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Three Extracellular Proteases from *Cochliobolus carbonum:* Cloning and Targeted Disruption of *ALP1*

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Three extracellular serine proteases (Alp1a, Alp1b, Alp2) from *Cochliobolus carbonum* were purified and characterized. Of eight carbon/protein substrates tested, total protease activity was highest when the fungus was grown on medium containing collagen. Alp1a and Alp1b are members of the trypsin family (EC 3.4.21.4), and Alp2 is a member of the subtilisin family (EC 3.4.21.62). Alp1a, Alp1b, and Alp2 have monomer molecular masses of 25, 30, and 38 kDa respectively. Alp1b is glycosylated, whereas Alp1a is not. The gene encoding Alp1a, *ALP1*, was isolated using PCR primers based on two amino acid sequences: One obtained directly from the N-terminus of Alp1a and another that is highly conserved in other trypsins. The transcriptional start site was determined using RACE and the intron structure and polyadenylation site were determined from a cDNA clone. An internal fragment of *ALP1* was used to create Alp1a null mutants by transformation-mediated gene disruption. Total protease activity in the mutants was reduced by 35% to 45%. By chromatographic analysis, the mutants had lost two peaks of UV absorption and the two protease activities corresponding to Alp1a and Alp1b, which, together with the biochemical data, indicates that Alp1a and Alp1b are products of the same gene. The in vitro growth and disease phenotypes of the *ALP1* mutants were indistinguishable from the wild-type strain; therefore, ALP1 is not by itself required for pathogenicity.

Additional keywords: cell wall degrading enzyme, *Helminthosporium*, maize, trypsin, virulence factor.

The first barrier a phytopathogenic microbe encounters on its host is the plant cell wall, and cellular pathogens secrete a variety of wall-depolymerizing enzymes. The role, if any, of these enzymes in the process of pathogenesis has been the subject of intensive research (Bateman and Basham 1976; Cooper 1983; Walton 1994).

Although the plant cell wall is mainly composed of polysaccharides, at least five classes of structural proteins and numerous classes of enzymes are present (McNeil et al. 1984; Showalter 1993). During pathogenesis the expression of many structural proteins and enzymes are up-regulated and secreted into the plant cell wall as part of a general defense response (Showalter 1993; Alexander et al. 1994). Insofar as these wall proteins are important to plant defense, effective pathogens may require extracellular proteases to degrade them. The proteases secreted by a pathogen might also be important as activators of any of its cell wall degrading enzymes that are secreted as zymogens (Drapeau 1978; Rypniewski et al. 1993; Moormann et al. 1993), or as processors of toxins (Howard and Buckley 1985) and elicitors (van den Ackerveken et al. 1993). Proteases might also have a role in pathogenesis by increasing the permeability of the plant plasma membrane (Tseng and Mount 1974). Many plants produce protease inhibitors, which suggests that plants have evolved mechanisms to counter pathogen proteases (Ryan 1990).

Extracellular proteases are produced by many phytopathogenic bacteria and fungi, e.g., *Xanthomonas alfalfae* (Reddy et al. 1971), *Monilinia fructigena* (Hislop 1982), *Colletotrichum lindemuthianum* (Ries and Albersheim 1973), and others (Porter 1966). An aspartic protease gene has been isolated from *Cryphonectria parasitica* (Choi et al. 1993). A protease mutant of *Cladosporium cucumerinum* had wild-type symptom development on cucumber seedlings, but residual protease activity was present (Robertsen 1984). No reduction in virulence was observed in a metalloprotease mutant of *Erwinia chrysanthemi* EC16 when inoculated on either potato tubers or chrysanthemum stems (Dahler et al. 1990). A protease-deficient mutant of *Xanthomonas campestris* pv. *campestris* had reduced virulence when introduced into the cut vein endings of turnip leaves (Dow et al. 1990). A UV-induced mutant of the fungus *Pyrenopeziza brassicae* that had lost both protease activity and pathogenicity could be complemented for both traits by a 40-kb genomic cosmid clone (Ball et al. 1991).

One definitive test of the role of any particular gene in pathogenesis is the construction of a null mutant using targeted gene disruption (e.g., Scott-Craig et al. 1990). To examine the role of proteases in plant pathogenicity, we report here the characterization of three proteases that *C. carbonum* makes when grown on collagen, and the sequence and disruption of *ALP1*, which encodes two of these proteases.

RESULTS AND DISCUSSION

Characterization of Alp1a, Alp1b, and Alp2.

To facilitate the study of proteases, we investigated which growth conditions maximized total protease activity. Collagen

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The sequence of *ALP1* has been submitted to GenBank with accession number U39500.

was tried as a substrate because—like the extensins of the plant cell wall—it is a hydroxyproline-rich glycoprotein. Stimulation of the de novo synthesis as well as cross-linking of structural wall proteins such as extensin in response to pathogen attack is thought to be an important plant defense response (Showalter 1993; Lawton and Lamb 1987). Therefore, fungal proteases that are induced in planta to degrade wall structural proteins might also be induced in culture by collagen. Preliminary experiments indicated that of the potential protease substrates, *C. carbonum* produced most total protease on medium supplemented with collagen. Other protein sources (casein, gelatin, and bovine serum albumin) yielded less total protease activity than collagen (Fig. 1). Low levels of protease activity were observed on media supplemented with pectin, corn bran, or 2.0% sucrose (Fig. 1). In the presence of collagen, 0.2% sucrose stimulates protease production (Fig. 1), an effect also seen with polygalacturonase (Walton and Cervone 1990) and xylanases (Holden and Walton 1992). High levels of protease production on protein substrates and low levels on other substrates indicate that total protease activity in *C. carbonum* is substrate-induced and partially catabolite repressed.

