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Endo-Xylogalacturonan Hydrolase, a Novel Pectinolytic Enzyme

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We screened an *Aspergillus tubingensis* expression library constructed in the yeast *Kluyveromyces lactis* for xylogalacturonan-hydrolyzing activity in microwell plates by using a bicinchoninic acid assay. This assay detects reducing carbohydrate groups when they are released from a carbohydrate by enzymatic activity. Two *K. lactis* recombinants exhibiting xylogalacturonan-hydrolyzing activity were found among the 3,400 colonies tested. The cDNA insert of these recombinants encoded a 406-amino-acid protein, designated XghA, which was encoded by a single-copy gene, *xghA*. A multiple-sequence alignment revealed that XghA was similar to both polygalacturonases (PGs) and rhamnogalacturonases. A detailed examination of conserved regions in the sequences of these enzymes revealed that XghA resembled PGs more. High-performance liquid chromatography and matrix-assisted laser desorption ionization–time of flight mass spectrometry of the products of degradation of xylogalacturonan and saponified modified hairy regions of apple pectin by XghA demonstrated that this enzyme uses an endo type of mechanism. XghA activity appeared to be specific for a xylose-substituted galacturonic acid backbone.

Pectin occurs as constituent of higher-plant cell walls, where it is embedded in the cellulose fibrils. The composition of pectin is different in different plant species and also depends on the age and maturity of the plant part. Most pectin polymers consist of smooth homogalacturonan regions and ramified hairy regions. The smooth regions consist of a linear homogalacturonan backbone. The hairy regions, as identified in apples (28), consist of three different subunits; subunit I is xylogalacturonan (xga) (a galacturonan backbone heavily substituted with xylose), subunit II is a short section of a rhamnogalacturonan backbone that has many relatively long arabinan, galactan, and/or arabinogalactan side chains (the hairs), and subunit III is rhamnogalacturonan composed of alternating rhamnose and galacturonic acid residues. Some of the rhamnose residues are substituted with single galactose residues. It is thought that subunit III connects the other two subunits. Isolated hairy regions are referred to as modified hairy regions (mhr), since the isolation procedure may alter the sugar compositions and degrees of methylation of the regions (27).

In industrial food processing (e.g., clarification of fruit juices), it is important that pectins are degraded completely (36). The smooth region (polygalacturonan) is readily degraded by pectinases, such as endo-polygalacturonase (endo-PG), endopectate lyase, endo-pectin lyase, and pectin methyl esterase. The corresponding genes have been cloned from various plants, fungi, and bacteria, including *Aspergillus niger* (5, 13, 15, 24). *A. niger* is of considerable economic importance, since it is employed to produce extracellular enzymes used in the food industry.

The hairy regions of apple pectin can be degraded by rhamnogalacturonases (RHGs), arabinases, and galactanases. Two enzymes, RHG-A and RHG-B, a hydrolase and a lyase, respectively (21, 26), which are able to split the rhamnogalacturonan backbone in mhr, have been identified and isolated from *A. niger* (32). In addition, workers have found two enzymes that degrade the rhamnogalacturonan backbone in an exo fashion; a rhamnohydrolase and a galacturonohydrolase catalyze the release of Rha and GalA residues, respectively, from rhamnogalacturonans (20, 22). Also, rhamnogalacturonan acetyl esterases hydrolyze the acetyl esters present in mhr (29). The side chains in the hairy regions are hydrolyzed by arabinases and galactanases, and the corresponding genes have also been cloned (6, 11).

Enzymic digestion of mhr with the enzymes that have been identified thus far leaves xga as an inert carbohydrate. Only one xga-degrading enzymes has been described to date; this exo-galacturonase is able to remove a Xyl-GalA disaccharide from xga (1). However, by analogy to the array of enzymes that degrade the smooth regions and subunits II and III of the hairy regions of pectin, there should be a set of enzymes that degrade xga. For example, in addition to an endo-xylogalacturonase, there could be a lyaselike enzyme which splits glycosidic linkages between methylated and xylosylated galacturonic acid residues. In addition, there could be a xylosidase which hydrolyzes the xylose residues from the galacturonic acid backbone, releasing a backbone sensitive to PG or pectate lyase activities. Such enzymes could be very valuable analytical tools for studying plant cell wall structures and for comparing xga polysaccharides in pectins from various plant sources.

The availability of sufficient amounts of purified xga from gum tragacanth (19) and the expression cloning technique (8) should allow cloning of an xga-hydrolyzing enzyme if it is present in *Aspergillus* sp. In contrast to expression cloning in *Escherichia coli* (34), the yeast *Kluyveromyces lactis* is able to secrete extracellular *Aspergillus* enzymes, and this allows screening for carbohydrolases in the supernatants of recombinant *K. lactis* clones. This allows screening in microwell plates instead of the commonly used plate assays. The bicinchoninic acid (BCA) assay (12) has been used to screen for carbohydrolase activities in microwell plates (18).

