of the *N*-glycosylated and non-*N*-glycosylated pectate lyase A were 40.5 and 41.1 units mg^{-1}, respectively, demonstrating that the *N*-glycosylation does not affect catalysis. To avoid any interference due to heterogeneous *N*-glycosylation in further studies of the enzyme, the *N*-glycosylation free enzyme was used for detailed characterization.

In Figure 1, panels A and B, the pH optimum for pectate lyase using polygalacturonic acid as a substrate is shown as determined in Tris/HCl/glycine and 2 amino-2 methyl-1 propanol/HCl. The profiles for the buffers, without 100 mM NaCl added, indicate that the decrease in activity coincides with the pK_a of the respective buffers. The addition of 100 mM NaCl has only a moderate effect on the pectate lyase activity in 2 amino-2 methyl-1 propanol/HCl buffer whereas a strong effect was observed in Tris/HCl/glycine buffer. Furthermore, with 100 mM NaCl added, the pH optimum is the same in both buffers. The effect of the addition of NaCl

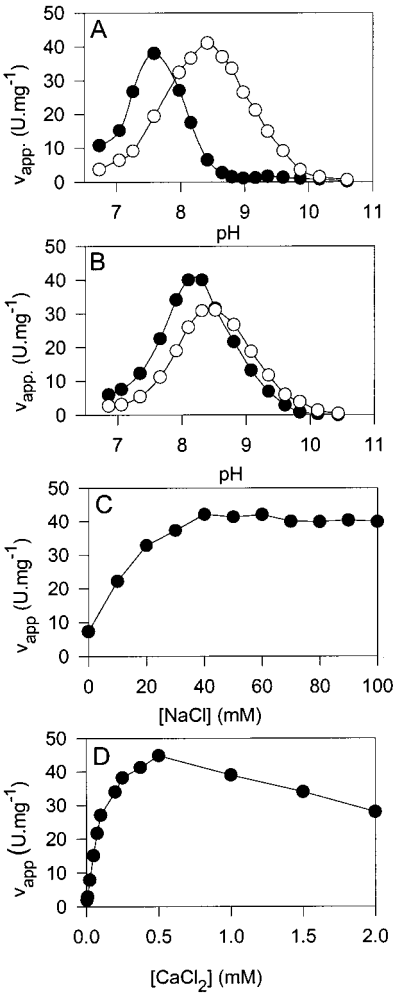

FIGURE 1: Biochemical properties of *Aspergillus niger* pectate lyase A. (A,B) The effect of 100 mM NaCl (○) on the pH optima in 50 mM Tris/HCl/glycine (●) and 50 mM 2-amino methyl propanol (●), respectively, using 0.25% (mass/vol) polygalacturonic acid as a substrate in the presence of 1.0 mM $CaCl_2$. (C) The effect of [NaCl] in Tris/HCl/glycine, 1.0 mM $CaCl_2$, pH 8.45, 0.25% (mass/vol) polygalacturonic acid. (D) The effect of [$CaCl_2$] in Tris/HCl/glycine, 50.0 mM NaCl, pH 8.45, and 0.25% (mass/vol) polygalacturonic acid.

to the Tris/HCl/glycine buffer at pH 8.45 is depicted in Figure 1C. For optimal catalysis, the addition of 50 mM NaCl is apparently sufficient. The influence of Ca^{2+} on catalysis was investigated in Tris/HCl/glycine buffer at pH 8.45, 50 mM NaCl (see Figure 1D). Optimal catalysis was found at 0.5−1.0 mM Ca^{2+}. At concentrations above 1.5 mM Ca^{2+}, gel formation of the polygalacturonic acid occurred which hampered catalysis.

Mode of Action of Pectate Lyase A from A. niger. The product distribution for pectate lyase A during polygalacturonic acid conversion appeared to be typical for an endo-acting enzyme, as initially a mixture of Δ4,5 unsaturated oligogalacturonides of chain length 2−7 was formed which was gradually converted into smaller Δ4,5 unsaturated oligogalacturonides (not shown).