Culture filtrates were concentrated by rotary evaporation, dialyzed, and then passed over a low-pressure anion-exchange column to remove acidic proteins and pigments. Alp1a, Alp1b, and Alp2 were then separated by cation-exchange HPLC. Alp1a was further purified by hydrophobic-interaction HPLC and sequenced at the N-terminus. By SDS-PAGE, Alp1a, Alp1b, and Alp2 had M_r's of 25, 30, and 38 kDa, respectively, similar to serine proteases (North 1982). Alp1a and Alp1b activities were inhibited strongly by aprotinin (77% and 85%, respectively) and leupeptin (76% and 96%, respectively) and weakly by phenylmethylsulfonyl fluoride (PMSF) (37% and 37%, respectively) (Fig. 2), suggesting that these proteases are related to trypsin (Gebhard et al. 1986; Powers and Harper 1986). Alp2 was more sensitive to PMSF (81% inhibition) than to aprotinin or leupeptin (40% and 47% inhibition, respectively) (Fig. 2), suggesting that it is related to the subtilisin family of proteases (Ottensen and Svendsen 1970). Alp1a, Alp1b, and Alp2 were less sensitive to other major classes of protease inhibitors (Fig. 2).

Periodic acid/Schiff staining indicated that Alp1b is a glycoprotein, whereas Alp1a lacks glycosylation (data not shown). All three enzymes were most active between pH 7 and 8, but each showed some activity over the pH range of 5 to 11. Temperature optima were similar for all three enzymes. Each enzyme was as active at 45°C as at 55°C but lost activity at 65°C and above. Alp1a and Alp1b seemed to be less stable than Alp2, based on the observation that after being stored at –20°C and then separated by SDS-PAGE, Alp1a and Alp1b were degraded, whereas Alp2 was not. To test the possibility that plant cell wall structural proteins might be substrates for these proteases, salt-extractable extensin was purified from maize stylar tissue (Murphy and Hood 1993). Neither crude culture filtrates nor the proteases individually or in combination could degrade extensin, whereas in parallel experiments casein was degraded to small peptide fragments (data not shown). Therefore, these proteases probably do not have a role in degradation of this class of maize cell wall structural proteins during pathogenesis.

Although no evidence for additional proteases was found, we cannot exclude the possibility that *C. carbonum* makes other proteases, which might have been overlooked because (i) they are unstable, (ii) they are not produced on the substrates tested, (iii) they are acidic and therefore retained by the DEAE-cellulose pre-treatment, or (iv) they are active only below pH 5, under which conditions the substrate, azocasein, precipitates.

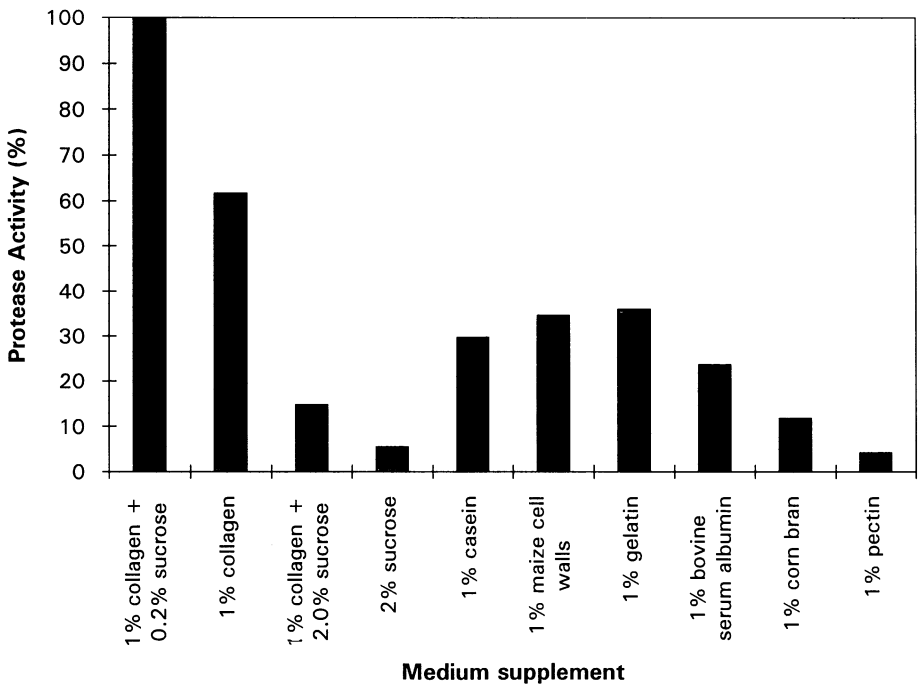

Fig. 1. Effect of different media supplements on total protease activity in culture filtrates of *Cochliobolus carbonum.* Protease activities are shown as a percentage of the highest activity measured. Experiment was repeated three times with similar results.

Isolation and characterization of *ALP1*.

The N-terminal amino acid sequence of Alp1a was determined to be IVGGTTAAAGEYPFIVS (indicated by double underlining in Fig. 3). A search of the nonredundant databases using BLASTP (Gish and States 1993) identified a 76% identity with the N-terminus of a 22-kDa trypsin-like protease from *Fusarium oxysporum*. A 48-fold degenerate oligonucleotide based on the amino acid sequence EYPFIV was used in conjunction with a 256-fold anti-sense degenerate oligonucleotide coding for the amino acid sequence VAGWGA (also indicated by double underlining in Fig. 3), which is a highly conserved internal amino acid sequence of many trypsins. Using these two primers and DNA isolated from a *C. carbonum* cDNA library as template in PCR, a 330-bp product was generated. BLASTX analysis of the PCR product showed a high degree of similarity with trypsin-like proteases. The PCR product was used as a probe to screen a *C. carbonum* cDNA library. A 1.0-kb cDNA clone (pC8-6.1) was isolated and sequenced, and also used to screen a library of *C. carbonum* genomic DNA. Oligonucleotide primers that had been used to sequence the cDNA copy of *ALP1* were used to sequence, on both strands, approximately 1.5-kb of genomic DNA covering *ALP1*.