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MATERIALS AND METHODS

Construction of the expression library in *K. lactis*. The *Aspergillus tubingensis* expression library was constructed from poly(A)$^+$ RNA from mycelia cultivated for 10, 16, and 24 h in minimal medium (33) containing 0.5% soyoptim (Société Industriel Oléagineux, Saint Laurent Blangy, France). The cDNA was ligated to the expression vector pCVlacK (33). The primary library in *E. coli* consisted of 7,400 colonies (33). After amplification, the library was transferred to *K. lactis* by using the method of Faber et al. (10).

Cultivation of *K. lactis* (i) In microwell plates. Individual *K. lactis* transformants were transferred to 35 microwell plates (Nunc type 96F). They were cultivated for 2 days at 30°C in 200 μl of minimal medium I (adapted from the medium of Blondeau et al. [2]) containing mannitol as the sole carbon source and 80 ng of Geneticin G418 sulfate (Gibco BRL) per ml. Subsequently, cultures were replica plated on fresh microwell plates. Glycerol was added to the parental plates to a final concentration of 15% (vol/vol), and the plates were stored at −80°C. The replicates were incubated for 2 days at 30°C. The cells were pelleted by centrifugation at 3,000 rpm with a Hermle model zk380 centrifuge, and the supernatant was used in the BCA assay.

(ii) In Erlenmeyer flasks. *K. lactis* transformant 27E8 secreting xylogalacturonan hydrolase (XghA) was transferred from a glycerol stock preparation in a microwell plate to a reagent tube containing 2 ml of medium I supplemented with 80 μg of Geneticin G418 sulfate per ml. Wild-type *K. lactis* CBS2359 was cultivated in a tube containing 2 ml of medium. Both tubes were incubated for 2 days at 30°C, and the cultures were then used to inoculate 500-ml portions of the corresponding media in 1-liter Erlenmeyer flasks. After 2 days of incubation, the cells were pelleted, and each supernatant was dialyzed extensively against 50 mM sodium acetate buffer (pH 5.0) and then used for enzyme studies.

Microwell plate screening. The substrate xga, which was isolated from gum tragacanth (18), consisted of 2% rhamnose, 1% arabinose, 23% xylose, 5% galactose, 3% glucose, and 66% galacturonic acid (molar percentages). The composition was determined by using a Carlo Erba model 4200 gas-liquid chromatography system and a J&W DB225 column after hydrolysis with 2 M trifluoroacetic acid (1 h, 121°C) and conversion of the monomers to alditol acetates (9).

After incubation of the *K. lactis* expression library in microwell plates, 25 μl of supernatant was pipetted into a new microwell plate. Next, 25 μl of a 0.2% xga solution in 100 mM sodium acetate buffer (pH 5.0) was added. After incubation overnight at 30°C, the increase in the reducing carbohydrate content was measured by using a modified BCA assay (18). Ten microliters of the enzyme incubation preparation was mixed with 90 μl of water and 100 μl of BCA reagent in a microwell plate. The BCA reagent was freshly prepared by mixing stock solutions A and B (1:1, vol/vol). Solution A contained (per liter of distilled water) 54.3 g of Na_2CO_3, 24.2 g of $NaHCO_3$, and 1.9 g of Na_2BCA. Solution B contained (per liter of distilled water) 1.25 g of $CuSO_4 \cdot 5H_2O$ and 1.26 g of L-lysine.

Following incubation for 1 h at 80°C in a water bath, the plate was cooled on ice, and the absorbance at 550 nm was determined with a microwell plate reader. Transformants that produced absorbance which was 0.1 U higher than the absorbance of the blank were retested.

General cloning and sequencing techniques. Standard methods were used for DNA manipulation and Southern blotting (25). The blots were hybridized by using a digoxigenin-labelled probe and chemiluminescence detection as recommended by the manufacturer (Boehringer Mannheim). The pCVlacK expression plasmid from *K. lactis* was isolated by using the glass bead method of Sobanski and Dickinson (30). Sequencing was performed with a *Taq* DYE primer cycle sequencing kit (Applied Biosystems). The nucleotide sequences of both strands were determined. Sequence analysis and multiple alignment were performed by using the Wisconsin Package, version 9.0 (Genetics Computer Group, Madison, Wis.).

HPLC analysis of xga and mhr-s degradation. Dialyzed culture supernatant (250 μl) was incubated with 500 μl of substrate (1% xga or 1% saponified mhr of apple pectin [mhr-s] [27] in 50 mM sodium acetate [pH 5.0]) and 250 μl of 50 mM sodium acetate (pH 5.0) for 18 h at 30°C. The samples were heated in a boiling water bath for 5 min before the degradation products were analyzed by high-performance liquid chromatography (HPLC). A polygalacturonan digest was prepared in a similar way by incubating polygalacturonan (ICN Biomedicals) with endo-PG (EC 3.2.1.15) from *Kluyveromyces fragilis* purified in our laboratory (23).