The bond cleavage frequencies and turnover rates for pectate lyase A on purified oligogalacturonides are presented in Table 1. Both (Gal*p*A)$_2$ and (Gal*p*A)$_3$ were not cleaved by the pectate lyase, even after prolonged incubation. (Gal*p*A)$_4$ binds in only one productive binding mode, whereas for each Gal*p*A increase in chain length up to $n = 6$ an additional productive binding mode was observed. (Gal*p*A)$_7$ and (Gal*p*A)$_8$ were preferentially cleaved at the fifth glycosidic linkage counting from the reducing end. Increasing chain length also resulted in an elevated turnover. The turnover appeared dependent on the $CaCl_2$ concentration for (Gal*p*A)$_{4-6}$. At 10 mM $CaCl_2$, precipitation of the substrate occurred for (Gal*p*A)$_7$ and (Gal*p*A)$_8$. The $CaCl_2$ concentration not only influenced the turnover rate but for (Gal*p*A)$_5$ and (Gal*p*A)$_6$ (see Table 1), the bond cleavage frequencies were also affected. For (Gal*p*A)$_5$, a shift for the preferred bond was observed from the third to the fourth glycosidic linkage from the reducing end, and for (Gal*p*A)$_6$ the preferred bond shifted from the fourth glycosidic linkage from the reducing end to the fifth glycosidic linkage upon increasing [$CaCl_2$]. A similar analysis for (Gal*p*A)$_4$ revealed no change in bond cleavage frequency.

Effect of Ca^{2+}-Ions on Catalysis. To address the role of Ca^{2+} during catalysis of the pectate lyase, a kinetic analysis was performed. The substrates we used included (Gal*p*A)$_6$, for which the enzyme showed a shift of the preferred bond cleaved as a function of [$CaCl_2$], and (Gal*p*A)$_4$, which formed only one productive complex at various [$CaCl_2$] (see Experimental Procedures). NaCl greatly improved the activity on polygalacturonate. To determine whether NaCl should be included in the TrisHCl/glycine buffer, turnover was studied as a function of [(Gal*p*A)$_6$] in TrisHCl/glycine buffer containing 10 mM $CaCl_2$ with 0, 10, 50, and 125 mM NaCl added, respectively. Analysis of the data using a simple Michaelis−Menten equation resulted in a reasonable fit of the data and indicated that V_{max} remained constant whereas K_m increased upon increasing [NaCl] (see Table 2). For further studies 10 mM NaCl was included in the buffer.

The results of the kinetic analysis are presented in Figure 2 for (Gal*p*A)$_6$ and Figure 3 for (Gal*p*A)$_4$. Figures 2 and 3A show the apparent rates at fixed [$CaCl_2$] plotted against [(Gal*p*A)$_6$] and [(Gal*p*A)$_4$], respectively. In Figures 2 and 3B, the apparent rates at fixed [(Gal*p*A)$_6$] and [(Gal*p*A)$_4$], respectively, are plotted against [$CaCl_2$]. Panel C shows the low [$CaCl_2$] range of panel B. For clarity, not all plots for the fixed substrate concentrations are shown. Those plots not shown are qualitatively the same as those shown.

Figures 2 and 3A show that at fixed [$CaCl_2$] the plots of the dependence on [(Gal*p*A)$_6$] and [(Gal*p*A)$_4$] are not aligned as would be expected by a simple Michaelis−Menten dependence on the second substrate. This is reflected in panels B and C of Figures 2 and 3. These plots show a clear sigmoidal relationship between the apparent rate on [$CaCl_2$] at fixed [(Gal*p*A)$_6$] and [(Gal*p*A)$_4$].

DISCUSSION

In this paper, we have described the cloning, sequencing, and overexpression of the *ply*A gene encoding pectate lyase A from *A. niger*, and the purification and a kinetic characterization of the enzyme. Homologous expression of the gene under the direction of a strong glycolytic promoter results in the production of two isoforms of which one is *N*-glycosylated (*36*). Both isoforms migrate at different positions in SDS−PAGE than might be expected on the basis of their molecular mass. Similar behavior has been observed for other pectinases such as pectin lyase A and several

Table 1: Bond Cleavage Frequencies and Reaction Rates of *Asperillus niger* Pectate Lyase A Acting on Oligogalacturonides of Defined Length[a]

n	$CaCl_2$ (mM)																rate (units/mg)
2	1.0													G	–	**G**	nd
3	1.0										G	–	G	–	**G**		nd
4								G	–	G	–	G	–	**G**			
	0.1								100								0.04
	1.0								100								0.8
	10.0								100								9.1
5						G	–	G	–	G	–	G	–	**G**			
	0.1						2.4		97.6								1.2
	1.0						8.2		91.8								16.8
	10.0						17.6		82.3								106.0
6					G	–	G	–	G	–	G	–	G	–	**G**		
	0.1					10.8		60.3		29.1							6.2
	1.0					36.2		41.6		22.3							95.4
	10.0					59.0		25.6		15.4							332.1
7			G	–	G	–	G	–	G	–	G	–	G	–	**G**		
	1.0					51.7		27.0		21.3							164.3
8		–	G	–	G	–	G	–	G	–	G	–	G	–	**G**		
	1.0			20.1		47.8		32.1									153.5