Figure 3 shows the sequence and structure of *ALP1* and its deduced amino acid sequence. The start of the *ALP1* message was determined by sequencing three independent RACE products (Frohman et al. 1988). The context of the first ATG, 91 bp downstream of the transcription start site (CACCATGCGT) (Fig. 3), is in good agreement with the 5′ end of the consensus sequence for *Neurospora* translation initiation (CAMMATGGCT where M = C or A) (Edelmann and Staben 1994). Sequences typical of promoters of lower eukaryotes, TATAA and CAAC (Gurr et al. 1987), are located 34 and 72 bp, respectively, upstream of the transcription start site (Fig. 3). A single 74-bp intron deduced by comparing the cDNA and genomic sequences of *ALP1* is indicated by lowercase letters (Fig. 3). The 5′ (G^GTAAGTTCACTCA; consensus G^GTAAGTNNYCNYY, where Y = T or C) and 3′ (AACAG; consensus WACAG, where W = A or T) donor sites as well as the splice branch site (AACTAACA; consensus WRCTRACM, where R = A or G) and intron length are consistent with other introns of *C. carbonum* and other fungi (Apel et al. 1993; Scott-Craig et al. 1990; Sposato et al. 1995; Edelmann and Staben 1994). No AATAAA polyadenylation signal sequence (Gurr et al. 1987) could be identified before the polyadenylation site 229 bp downstream of the stop codon (Fig. 3).

ALP1 is predicted to encode a mature protein of 261 amino acids and a mass of 24.5 kDa, which is in agreement with the size of Alp1a estimated by SDS-PAGE. A single predicted N-glycosylation site (NKT) occurs at amino acid 225. Compari-

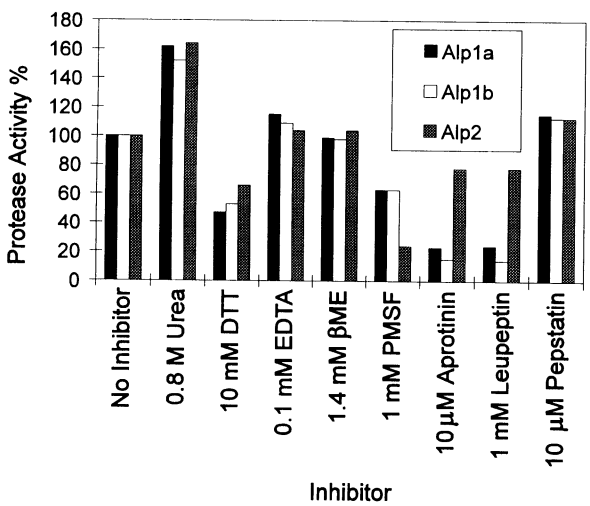

Fig. 2. Effect of protease inhibitors on Alp1a, Alp1b, and Alp2. Each enzyme was incubated with azocasein in the presence of the inhibitor being tested for 30 min at 45°C. Protease activities are expressed as a percentage of activity obtained in the control. Data are the average of two experiments. DTT, dithiothreitol; EDTA, ethyelenediaminetetracetic acid; βME, β-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride.