High-performance anion-exchange chromatography (HPAEC) was performed by using a Dionex system (Dionex, Sunnyvale, Calif.) which included a model BioLC quaternary gradient pump, a model EDM (He) deventilating unit, a Carbopac PA-1 column (4 by 250 mm) with a matching guard column, and a pulsed electrochemical detector operated in pulsed amperometric detection mode. A linear gradient of sodium acetate in 100 mM NaOH at a flow rate of 1 ml/min was used as follows: 0 to 50 min, 0 to 620 mM; 50 to 55 min, 620 to 1000 mM; and 55 to 65 min, 1,000 mM.

High-performance size exclusion chromatography (HPSEC) was performed by using a model SP8800 HPLC (Spectra Physics) equipped with three Bio-Gel TSK columns (each 300 by 7.8 mm) in series (40XL, 30XL, and 20XL; Bio-Rad Laboratories) in combination with a Bio-Gel TSK XL guard column (40 by 6 mm). The temperature of the columns was 30°C. The columns were eluted with 0.4 M sodium acetate buffer (pH 3.0) at a flow rate of 0.8 ml/min. During elution the refractive index was monitored with a Shodex model SE-61 refractive index detector.

MALDI-TOF mass spectrometry of the degradation products of xga. xga was incubated with XghA as described above. The molecular masses of the resulting oligomers were determined by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry. Each sample was analyzed with a Perspective Biosystems Inc. Voyager-DE RP instrument equipped with a nitrogen laser (wavelength, 337 nm; pulse width, 3 ns). The mass spectrometer was operated in the positive ion mode with a delayed extraction time of 200 ns. The ions were accelerated to an energy of 12 kV before they entered the time of flight mass spectrometer. The minimum laser power for obtaining a good spectrum was used, and 20 to 50 spectra were obtained for each analysis. Approximately 0.35 g of Dowex 50WX8 (50-100 mesh; Fluka Chemika-BioChemika, Buchs, Switzerland) was added to the mixture of oligomers. The sample was thoroughly mixed and centrifuged to pellet the Dowex material. One microliter of the clear supernatant was applied to a matrix-assisted laser desorption ionization plate and mixed on the plate with 1 μl of matrix suspension, and then the preparation was dried with a gentle stream of air at room temperature. The matrix suspension was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid and 3 mg of isocarbostyril in 1 ml of a water-acetonitrile mixture (7:3). The mass spectrometer was calibrated externally with a polygalacturonic acid endo-PG digest (7).

Nucleotide sequence accession number. The nucleotide sequence of the *xghA* gene has been deposited in the EMBL database under accession no. AJ 249460.

RESULTS

Screening of the expression library. An *A. tubingensis* expression library constructed in *K. lactis* was initially designed to screen for arabinogalactan-modifying enzymes (33). The shuttle vector pCVlacK used for expression of the cDNA library in *K. lactis* also facilitated isolation and analysis of the cDNA insert in *E. coli*.

Screening of expression libraries on plates by using chromogenic substrates or overlays and Congo red staining has been used extensively. Here we describe screening of a library in microwell plates by using a modified BCA assay (18). This assay is based on reduction of Cu(II) to Cu(I) by reducing carbohydrate mono- and oligomers. A purple complex consisting of BCA and Cu(I) is formed, and this complex can be measured spectrophotometrically. Screening 35 microwell plates (about 3,400 *K. lactis* transformants) by using xga as the substrate yielded two transformants (27E8 and 42B4) with xga-hydrolyzing activity. The pCVlacK plasmids were isolated from both *K. lactis* recombinants. After transformation and propagation of the plasmids in *E. coli*, the cDNA inserts were excised from pCVlacK by *Hin*dIII-*Xho*I digestion. This digestion released 1.0- and 0.4-kb fragments due to an internal *Hin*dIII site, as determined from the nucleotide sequence. The plasmid inserts of the transformants were identical, as determined by restriction analysis.

Structural analysis of XgaA. The DNA sequences of both strands of the cDNA insert derived from *K. lactis* transformant 27E8 were determined by using 5′- and 3′-specific primers flanking the cDNA insert and specific primers based on the cDNA sequence obtained with the former primers. The cDNA sequence, designated *xghA*, contained a 1,218-bp open reading frame that began with an ATG start codon and ended with a TAA stop codon (Fig. 1). It encoded a 406-residue polypeptide designated XghA that had a calculated molecular mass of 42,070 Da. The open reading frame was preceded by a 20-bp 5′ noncoding region and was followed by a 130-bp 3′ noncoding region and a poly(A) tail. The TCATCATGGC sequence covering the ATG start codon closely resembled the consensus sequence for initiation of translation in higher eukaryotes (17). The *xghA* cDNA encoded an apparent signal sequence consisting of 18 amino acids at the NH_2 terminus, and there was a typical signal peptidase cleavage site between Ala-18 and Ala-19 (35). Two potential N-glycosylation sites were present at Asn-278–Ser–Thr and Asn-301–Val–Thr (Fig. 1).