[a] For conditions and analyses see Experimental Procedures. Bond cleavage frequencies are given in percentages. The reducing end of the oligogalacturonides is printed in bold. *n* = Degree of Polymerization. G = Gal*p*A. nd = not detectable

Table 2: Ionic Strength Dependence of $(Gal pA)_6$ Cleavage by *Aspergillus niger* pectate lyase A[a]

[NaCl] (mM)	K_m (mM)	V_{max} (units/mg)
0	0.29	314
10	0.29	293
50	0.56	335
125	1.16	303

[a] Conditions: 50 mM Tris/HCl/glycine, pH 8.45, 10 mM $CaCl_2$, [NaCl] as indicated. Initial rates were determined spectrophotometrically with $(Gal pA)_6$ as the varied substrate. Data were fitted to $v = V_{max}[S]/(K_m + [S])$.

endopolygalacturonases from *A. niger* (*38*, *39*), which all adopt similar 3-D structures, a parallel β-helix (*9*−*15*).

Determination of the pH optimum of the enzyme using polygalacturonate as a substrate in two different buffers, viz., Tris/HCl/glycine and 2 amino-2 methyl-1 propanol/HCl, resulted in two different pH optima. The drop in activity at the basic side in both buffers coincides with the pK_a of Tris and 2-amino-2 methyl-1 propanol, respectively. Below the pK_a, the buffers are positively charged and may act as a swamping electrolyte. Such electrolytes have been shown to liberate loosely bound Ca^{2+} from pectate and to limit interchain association to dimers of chains (*40*). The fact that by the addition of NaCl at 100 mM the pH optima merged in the two buffers strengthens the hypothesis of the swamping effect of the buffers. The positive effect of NaCl on the activity can be explained by the fact that more single pectate chains become available for the enzyme and/or more free Ca^{2+} becomes available to interact with the substrate and the enzyme (see below). Kohn (*41*) pointed out that the activity coefficient of Ca^{2+} in the presence of oligogalacturonates with degree of polymerization of <12 exactly followed the theoretical curve for solely electrostatic interactions. Powell et al. (*42*) as well as Kohn (*41*) determined that cooperative binding of Ca^{2+} to pectate, forming (soluble) aggregates of pectate chains, occurred when the degree of polymerization exceeded 25. Thus, for smaller oligogalacturonides no swamping effect is expected. The data in Table 2 show indeed that no positive effect was observed for cleavage of $(Gal pA)_6$. Rather, an adverse effect was recorded. Therefore, it is concluded that the effect of the buffers and NaCl is exerted on the substrate polygalacturonate rather than

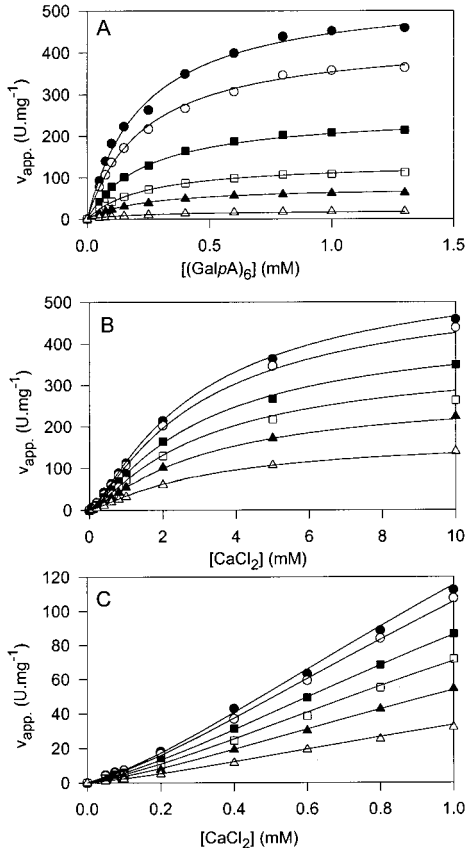

FIGURE 2: *Aspergillus niger* pectate lyase A kinetics for $(Gal pA)_6$ and $CaCl_2$. (A) $(Gal pA)_6$ as the varied substrate at fixed [$CaCl_2$]: (△) 0.2 mM; (▲) 0.6 mM; (□) 1.0 mM; (■) 2.0 mM; (○) 5.0 mM; (●) 10.0 mM. (B) $CaCl_2$ as the varied substrate at fixed [$(Gal pA)_6$]: (△) 0.075 mM; (▲) 0.15 mM; (□) 0.25 mM; (■) 0.4 mM; (○) 0.8 mM; (●) 1.3 mM. (C) Enlargement of the low [$CaCl_2$] region of panel B.