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   1 GTAGATAAGGCAGCTGCTAGGCTCGGGGTAATTGGTCTCCACTGCTTGAT
  51 CTAAGGCACACGGCGGACTACAGGTTAAGACTTTGCCAAGCCATAATAGG
 101 TCCCAAACTGGAAGGACAAATCGTACTCCTAGTAGATCAGTCTTGGTATA
 151 CCCTAAAGCACGCCATGACAAGGTCGGCCTTCTACCATCTACACAACCAT
 201 TGGAGTGTCCATGACCGACAACGACATTCACTATAAGTATCCAGCAATCT
                    #
 251 GCCCTCTGTAATCATCATCAGCCAGCTCATCCAGTCGCTTGTCTCTTCAA
 301 ACCATTCCATCGCTTCTCTCCAACAGTCGCTGCCTTTGCAAAGCCCATCA
 351 TTCACCATGCGTTTCCAGTCTATGATCACTGCTGCGCTTCCTGCGCTCGT
           M  R  F  Q  S  M  I  T  A  A  L  P  A  L  V
 401 CCTCTCCGCTCCTACTCCCCAGTGGGATGATGTTCCTGAGGACTCCATTG
  16   L  S  A  P  T  P  Q  W  D  D  V  P  E  D  S  I
 451 TTGGTGGAACCACCGCTGCTGCCGGCGAGTACCCCTTCATCGTCTCTATC
  32 V  G  G  T  T  A  A  A  G  E  Y  P  F  I  V  S  I
 501 CAGCTTGGCGGTCGCCACAACTGCGGTGGTACCCTCATCAACGGCAACAC
  49  Q  L  G  G  R  H  N  C  G  G  T  L  I  N  G  N  T
 551 CGTTGTCACTGCTGCCCACTGCTCCGTCAGCAGCGCCATTGGCGGCTCCA
  66   V  V  T  A  A  H  C  S  V  S  S  A  I  G  G  S
 601 TCAACAACGTCGCTGTCCGCGTCGGCTCCTTGgtaagttcactcatctga
  82 I  N  N  V  A  V  R  V  G  S  L
 651 tacagtactttatgcacttggcaaaggacaaagaaactaacacaagttct
 701 aaacagAGCGCCAACTCTGGTGGCCAAGTCATCAAGGTCTCCAAAATCAT
  93        S  A  N  S  G  G  Q  V  I  K  V  S  K  I  I
 751 CATCCACCCCAGCTACCAGGCAAGCACCTCCAACAACGACATTGCCATCT
 108   I  H  P  S  Y  Q  A  S  T  S  N  N  D  I  A  I
       HindIII
 801 GGAAGCTTTCCAGCACCGTCACTGCCGGTGGCAACATCGGCTTTGCTTCC
 124 W  K  L  S  S  T  V  T  A  G  G  N  I  G  F  A  S
                                   BamHI
 851 CTCGCCGCCTCTGGCTCTGATCCCGCCAGCGGATCCACCACCTCCGTTGC
 141  L  A  A  S  G  S  D  P  A  S  G  S  T  T  S  V  A
 901 TGGATGGGGAGCTACCCGTGAGGGTGGCGGCGCCAACAACGCTCTCCTCA
 158   G  W  G  A  T  R  E  G  G  G  A  N  N  A  L  L
 951 AGGTCAGCGTCCCCATTGTTGCCCGCTCCACCTGCGTGTCCAACTACAAC
 174 K  V  S  V  P  I  V  A  R  S  T  C  V  S  N  Y  N
1001 GCCGTCGGTCTCACCGTCACCACCAACATGGTCTGCGCTGGTGTCACTGC
 191  A  V  G  L  T  V  T  T  N  M  V  C  A  G  V  T  A
                                                  SalI
1051 TGGTGGCCGCGACTCTTGCCAGGGCGACTCTGGCGGCCCTCTCGTCGACG
 208   G  G  R  D  S  C  Q  G  D  S  G  G  P  L  V  D
1101 CCAACAAGACCCTCATCGGCGTCGTCTCCTGGGGAACCGGCTGCGCTCGC
 224 A  N  K  T  L  I  G  V  V  S  W  G  T  G  C  A  R
1151 CCCAACCTCCCCGGTGTCTACTCCCGCGTCGGCACCCTCCGCAGCTTCAT
 241  P  N  L  P  G  V  Y  S  R  V  G  T  L  R  S  F  I
1201 CGACCAGAACGCTTAAGCGCGTACATCTTGAAAGCGAGTTGGATATGATT
 258   D  Q  N  A  *
1251 TGGAAACGGTCGACTTTGGATATGAAAAGAGCAATGGCTTTGATGAGTAT
1301 GGTATGGGGGAGACCCTGAAGTTGGGAGGGAAAACGGTGATGATGGACTT
1351 TGCTTTTTTACTTTACCTCTTCTCCCTCCTTAATTTCGGTGACGGCATCT
1401 TGTAAATAGGTCTAGCCTCCCACGATTATTTTTTGTCTGTACCTTATTTT
                                                      +
1451 TTTCCTTTGTGTAGCTAGGAAATCGCATTGTGTGTTGGAACAACATCCCT
1501 TTTTTGTGCTCTC
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Fig. 3. Sequence of *ALP1*. The amino acid sequence of the mature N-terminus obtained directly from Alp1a, IVGGTTAAAGEYPFIVS, is indicated by double underlining. The sequence conserved among other trypsin-like proteases and used to design the second oligonucleotide primer for PCR amplification, VAGWGA, is also indicated by double underlining. The intron is indicated in lowercase letters. The indicated *Hind*III, *Sal*I, and *Bam*HI restriction sites are those used to construct and linearize the disruption vector pJM9. The transcriptional start site is indicated by # and the polyadenylation site by + (symbols refer to nucleotides underneath and amino acid codes refer to nucleotides above). The predicted glycosylation site (amino acid 225) is indicated in bold lettering.

son of the predicted N-terminal amino acid sequence with the experimentally determined sequence of the mature N-terminus (double underlined in Fig. 3) reveals a 30-amino acid signal peptide with a potential signal peptide cleavage site that violates the (−3, −1) rule (von Heijne 1986). Many trypsin-like proteases are synthesized as prepropeptides (trypsinogens) that undergo a second proteolytic processing after removal of the signal peptide, e.g., mammalian trypsins (Walsh and Wilcox 1970). Alp1 might also be synthesized as a prepropeptide.

The predicted amino acid sequence of Alp1a has a high degree of similarity with other trypsin-like proteases from a variety of species (Fig. 4). Alp1a contains the catalytic triad common to all serine proteases (His-71, Asp-120, and Ser-217) (Neurath 1984). The sequence flanking the active site serine (CQGDSGGP) is completely conserved in the other trypsin-like proteases (Fig. 4).

N-terminal sequencing of Alp2.

Fractions from the cation-exchange HPLC step that had Alp2 activity were run on SDS-PAGE, blotted, and three proteins that were visible with Coomassie R-250 staining of the blot were excised and sequenced from their N-termini. Two of the proteins had N-terminal sequences with no strong similarity to any sequences in the nonredundant databases, whereas one of the proteins, which showed the best correlation between staining intensity and Alp2 activity among the HPLC fractions, gave the sequence AYTTQSSAPWGL-ARISSQXRGTTGYXXDD, where X indicates an unknown amino acid. Analysis of this sequence by TBLASTN (Gish and States 1993) showed that it has strong similarity to numerous serine proteases, including subtilisin-like proteases from the fungi *Trichoderma album* (GenBank M54901) (69% identity), *Paecilomyces lilacinus* (GenBank L29262) (57% identity), and *Beauveria bassiana* (GenBank U16305) (58% identity). We are currently testing the hypothesis that this protein is Alp2 by cloning and disrupting the corresponding gene.

Transformation-mediated gene disruption of *ALP1*.