A comparison of the XghA amino acid sequence with se-

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   1  gaattccgaaatcgctcatcATGGCGCTATATCGTAACCTCTACCTTCTGGCCAGCCTTGGGCTAAGCAGTGCTGCTCCCTCCAAGGTCC
   1                     M  A  L  Y  R  N  L  Y  L  L  A  S  L  G  L  S  S  A↓ A  P  S  K  V  Q
  91  AGCGAGCCCCGGATTCTTCCATTCATGCTCGCGCTGTCTGTACCCCGACCGCAGGAGGCGATTCGTCCACCGACGATGTCCCCGCCATCA
  25    R  A  P  D  S  S  I  H  A  R  A  V  C  T  P  T  A  G  G  D  S  S  T  D  D  V  P  A  I  T
 181  CCGAGGCCCTCAGCTCGTGCGGAAATGGTGGCACCATCGTCTTCCCCGAGGGCAGCACCTACTACCTCAACAGTGTGCTGGACTTGGGCA
  55    E  A  L  S  S  C  G  N  G  G  T  I  V  F  P  E  G  S  T  Y  Y  L  N  S  V  L  D  L  G  S
 271  GCTGCAGTGATTGCGACATCCAGGTGGAAGGTCTTCTGAAGTTCGCCAGCGATACCGATTACTGGAGCGGTCGCACTGCCATGATCAGTG
  85    C  S  D  C  D  I  Q  V  E  G  L  L  K  F  A  S  D  T  D  Y  W  S  G  R  T  A  M  I  S  V
 361  TTTCCAATGTAGATGGTTTGAAGCTGCGCTCATTGACTGGATCTGGTGTCATTGATGGCAATGGCCAGGATGCGTGGGATCTCTTTGCTT
 115    S  N  V  D  G  L  K  L  R  S  L  T  G  S  G  V  I  D  G  N  G  Q  D  A  W  D  L  F  A  S
 451  CGGACAGTAGTTACTCACGCCCGACGCTCTTGTACATCACTGGCGGCAGCAACCTAGAAATCTCCGGGCTGCGTCAAAAGAATCCACCTA
 145    D  S  S  Y  S  R  P  T  L  L  Y  I  T  G  G  S  N  L  E  I  S  G  L  R  Q  K  N  P  P  N
 541  ACGTGTTCAACTCGGTCAAGGGTGGCGCCACTAATGTCGTCTTCTCCAACCTGAAGATGGATGCCAACTCCAAGTCGGACAATCCGCCCA
 175    V  F  N  S  V  K  G  G  A  T  N  V  V  F  S  N  L  K  M  D  A  N  S  K  S  D  N  P  P  K
 631  AGAACACTGATGGGTTCGACATTGGCGAGAGTACCTATGTGACCATCACCGAGGTCACCGTAGTCAACGATGACGACTGTGTCGCCTTCA
 205    N  T  D  G  F  D  I  G  E  S  T  Y  V  T  I  T  E  V  T  V  V  N  D  D  D  C  V  A  F  K
 721  AGCCCAGTTCCAACTACGTGACAGTGGACACGATCAGCTGCACCGGCTCCCATGGAATTTCCGTGGGATCATTAGGAAAGTCGAGCGACG
 235    P  S  S  N  Y  V  T  V  D  T  I  S  C  T  G  S  H  G  I  S  V  G  S  L  G  K  S  S  D  D
 811  ACTCGGTCAAGAACATTTATGTCACGGGCGCAACTATGATCAACTCCACCAAAGCCGCCGGGATCAAGACTTATCCGAGTGGAGGCGACC
 265    S  V  K  N  I  Y  V  T  G  A  T  M  I  N  S  T  K  A  A  G  I  K  T  Y  P  S  G  G  D  H
 901  ACGGTACCTCCACGGTCAGCAATGTGACCTTCAACGATTTCACTGTGGACAACTCCGACTATGCCTTCCAGATCCAGAGCTGCTATGGCG
 295    G  T  S  T  V  S  N  V  T  F  N  D  F  T  V  D  N  S  D  Y  A  F  Q  I  Q  S  C  Y  G  E
 991  AGGACGATGACTATTGCGAGGAAAACCCGGGCAACGCCAAACTGACTGATATAGTCGTGTCAAGCTTCAGTGGGACAACCAGTGACAAGT
 325    D  D  D  Y  C  E  E  N  P  G  N  A  K  L  T  D  I  V  V  S  S  F  S  G  T  T  S  D  K  Y
1081  ACGATCCGGTCGTGGCCAACCTCGACTGCGGTGCGGATGGAACTTGTGGCATCTCCATCAGTGGGTTCGATGTCAAGGCGCCATCGGGCA
 355    D  P  V  V  A  N  L  D  C  G  A  D  G  T  C  G  I  S  I  S  G  F  D  V  K  A  P  S  G  K
1171  AGTCTGAAGTGTTGTGCGCCAACACCCCGTCTGATTTGGGCGTCACTTGCACTTCGGGGGCTTCGGGCTAAatagctttggccgggttgc
 385    S  E  V  L  C  A  N  T  P  S  D  L  G  V  T  C  T  S  G  A  S  G  *
1261  tttctgaatccactgagtggaggtcttcttcgggtttgatattttgtatggtcgtgtgtatagcagaatgtgacaatagaattagtgaaa
1351  ttgccattcttttcgaaagacaaaaaaaaaaaaaaaaaaaaaaaaaaa
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FIG. 1. Nucleotide sequence of the cDNA encoding XghA (*xghA*) and the deduced amino acid sequence of the encoded protein (XghA). The arrow indicates the potential cleavage site of the signal sequence. Two potential glycosylation sites are underlined.