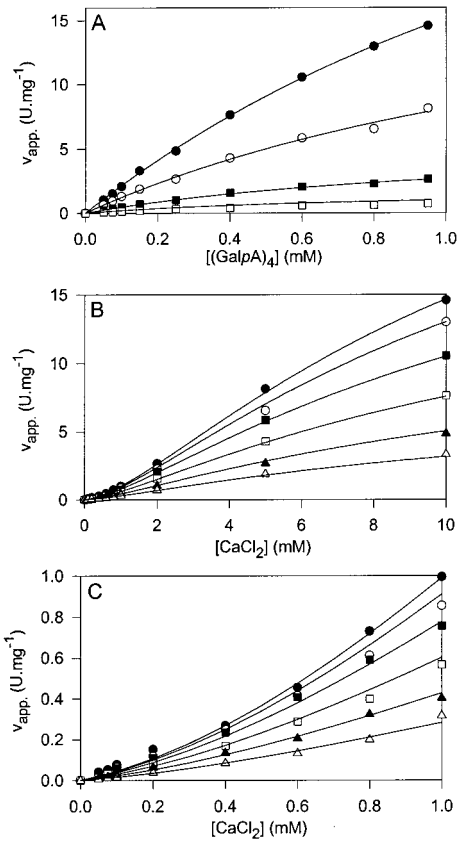

FIGURE 3: *Aspergillus niger* pectate lyase A kinetics for (Gal*p*A)$_4$ and $CaCl_2$. (A) (Gal*p*A)$_4$ as the varied substrate at fixed [$CaCl_2$]: (□) 1.0 mM; (■) 2.0 mM; (○) 5.0 mM; (●) 10.0 mM. (B) $CaCl_2$ as the varied substrate at fixed [(Gal*p*A)$_4$]: (△) 0.075 mM; (▲) 0.15 mM; (□) 0.25 mM; (■) 0.4 mM; (○) 0.8 mM; (●) 1.3 mM. (C) Enlargement of the low [$CaCl_2$] region of panel B.

on the enzyme. Polygalacturonate formed a gel in the presence of >1 mM $CaCl_2$, even with 50 mM NaCl added. The interaction/gel formation of polygalacturonate with Ca^{2+} ions may also explain the hyperbolic activity curve seen with polygalacturonate as a fixed substrate with $CaCl_2$ varied (see Figure 1D), versus the sigmoidal activity curves obtained for (Gal*p*A)$_4$ and (Gal*p*A)$_6$.

The observation that in the presence of increasing amounts of NaCl V_{max} for (Gal*p*A)$_6$ cleavage remained constant (Table 2) whereas K_m increased demonstrates that NaCl influences the substrate binding. The level at which inhibition by NaCl is excerted is most likely by interfering with the Ca^{2+} ions which are necessary for binding (see below). The fact that inhibition by NaCl was not observed using polymer substrate may reside in the much stronger binding of Ca^{2+} ions by the polymer substrate.

Atallah and Nagel (*17*) have argued that for *Cephalosporium* pectate lyase Ca^{2+} might act as an activator. Indeed, such a role for Ca^{2+} could explain the sigmoidicity of the plots as shown in Figures 2 and 3, panels B and C. Also, a Ca^{2+}-ion has been observed in the crystal structure of *B. subtilis* pectate lyase at the bottom of the substrate binding cleft. However, this Ca^{2+} is bound quite firmly (K_D = 2.3 μM)[2] and is coordinated by three acidic amino acid residues (*11*). Modeling of the *A. niger* pectate lyase A structure based on the *Erwinia chrysanthemi* pectate lyase E structure (*10*), which shares the highest sequence identity with the *A. niger* pectate lyase A of all pectate lyases for which structures are available, revealed the conservation of the three acidic residues involved in Ca^{2+} binding.[3] On the basis of this it is expected that the "activating" Ca^{2+} in the *A. niger* pectate lyase A will bind with a similar affinity as observed for the *B. subtilis* enzyme and that at the [$CaCl_2$] used for the kinetic analyses the enzyme will be completely in the activated state (thus 10 μM $CaCl_2$ was included in the enzyme dilution buffer).