Plasmid pJM9 containing an internal 250-bp *Hin*dIII/*Sal*I fragment of *ALP1* (Figs. 3 and 5A) plus the gene for hygromycin resistance was linearized at a unique *Bam*HI restriction site and transformed into *C. carbonum* wild-type strain 367-2A. Two single-spored, hygromycin-resistant transformants (T515-1A and T515-3A) were analyzed. The restriction map

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             1                                      #         50
C. carbonum  ......MRFQ SMITAALPAL VLSAPTPQWD DVPEDSIVGG TTAAAGEYPF
F. oxysporum .......... ..MVKFASVV ALVAPLAAAA PQEIPNIVGG TSASAGDFPF
B. mori      .......... MTNSLLICFT ILGLAASSPK PIGDIRIVGG EDIVITEAPY
B. taurus    .......... .......... .......... VDDDDKIVGG YTCGANTVPY
S. griseus   MKHFLRALKR CSVAVATVAI AVVGLQPVTA SAAPNPVVGG TRAAQGEFP.
                                                    **** * * *** *
             51                                                100
C. carbonum  IVSIQLGGRH NCGGTLINGN TVVTAAHCSV SSAIGGSINN VAVRVGSLSA
F. oxysporum IVSISRNGGP WCGGSLLNAN TVLTAAHCVS GYAQSG.... FQIRAGSLSR
B. mori      QVSVMFRGAH SCGGTLVAAD IVVTAAHCVM SFAPED.... YRIRVGSSFH
B. taurus    QVSLN.SGYH FCGGSLINSQ WVVSAAHCYK S........G IQVRLGEDNI
S. griseus   MVRLSMG... .CGGALYAQD IVLTAAHCVS GSGNNTS... ..ITATGGVV
              **    * *  *** * *    *******   * *           * **
             101                                               150
C. carbonum  N...SGGQVI KVSKIIIHPS YQASTSNNDI AIWKLSSTVT AGGNIGFASL
F. oxysporum T...SGGITS SLSSVRVHPS Y..SGNNNDL AILKLSTSIP SGGNIGYARL
B. mori      Q...RDGMLY DVGDLAWHPD FNFASMDNDI AILWLPKPVM FGDTVEAIEM
B. taurus    NVVEGNEQFI SASKSIVHPS YNSNTLNNDI MLIKLKSAAS LNSRVASISL
S. griseus   DLQSSSAVKV RSTKVLQAPG YN..GTGKDW ALIKLAQP.. ....INQPTL
                 * *      **   *** *     **** ** **         *  *    *
             151                                               200
C. carbonum  AASGSDPASG STTS.VAGWG ATREGGGANN .ALLKVSVPI VARSTCVSNY
F. oxysporum AASGSDPVAG SSAT.VAGWG ATSEGGSSTP VNLLKVTVPI VSRATCRAQY
B. mori      VETNSEIPDG DITI.VTGWG HMEEGGG.NP SVLQRVIVPK INEAACAEAY
B. taurus    PTSCA..SAG TQCL.ISGWG NTKSSGTSYP DVLKCLKAPI LSDSSCKSAY
S. griseus   KIATTTAYNQ GTFTGVAGWA NR.EGGSQQR Y.LLKANVPF VSDAACRSAY
               * *   *      *****    ***        **** *** *    * * *
             201                                               250
C. carbonum  NAVGLTVTTN MVCAGV.TAG GRDSCQGDSG GPLVDANKT. ...LIGVVSW
F. oxysporum GTSA..ITNQ MFCAGV.SSG GKDSCQGDSG GPIVDSSNT. ...LIGAVSW
B. mori      SPI.YAITPR MLCAGT.PEG GKDACQGDSG GPLVH.KKK. ...LAGIVSW
B. taurus    PG...QITSN MFCAGY.LEG GKDSCQGDSG GPVV.CSGK. ...LQGIVSW
S. griseus   GNE.LVANEE I.CAGYPDTG GVDTCQGDSG GPMFRKDNAD EWIQVGIVSW
                   *    * ***   *  * ******** ** *          * * ***
             251                                    290
C. carbonum  GTGCARPNLP GVYSRVGTLR SFIDQNA$.. ..........
F. oxysporum GNGCARPNYS GVYASVGALR SFIDTYA$.. ..........
B. mori      GLGCARPEYP GVYTKVSALR EWVDENITNL RLKHILRRF$
B. taurus    GSGCAQKNKP GVYTKVCNYV SWIKQTIASN $.........
S. griseus   GYGCARPGYP GVYTEVSTFA SAIASAARTL $.........
             * ****** * ***  *  ** * **  *
                  %Similarity   %Identity
F. oxysporum           80            57
B. mori                60            39
B. taurus              62            41
S. griseus             55            33
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Fig. 4. Comparison of the predicted amino acid sequences of *ALP1* and four related trypsin-like proteases using PILEUP (Devereux et al. 1984). Sequence references: *Fusarium oxysporum*, SwissProt P35049, Rypniewski et al. (1993); *Bombyx mori*, PIR S32794, Ikeda et al. (1991); *Bos taurus*, PIR A90164, Walsh and Neurath (1964); *Streptomyces griseus*, PIR JQ1302, Olafson et al. (1975). Amino acids conserved between Alp1 and at least two of the other proteins are indicated by asterisks. Putative signal peptides were not included in the PILEUP analysis. The sequence for *B. taurus* is trypsinogen. The mature N-terminus of Alp1a is indicated by # and the stop codons by $.