quences in the EMBL data library revealed homology to PG (EC 3.2.1.15) sequences of prokaryotes, fungi, and plants and to RHG-A (EC 3.2.1.-) and RHG-B (EC 4.2.2.-) sequences of *Aspergillus aculeatus* (16, 31) and *A. niger* (32). Many PGs have been cloned from members of the genus *Aspergillus*, but only the *A. niger* and *A. tubingensis* sequences (Fig. 2) were used for a detailed sequence comparison. XghA exhibited 31 to 39% similarity to the endo-PGs and 44% similarity to the exo-PG of

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                        I                                  II                                  III
Atub-PgaII 173  TLGGH...A. .V.N.VG.N. IKPW.H.Q.. .L.IN-.GEN IWFTSGT.I. G..L.I..V.
Anig-PgaII 173  TQGGH...A. .V.N.VG.N. IKPW.H.Q.. .L.VN-.GEN IWFTGGT.I. G..L.I..V.
Anig-PgaI  179  ..GGH..... ..SE..G.Y. SGA..K.Q.. .I.IN-.GES ISFTGGT.S. G..L.I..V.
Anig-PgaC  193  .DLAA..... .......I.. .GAEIY.Q.. ...IN-.GEN IYFSASV.S. G..L.I..V.
Atub-PgaX  216  ..EAK....W .TYR.NNIV. QNSVIN.G.. ..S...NSTN IL.QNLH.N. ..........

Atub-XghA  200  DNPPKNTDGF DIGESTYVTI TEVTVVNDDD CVAFKPSSNY VTVDTISCTG SHGISVGSLG

 Aac-RhgA  188  GGNEGGL..I .VWG.N-IWV HD.E.T.K.E ..TV.SPA.N IL.ES.Y.NW .G.CAM....
Anig-RhgA  189  GGNSGGL..I .VWG.N-IWV HD.E.T.K.E ..TV.GPA.N IL.ES.Y.NW .G.CAM....
Anig-RhgB  192  GGNHGGL..I .VWSNN-IWV HD.E.T.K.E .VTVKSP.KN ILIES.Y.NW .G.CGM..F.

                                    IV
Atub-PgaII 232  GR.N--NV.. .VTIEHS.VS ..EN.VR... VSGATGSVSE ITYSNIVMSG IS.YGVVIQ.
Anig-PgaII 232  DR.N--NV.. .VTIEHS.VS ..EN.VR... ISGATGSVSE ITYSNIVMSG IS.YGVVIQ.
Anig-PgaI  238  GRD.--NT.. .VTISDS.VS .SANGVR... IYKETGDVSE ITYSNIQLSG IT.YGIVIE.
Anig-PgaC  252  GRD.--NT.. .VTFYDVNVL K.QQ.IR... IYGDTGSVSE VTYHEIAFSD AT.YGIVIE.
Atub-PgaX  276  QYK.EV.I.E .V..YNISMF .ASDM.R..V W.GTPSALSA DLQGGGGSGS VKN--ITYDT