Recently, the 3-D structure of the enzyme−substrate complex of *E. chrysanthemi* EC16 pectate lyase C complexed with (Gal*p*A)$_5$ has been solved (*43*). In this structure, four Gal*p*A units are clearly visible with each of the four carboxylic functions interacting with the enzyme mediated by a bridging Ca^{2+} ion. In view of this and the fact that (Gal*p*A)$_6$ binding is rate limiting (Table 2), it is concluded that the sigmoidal character of the $CaCl_2$ dependent activity profiles is a result of the binding of several Ca^{2+} ions to the enzyme−substrate complex or the binding of a substrate−Ca^{2+} complex to the enzyme. Our finding that the bond cleavage frequencies for (Gal*p*A)$_5$ and (Gal*p*A)$_6$ change as a function of [$CaCl_2$] (Table 1) does not support the binding of Ca^{2+} to an already existing enzyme−substrate complex. In such a case the enzyme−substrate complex exists in the absence of Ca^{2+} and displays the same binding modes irrespective of [Ca^{2+}]. Rather, a substrate−Ca^{2+} complex binds to the enzyme and the preferred mode depends on the number of Ca^{2+} ions bound to the substrate, the degree of polymerization of the substrate, and the actual architecture of the individual subsites.

In the 3-D structure of the *E. chrysanthemi* pectate lyase C−substrate complex the Ca^{2+} ions appear to be in positions very different from those postulated for inter-strand Ca^{2+} ions linking polygalacturonate helices (*40*, *42*, *43*). Also, the substrate has adopted a partial 3_1 and partial 2_1 helical structure which is different from the general 2_1 helical structure in solution (*40*). Assuming a similar situation holds for the *A. niger* pectate lyase A we propose that the actual rate-limiting step is the distortion of the helical structure of the substrate and the concomitant rearrangement of the Ca^{2+} ions upon binding of the substrate−Ca^{2+} complex to the enzyme.

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REFERENCES

1. O'Neill, M. A., Albersheim, P., and Darvill, A. G. (1991) *Plant Biochem. 2*, 415−441.
2. Albersheim, P., Darvil, A. G., O'Neill, M. A., Schols, H. A., and Voragen, A. G. J. (1995) in *Pectins and Pectinases, Progress in Biotechnology* (Visser, J., and Voragen, A. G. J., Eds.) pp 47−55, Elsevier, Amsterdam, The Netherlands.
3. De Vries, J. A., Hansen, M., Søderberg J., Glahn, P. E., and Pedersen, J. K. (1986) *Carbohydr. Polym. 6*, 165−176.
4. Hugouvieux-Cotte-Pattat, N., Condemine, G., Nasser, W., and Reverchon, S. (1996) *Annu. Rev. Microbiol. 50*, 213−257.
5. Benen, J. A. E., Pařenicová, L., Kester, H. C. M., and Visser, J. (1996) in *Pectins and Pectinases, Progress in Biotechnology* (Visser, J., and Voragen, A. G. J., Eds.) pp 331−346, Elsevier, Amsterdam, The Netherlands.

[2] R. Pickersgill, personal communication.
[3] J.A.E.B., unpublished material.