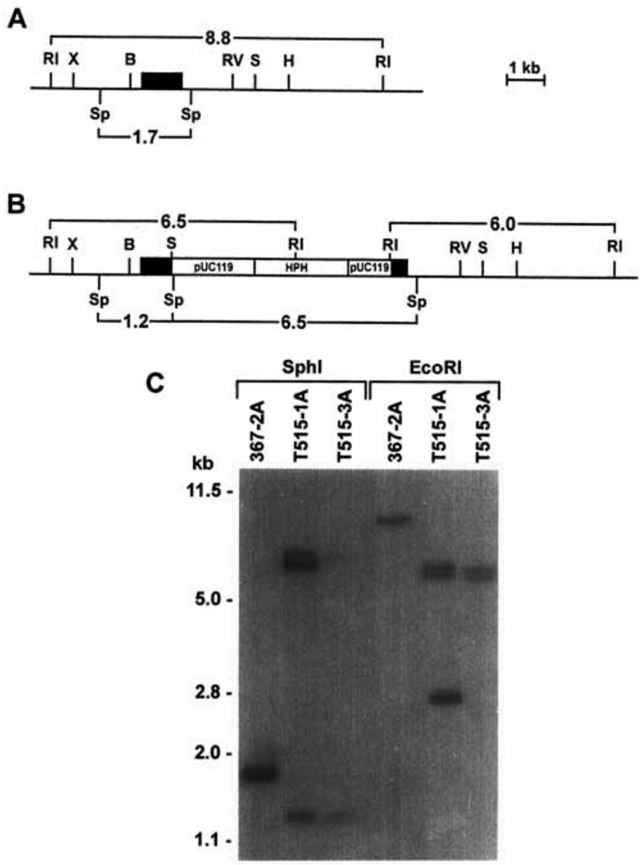

Fig. 5. A, Restriction map of wild-type locus of *ALP1* showing the location of the *ALP1* transcript. **B,** Predicted restriction map of *ALP1* with insertion of a single copy of pJM9. Predicted *Eco*RI and *Sph*I fragment sizes are indicated (in kb). **C,** DNA blot comparing wild type (367-2A) and two transformants (T515-1A and T515-3A). Isolated DNA was cut with *Sph*I (lanes 1 to 3) or *Eco*RI (lanes 4 to 6) and the blot was probed with a cDNA copy of *ALP1* (C8-6.1). The extra bands in the digests of T515-1A are as predicted for tandem multiple insertions of pJM9. The shaded boxes indicate *ALP1* sequences. RV, *Eco*RV; RI, *Eco*RI; X, *Xba*I; B, *Bam*HI; Sp, *Sph*I; H, *Hin*dIII; S, *Sal*I. Not all sites are shown. Additional sites within *ALP1* are shown in Figure 3.

of the wild type *ALP1* locus and the predicted map resulting from integration of a single copy of pJM9 at *ALP1* are shown in Figure 5A and B, respectively. The pattern of hybridization of pC8-6.1 indicates that pJM9 has integrated at *ALP1* in single and multiple copies in T515-3A and T515-1A, respectively (Fig. 5C).

Both *ALP1* mutants were analyzed for their protease profiles and pathogenicity. Total extracellular protease activity was reduced by 35 to 45% in the *ALP1* mutants. Growth of the mutants on 1% collagen was not significantly altered based on appearance or yield of DNA and total extracellular protein (data not shown). Therefore, we conclude that *ALP1* by itself is not required for growth in vitro on collagen. Activities of other extracellular enzymes of *C. carbonum* (cellulase, endo-β1,4-xylanase, endo-polygalacturanase, β1,3-1,4-glucanase, and exo-β1,3-glucanase—Scott-Craig et al. 1990; Apel et al. 1993; Sposato et al. 1995; unpublished results) were unaltered in the *ALP1* mutant, indicating that *ALP1* is also not required for functional processing of these enzymes.

Protease activities from wild type and *ALP1* mutant strains were purified as described above through cation exchange HPLC. The *ALP1* mutants lacked two peaks of activity and UV absorption corresponding to Alp1a and Alp1b (shown for mutant T515-1A in Fig. 6B). Taking into account that (i) Alp1b is about 5 kDa larger than Alp1a, (ii) Alp1b but not Alp1a is glycosylated, (iii) Alp1a and Alp1b are similarly inhibited by aprotinin and leupeptin, (iv) the product of *ALP1* has one predicted glycosylation site, and (v) disruption of *ALP1* results in the disappearance of both Alp1a and Alp1b, we conclude that Alp1a and Alp1b are products of the same gene, *ALP1*. Since Alp2 is not affected by disrupting *ALP1* (Fig. 6), we conclude that it is the product of another gene.

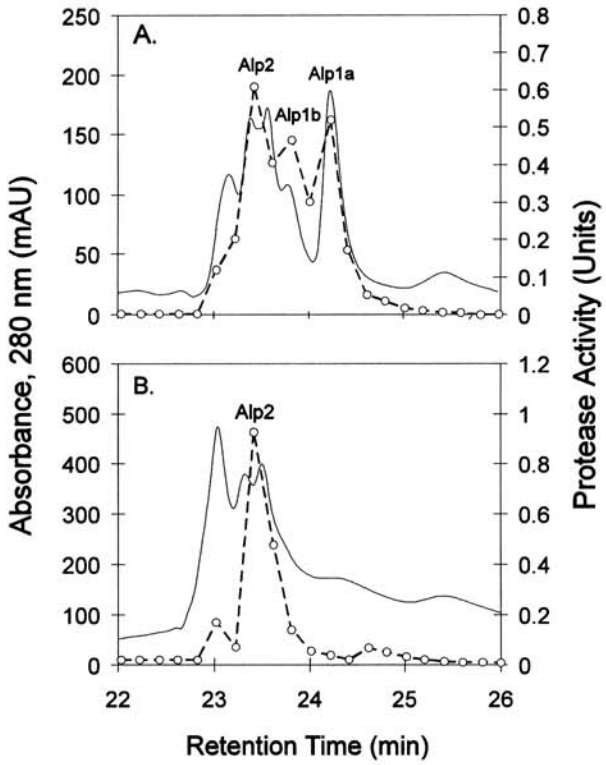

Fig. 6. Cation exchange HPLC analysis of proteins from culture filtrates of **(A)** wild type and **(B)** *ALP1* mutant T515-1A. Solid lines: OD_{280}; dashed lines: protease activity.