Atub-XghA  260  KSSD--DSVK NIYVTGATMI NSTKAAGIKT YPSGGDHGTS TVSNVTFNDF TVDNSDYAFQ

 Aac-RhgA  247  --A.--TD.T D.VYRNVYTW S.NQMYM..S -----NG.SG .....LLEN. IGHGNA.SLD
Anig-RhgA  248  --A.--TDIT D.LYRNVYTW S.NQMYM..S -----NG.SG ..N.TLLEN. IGRGNR.SLD
Anig-RhgB  251  --S.--TN.S D.TYRNIYTW S.NNMML..S -----NG.SG F.E..LLEN. IGHGNA.SLD
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FIG. 2. Part of a multiple alignment of PG, XghA, and RHG amino acid sequences. Amino acids identical to the amino acids in the XghA sequence are indicated by dots, and gaps that were introduced to obtain the optimal alignment are indicated by dashes. Amino acids conserved in all plant, fungal, and prokaryotic PGs are shaded. An HG sequence (domain III in PG and XghA) is underlined in the sequences of *A. aculeatus* RHG-A and *A. niger* RHG-B. Abbreviations: *A. tubingensis* PGAII Atub-PgaII, (EMBL accession no. P19805 [3]); Anig-PgaII, *A. niger* PGAII (P26214 [3]); Anig-PgaI, *A. niger* PGAI (P26213 [4]); Anig-PgaC, *A. niger* PGAC (X64356 [5]); Atub-PgaX, *A. tubingensis* exo-PG (X99795 [14]); Aac-RhgA, *A. aculeatus* RHG-A (A544425 [16] and S80208 [31]); Anig-RhgA, *A. niger* RHG-A (X94220 [32]); Anig-RhgB, *A. niger* RHG-B (X94221 [32]); Atub-XghA, *A. tubingensis* XghA. The *A. niger* PG sequence (24) deposited under EMBL accession no. X54146 is identical to the *A. tubingensis* PGAII sequence (3).

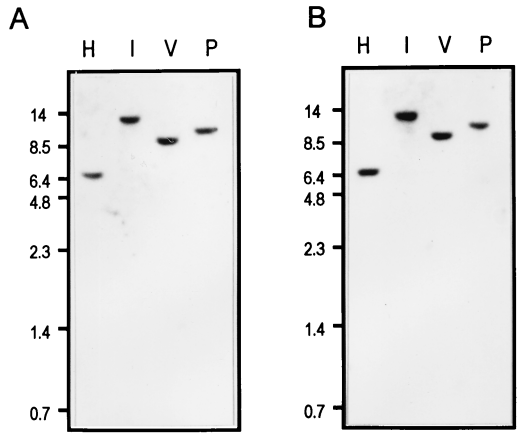

FIG. 3. Autoradiographs of Southern blots containing genomic DNA of *A. tubingensis* digested with four restriction enzymes (lanes H, *Hin*dIII; lanes I, *Eco*RI; lanes V, *Eco*RV; lanes P, *Pst*I). The positions of molecular size markers (in kilobases) are indicated on the left. (A) Blot hybridized under low-stringency conditions (60°C, 1× SSC). (B) Blot hybridized under high-stringency conditions (65°C, 0.2× SSC). A 1.0-kb *Hin*dIII fragment of *xghA* was used as a probe.

A. tubingensis; these values are higher than the levels of similarity between the endo-PGs and the exo-PG (26 to 33%). The levels similarity of XghA to the two RHG-As were 30% (*A. niger* RHG-A) and 32% (*A. aculeatus* RHG-A), whereas the level of similarity to RHG-B of *A. niger* was very low (for the whole sequence).

Based on the values given above, the levels of similarity of XghA to the PGs and the RHG-As seem to be comparable. However, an analysis of a multiple alignment of the enzymes produced different results. Figure 2 shows part of a multiple alignment of four domains of conserved amino acids which were first described for PGs of plant, fungal, and bacterial origin (3). When all of the PGs recovered from a database search were aligned, only the following four short stretches of amino acids were conserved: NXD, DD, HG, and RXK (14) (Fig. 2). Possible candidates for the essential amino acids involved in the hydrolysis reaction were the three aspartic acid residues of domains I and II and the histidine residue of domain III (3). These domains were completely conserved in XghA. The fourth domain contained amino acids that may be involved in substrate binding. The arginine residue of this domain was replaced by a glycine residue in XghA. The domains were less conserved in the RHG sequences. Only two of the three aspartic acid residues were conserved, and the histidine in domain III was replaced by glycine. It has been suggested that the HG sequence is present further along the sequence (31) (Fig. 2), but it is not present in the RHG-A sequence of *A. niger*.

Southern blot analysis. The copy number of the *xghA* gene was determined by performing a Southern blot analysis of the genomic DNA of *A. tubingensis* digested with several enzymes (Fig. 3). Hybridization under stringent conditions (65°C, 0.2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) and less stringent conditions (60°C, 1× SSC) with a 1.0-kb *Hin*dIII fragment of *xghA* clearly revealed single hybridizing fragments (Fig. 3). This demonstrated that a single copy of the *xghA* gene is present in the *A. tubingensis* genome.

Characterization of the enzymatic activity of XghA. Degradation of xga or mhr-s by XghA was monitored by HPAEC and HPSEC. Figure 4 shows HPAEC elution profiles for xga and mhr-s incubated with a wild-type *K. lactis* culture supernatant (Fig. 4A) or with an XghA-containing supernatant of transformant 27E8 (Fig. 4B). A polygalacturonan digest (Fig. 4C) was included as a reference (7). Incubation with the wild-type *K. lactis* culture supernatant did not result in breakdown of xga or mhr-s, whereas incubation with the XghA-containing supernatant yielded oligomers with both substrates. When the HPAEC elution times of these products were compared with the elution times of the polygalacturonic acid standards (Fig. 4C), it was obvious that the xga and mhr-s products eluted at different times, indicating that none of the degradation products was a galacturonic acid oligomer. Considering the sugar composition of the substrates, we concluded that xga oligomers were produced. The molecular masses of the oligomers released from xga after incubation with XghA were determined by MALDI-TOF mass spectrometry. The molecular weights of the resulting oligomers corresponded to the molecular weights of sodium adducts of oligomers consisting of xylose and galacturonic acid monomers. The compositions of these oligomers as related to the detected masses are shown in Table 1.