6. Whitaker, J. R. (1990) in *Microbial enzymes and biotechnology* (Fogarty, W. M., and Kelly, C. T., Eds.) pp 133−176, Applied Science Publishers, New York.
7. Mutter, M., Colquhoun, I. J., Schols, H. A., Beldman, G., and Voragen, A. G. J. (1996) *Plant Physiol. 110*, 73−77.
8. Suykerbuyk, M. E. G., Kester, H. C. M., Schaap, P. J., Stam, H. Musters, W., and Visser, J. (1997) *Appl. Environ. Microbiol. 63*, 2507−2515.
9. Yoder, M. D., and Jurnak, F. (1995) *Plant Physiol. 107*, 349−364.
10. Lietzke, S. E., Scavetta, R. D., Yoder, M. D., and Jurnak, F. (1996) *Plant Physiol. 111*, 73−92.
11. Pickersgill, R., Jenkins, J., Harris, G., Nasser, W., and Robert-Baudouy, J. (1994) *Nat. Struct. Biol. 1*, 717−723.
12. Mayans, O., Scott, M., Connerton, I., Gravesen, T., Benen, J., Visser, J., Pickersgill, R., and Jenkins, J. (1997) *Structure 5*, 677−689.
13. Petersen, T. N., Kauppinen, S., and Larsen, S. (1997) *Structure 5*, 533−544.
14. Vitali, J., Schick, B., Kester, H. C. M., Visser, J., and Jurnak, F. (1998) *Plant Physiol. 116*, 69−80.
15. Pickersgill, R., Smith, D., Worboys, K., and Jenkins, J. (1998) *J. Biol. Chem. 273*, 24660−24664.
16. Rombouts, F. M., and Pilnik, W. (1972) *Crit. Rev. Food Technol. 3*, 1−26.
17. Attalah, M. T., and Nagel, C. W. (1979) *J. Food Biochem. 1*, 185−206.
18. Jarvis, M. C. (1984) *Plant Cell Environ. 7*, 153−164.
19. Pontecorvo, G., Roper, J. A., Hemmons, L. J., MacDonald, K. D., and Bufton, A. W. J. (1953) *Adv. Genet. 5*, 141−238.
20. Vishniac, W., and Santer, M (1957) *Bacteriol. Rev. 21*, 195−213.
21. Woodcock, D. M., Crowther, P. J., Doherty, J., Jefferson, S., DeCruz, E., Noyer-Weidner, M., Smith, S. S., Michael, M. Z. and Graham, M. W. (1989) *Nucleic Acids Res. 17*, 3469−3478.
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Plainview, NY.
23. Pařenicová, L., Benen, J. A. E., Kester, H. C. M., and Visser, J. (1998) *Eur. J. Biochem. 251*, 72−80.
24. Gilliland, G., Perrin, S. and Bunn, H. F. (1990) in *PCR protocols: A guide to methods and applications* (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., Eds.) pp 60−69, Academic Press Inc., San Diego.
25. Feinberg, A. P., and Vogelstein, B. (1983) *Anal. Biochem. 132*, 6−13.
26. Dean, R. A., and Timberlake, W. E. (1989) *Plant Cell 1*, 275−284.
27. Harmsen, J. A. M., Kusters-van Someren. M. A., and Visser, J. (1991) *Curr. Genet. 18*, 161−166.
28. de Graaff, L. H., van den Broeck, H. C., and Visser, J. (1992) *Curr. Genet. 22*, 21−27.
29. Kusters-van Someren, M. A., Harmsen, J. A. M., Kester, H. C. M., and Visser, J. (1991) *Curr. Genet. 20*, 293−299.
30. MacMillan, G. P., and Vaughn, R. H. (1964) *Biochemistry 3*, 564−572.
31. Edelhoch, H. (1967) *Biochemistry 6*, 1948−1954.
32. Kester, H. C. M., and Visser, J. (1990) *Biotechnol. Appl. Biochem. 12*, 150−160.
33. Ahmem El Rayah, A., and Labavitch, J. M. (1977) *J. Food Biochem. 1*, 361−365.
34. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) *Gene 33*, 103−119.
35. Moldano, M. C., Strasser de Saad, M. A., and Callieri, D. (1989) *Curr. Microbiol. 18*, 303−306.
36. Colangelo, J., Licon, V., Benen, J., Visser, J., Bergmann, C., and Orlando, R. (1999) *Rapid Commun. Mass Spectrom. 13*, 2382−2387.
37. Segel, I. H. (1975) *Enzyme Kinetics*, J. Wiley & Sons Inc., New York.
38. Van Houdenhoven, F. A. E. (1975) Ph.D. Thesis, Wageningen University, Wageningen, The Netherlands.
39. Benen, J. A. E., Kester, H. C. M., and Visser, J. (1999) *Eur. J. Biochem. 259*, 577−585.
40. Morris, E. R., Powell, D. A., Gidley, M. J., and Rees, D. A. (1982) *J. Mol. Biol. 155*, 507−516.
41. Kohn, R. (1975) *Pure Appl. Chem. 42*, 371−397.
42. Powell, D. A., Morris, E. R., Gidley, M. J., and Rees, D. A. (1982) *J. Mol. Biol. 155*, 517−531.
43. Scavetta, R. D., Herron, S. R., Hotchkiss, A. T., Kita, N., Keen, N. T., Benen, J. A. E., Kester, H. C. M., Visser, J., and Jurnak. F. (1999) *Plant Cell 11*, 1081−1092.

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