Pathogenicity of T515-1A and T515-3A were compared to 367-2A on both resistant (cv. Great Lakes) and susceptible (Pr × K61) cultivars of maize in the greenhouse. Rate of lesion development, lesion size, and lesion morphology were examined daily for 14 days, at which point the plants had been killed by both the wild type and the *ALP1* mutants. No differences in lesion morphology, size, color, or rate of formation between the wild type and the two mutants were observed (data not shown). Leaves with both low and high lesion densities were observed. The fungus was reisolated from the maize leaves and tested for hygromycin sensitivity. Twenty-six isolates from wild-type lesions were all hygromycin-sensitive, 50 isolates from T515-1A lesions were all resistant to hygromycin, and 35 isolates from T515-3A lesions were all resistant to hygromycin. Thus we conclude that the pathogenicity of T515-1A and T515-3A was not due to restoration of *ALP1* activity by simple excision of pJM9.

We conclude that *ALP1* is neither an essential pathogenicity factor nor a major virulence factor for *C. carbonum* race 1. Since this pathogenicity assay is not quantitative, we cannot exclude the possibility that *ALP1* makes a small contribution to virulence. Since *C. carbonum* makes at least one additional extracellular protease, it cannot be concluded from this study that proteases have no role in pathogenicity or virulence. A similar conclusion was drawn from studies of the role of *ALP*, encoding an alkaline protease, in murine respiratory mycosis caused by *Aspergillus fumigatus*; *ALP* mutants were still fully pathogenic but residual protease activity remained (Monod et al. 1993; Tang et al. 1993).

MATERIALS AND METHODS

Fungal culture growth and maintenance.

Conidia of *C. carbonum* race 1, strain 367-2A, were stored at –80°C in 25% glycerol and grown on V8 juice agar plates. For enzyme production, approximately 5×10^5 spores were inoculated into a 1-liter Erlenmeyer flask containing 200 ml of mineral salts supplemented with 0.1% yeast extract and trace elements (van Hoof et al. 1991). Substrate supplements were: Type I collagen (Sigma C-9879), casein (Sigma C-0376), maize cell walls (Sposato et al. 1995), Type A gelatin (Sigma G-2625), bovine serum albumin (Sigma A-7906), corn bran, and pectin (Sigma P-9135). For routine protease production, the fungus was grown on 1.0% collagen. Cultures were incubated at 21 to 23°C with shaking at 125 rpm for 3.5 to 4 days.

Enzyme assays.

Proteases were assayed using azocasein (Sigma A-2765) (Ansari and Stevens 1983). Azocasein (0.5 ml of a 25 mg/ml solution in 50 mM sodium phosphate, pH 7.5) was incubated with 5 to 10 µl of enzyme fraction at 45°C for 30 min. To stop the enzyme reaction, 20 µl of 50% (w/v) trichloroacetic acid was added and the solution vortexed vigorously for about 5 s. The sample was centrifuged for 5 min at 14,000 × *g* in a microcentrifuge and the OD_{410} of 200 µl measured in an ELISA plate reader (Bio-Tek). Protease inhibitors were from Sigma. Units of enzyme activity are defined as ΔOD_{410} per 10 µl enzyme fraction under the conditions described.

Protein purification and characterization.

Culture filtrates (typically 200 to 300 ml per batch) were concentrated to about 10% of the original volume by rotary evaporation under vacuum at 37°C. After centrifugation to remove insoluble material, the filtrates were dialyzed for 16 h at 4°C in cellulose dialysis tubing (SpectraPor, MWCO 12,000 to 14,000) against 25 mM sodium acetate, pH 5.0, and applied to a column (10 to 20 ml bed volume in a 60 cc disposable syringe) of DEAE-cellulose (Sigma) equilibrated in 25 mM sodium acetate, pH 5.0. The column was washed with one bed volume of 25 mM sodium acetate, pH 5.0, and all material that was eluted from the column was pooled. The samples were again concentrated by rotary evaporation to about 5 ml and dialyzed against 25 mM sodium acetate, pH 5.0. After clarification by centrifugation, the samples were fractionated on a cation exchange high-performance liquid chromatography (HPLC) column (polysulfoethylaspartamide, The Nest Group, Southboro, MA). Running conditions were a linear gradient of buffer A (25 mM sodium acetate, pH 5.0) to buffer B (25 mM sodium acetate, pH 5.0, plus 0.4 M KCl) in 20 min at 1 ml/min. The peak of UV (280 nm) absorbance corresponding to Alp1a activity was collected and further purified by hydrophobic interaction chromatography (Biogel TSK-Phenyl-5PW, Bio-Rad, Richmond, CA) after adding ammonium sulfate to a final concentration of 1.7 M. Proteins were eluted with a 20 min linear gradient of 0.1 M KH_2PO_4, pH 7.0, plus 1.7 M ammonium sulfate to water at a flow rate of 0.9 ml/min. Fractions containing Alp1a were desalted, lyophilized, and sequenced by automated Edman degradation. Alp2 was purified using the same procedure as for Alp1a through cation exchange HPLC, taking 0.2-ml fractions (Fig. 6), and then separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins in the gel were transferred to ProBlot (Applied Biosystems, Foster City, CA) (Matsudaira 1987) and the blot was stained briefly with 0.1% Coomassie R-250 in 40% methanol and destained with 50% methanol. Three proteins were visible on the blot; all three were excised and sequenced.

SDS-PAGE was carried out in 12% (w/v) acrylamide resolving gel with 5% (w/v) stacking gels (Hames and Rickwood 1981). Glycoproteins were detected by periodic acid/Schiff staining (Strömqvist and Gruffman 1992).