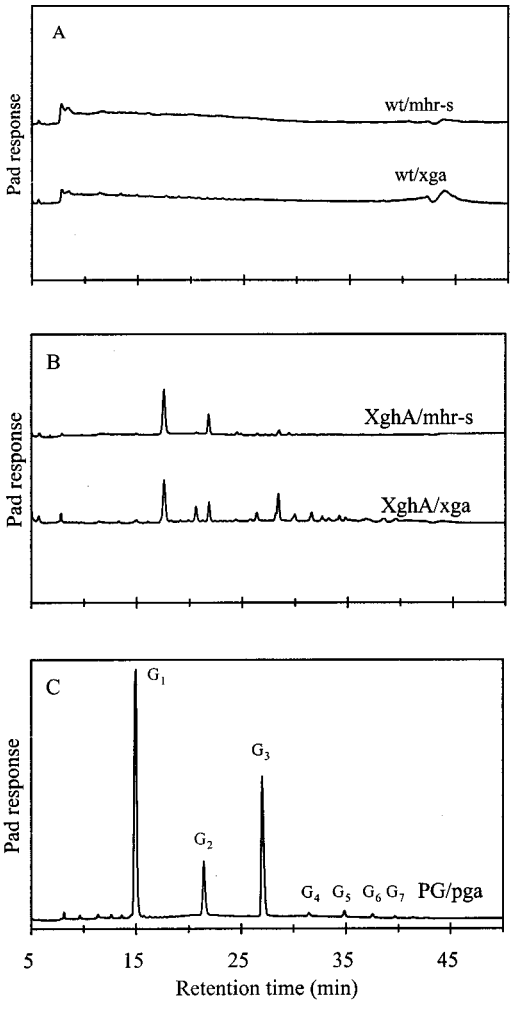

FIG. 4. HPAEC elution profiles of digests of xga and mhr-s incubated with *K. lactis* wild-type (wt) culture supernatant (A) or XghA-containing supernatant of *K. lactis* transformant 27E8 (B). (C) Polygalacturonic acid (pga) digested with PG. The subscript numbers indicate the form of galacturonic acid (1, monomer; 2, dimer; etc.) (7). Pad, pulsed amperometric detection.

Only two products were formed when mhr-s was incubated with XghA. The same two products plus some additional products whose elution times were greater than the elution time of

TABLE 1. Molecular weights and compositions of the oligomers released from xga after incubation with XghA, as determined by MALDI-TOF mass spectrometry

Oligomer	Detected mass (Da)
GalA-Xyl	349.1
$GalA_2$-Xyl	525.1
$GalA_2$-Xyl_2	657.3
$GalA_3$-Xyl	701.3
$GalA_3$-Xyl_2	833.4
$GalA_4$-Xyl	877.4
$GalA_4$-Xyl_2	1,009.5
$GalA_4$-Xyl_3	1,141.6
$GalA_5$-Xyl_2	1,185.5
$GalA_6$-Xyl_2	1,361.5

the galacturonic acid trimer standard were formed when xga was degraded by XghA. These larger oligomers appeared even after a short incubation time (results not shown), indicating that the mechanism of the enzyme was an endo type of mechanism.

XghA activity has also been tested with other substrates, such as polygalacturonic acid, linear arabinan, soy galactan, and xylan from oat spelt. No breakdown was detected (data not shown). Apparently, XghA activity requires a galacturonic acid backbone substituted with xylose.

The HPSEC results obtained show that there was a dramatic decrease in the molecular weight of xga after incubation with XghA (Fig. 5A). This decrease appeared after incubation for 1 h (results not shown), and developed rapidly, again indicating the endo nature of the enzyme. Degradation of mhr-s (Fig. 5B) resulted in a shift of the high-molecular-weight fraction to a lower molecular weight. However, this shift was less prominent, which could be explained if the xga were present at the ends of the mhr-s chains.