The pH optima for the purified enzymes was measured using buffers composed of 10 mM citric acid/20 mM sodium phosphate (pH range 5 to 7), 50 mM Tris-HCl (pH range 7 to 9), and 50 mM CAPS (3-cyclohexylamino-1-propanesulfonic acid)-HCl (pH range 9 to 11).

Extensin was purified from maize stylar tissue as described (Murphy and Hood 1993) and incubated with Alp1a, Alp1b, and Alp2 alone and in combination for 60 min or overnight at 45°C. Degradation was evaluated by SDS-PAGE. Casein was used as a control.

Nucleic acid manipulations and sequencing.

DNA was isolated as described by Pitkin et al. (1996) and RNA was isolated as described by Chomczynski and Sacchi (1987). The transcription start site of *ALP1* was determined using the Amplifinder RACE kit (Clontech, Palo Alto, CA) (Frohman et al. 1988). Reverse transcription was primed with the oligonucleotide CGTCGCTGTCCGCGTCGG (starting at nucleotide 608, see Fig. 3). PCR amplification was done using the primer sequence GTTGGTGGAACCACCGCTGCTGCCG (starting at nucleotide 450, see Fig. 3) and the "anchor" primer supplied with the RACE kit. *ALP1* was sequenced using specific oligonucleotides spaced about 250 bp apart. Sequencing was performed by automated fluorescent sequencing at the MSU-DOE-PRL Plant Biochemistry Facility using an Applied Biosystems (Foster City, CA) Catalyst 800 for Taq cycle sequencing and an Applied Biosystems 373A Sequencer for analysis of the products.

Isolation of *ALP1*.

PCR amplification of the 330-bp 5′ region of *ALP1* was performed as follows: 1× PCR reaction buffer (Gibco-BRL, Gaithersburg, MD); 0.15 mM each of dATP, dCTP, dATP, and dGTP; 1.5 mM $MgCl_2$; 2 U *Taq* polymerase (Gibco-BRL); 100 μM oligonucleotide primer; 50 ng of DNA isolated from phage lysate of a cDNA library prepared from polyA$^+$-RNA extracted from *C. carbonum* grown on maize cell walls (Pitkin et al. 1996). PCR reactions were performed in a Perkin-Elmer thermocycler model 480 under the following conditions: 1 min of denaturation at 94°C; 35 cyles of 1 min denaturation at 94°C, 2 min of annealing at 55°C, and 3 min of primer extension at 72°C; 7 min of primer extension at 72°C. PCR primers used were GAGTAYCCNTTYATHGT and GTNGCNGGNTGGGGNGC (N = any nucleotide; Y = T or C; H= A,T, or C) corresponding to the amino acid sequences EYPFIV and VAGWGA, respectively (Fig. 3). After one extraction with one volume chloroform, PCR products were precipitated with two volumes ethanol plus one-tenth volume 3 M sodium acetate, pH 5.2, by incubating for 10 min at −20°C and centrifuging 10 min in a microcentrifuge (14,000 × *g*). The resulting pellet was dried, redissolved in water, treated with T4 DNA Polymerase (Boehringer-Mannheim, Indianapolis, IN) in the presence of 1 mM dNTP's for 5 min at 37°C, and fractionated on a 1.0% agarose gel in TAE buffer (Maniatis et al. 1982). The unique product corresponding to *ALP1* was excised from the gel and purified using Gene-Clean (Bio 101, Vista, CA). The blunt-ended *ALP1* PCR product was ligated into pBluescript II SK+ at the *Sma*I site and sequenced.

Screening of the cDNA and genomic libraries, DNA blotting, probe labeling, and hybridization have been described (Scott-Craig et al. 1990; Sposato et al. 1995). DNA blotting was done with Nytran (Schleicher & Schuell, Keene, NH) and hybridizations were done in in 5× SSPE (Maniatis et al. 1982), 7% SDS, and 0.5% nonfat dry milk at 65°C for 16 h. Blots were washed twice at 22°C with 2× SSPE and 0.1% SDS for 15 min each time, and twice at 65°C in 0.1× SSPE and 0.1% SDS for 15 min each time.

Disruption of *ALP1*.

The transformation vector was constructed by cloning the 2.5-kb *Sal*I/*Hind*III fragment of pHYG1 (Apel et al. 1993) containing the *C. heterostrophus* promoter 1 driving the expression of the *hph* gene encoding hygromycin phosphotransferase (Schäfer et al. 1989) into the *Aat*II site of pUC119 to create pHYG3. Concurrently, the plasmid harboring the *ALP1* cDNA clone, pC8-6.1, was digested with *Hind*III, treated with T4 polymerase, and digested with *Sal*I. The resulting 290-bp internal fragment of *ALP1* (Fig. 3) was ligated into pHYG3 that had been digested with *Sma*I and *Sal*I to create pJM9.

This vector was linearized at the unique *Bam*HI site located within the 290-bp *ALP1* sequence (Fig. 3) and used to transform strain 367-2A of *C. carbonum*.

Preparation and transformation of protoplasts was as described (Scott-Craig et al. 1990; Apel et al. 1993). Transformants able to grow on V8 juice agar containing 100 units/ml hygromycin (Calbiochem, La Jolla, CA) were single-spored twice to obtain nuclear homogeneity. Pathogenicity was evaluated by inoculating leaves of 2-week-old maize cultivars Pr × K61 (susceptible) and Great Lakes (resistant) with 10^4 conidia/ml in 0.1% Tween-20. The whole plants as well as different leaves with different infection densities were observed daily for 2 weeks. After 1 week, individual lesions were excised and after surface sterilization in 10% (v/v) commercial bleach plus 0.1% Tween-20 plated on V8 juice agar. After 2 days of growth, the fungi were transferred to V8 juice agar plates containing hygromycin and evaluted for resistance to hygromycin.

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