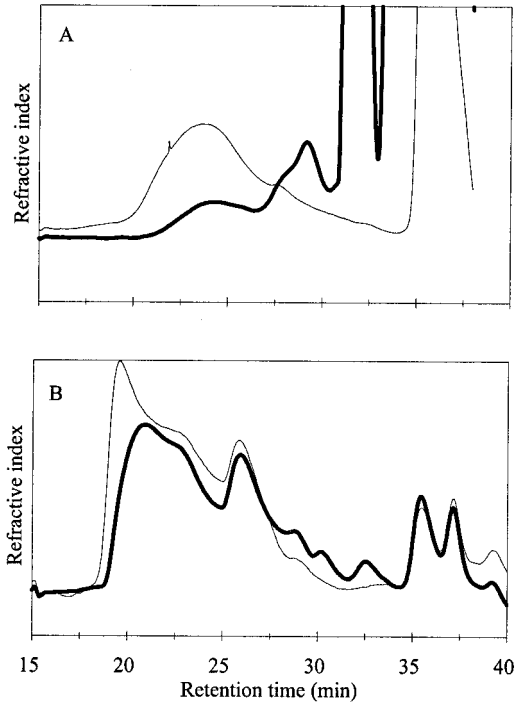

FIG. 5. Elution profiles of digests of xga (A) and mhr-s (B) incubated with XghA-containing supernatant of *K. lactis* transformant 27E8 (dark lines). The light lines indicate the results obtained with substrate incubated with water.

DISCUSSION

xga is an important constituent of the hairy regions of certain pectins. Although it has been suggested that an array of enzymes which are able to hydrolyze xga exist in nature, only an exogalacturonase which is able to remove a Xyl-GalA disaccharide from xga has been identified (1). Because of the availability of xga (19) as a very specific substrate and the availability of an *A. tubingensis* expression library in *K. lactis*, we performed the work described in this paper, which was aimed at finding enzymes that degrade xga in an endo fashion.

Expression libraries are most commonly screened by performing plate assays. In this paper we describe a BCA assay for detecting carbohydrolase activity in the supernatants of *K. lactis* recombinants in which microwell plates were used. The advantage of this method is that less substrate is required than the amount required for plate assays. Furthermore, it is very sensitive and fast.

Two identical *K. lactis* recombinants from the approximately 3,400 colonies screened exhibited hydrolyzing activity with xga. An analysis of the cDNA insert demonstrated that it codes for a 406-residue polypeptide with a signal sequence consisting of 18 amino acids. A single copy of the gene is present in the *A. tubingensis* genome. HPLC analysis and MALDI-TOF mass spectrometry of xga degradation by XghA-containing supernatant of the *K. lactis* recombinant clearly showed that the mechanism of the enzyme was an endo type of mechanism. To our knowledge, this is the first report of a true endo-xylogalacturonase. Since XghA exhibits no activity with polygalacturonic acid, linear araban, soy galactan, or xylan from oat spelt, we concluded that XghA requires a galacturonic acid backbone substituted with xylose.

When mhr-s was incubated with XghA, only two major products were produced, whereas several peaks were formed when xga was incubated with XghA (Fig. 4). The difference could have been the result of the different Xyl/GalA ratios; the Xyl/GalA ratio was two to three times higher in mhr-s than in xga obtained from gum tragacanth. Steric hindrance by other subunits of mhr-s could also have been the reason for the difference. Also, HPSEC analysis of mhr-s incubated with XghA revealed a shift to the lower-molecular-weight fraction, which was less significant than it was in the elution profile of xga with XghA. A possible explanation for this is the presence of xga at the ends of the mhr-s chains. The mhr-s degradation data obtained in the HPAEC and HPSEC analyses show that XghA is valuable for determining the structures of complex polysaccharide fractions, like mhr-s.

A search of the EMBL data library for sequences homologous to the XghA sequence revealed that the levels of similarity of XghA to PGs and RHGs were low. The percentages of similarity were approximately the same for both groups of enzymes. However, an analysis of a multiple alignment of conserved domains in these proteins showed that XghA resembled PGs more than RHGs. The putative active site residues aspartate and histidine (present in domains I to III [Fig. 2]) are conserved in the PGs and XghA. In RHGs, however, the histidine is replaced by a glycine, and only two aspartic acid residues are conserved. This suggests that the catalytic mechanism of RHGs may be different from the catalytic mechanisms of the PGs and XghA and that PGs and XghA might have similar catalytic mechanisms. This could also correspond to the cleavage sites in the backbones of the respective sub-

strates. RHGs cleave a rhamnosyl-galacturonic acid linkage, whereas the PGs and XghA cleave a galacturonic acid-galacturonic acid linkage.

The positively charged sequence Arg-Ile-Lys present in domain IV of PGs, postulated to play a role in substrate binding, is not fully conserved in XghA and RHGs. This arginine residue is not conserved in either XghA or RHGs, but the substrate for these enzymes is different from the substrate for PGs, which may account for the discrepancy (14). Although the substrate backbone for both PGs and XghA consists of GalA, XghA requires a substrate with a xylose-substituted backbone.

In conclusion, XghA is the first enzyme that has been reported to hydrolyze xga in an endo fashion. Based on sequence similarities of the active site residues, we propose that the catalytic mechanism of XghA is similar to that of PGs. This possibility must be investigated further, however. XghA should be a useful tool that allows workers to study plant cell wall structures. In practice, XghA can be a valuable component of tailor-made enzyme preparations that are used in fruit juice manufacturing.

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