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Biochemical and Antigenic Characterization of a New Dipeptidyl-Peptidase Isolated from *Aspergillus fumigatus**

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Anne Beauvais‡§, Michel Monod¶, Jean-Paul Debeaupuis‡, Michel Diaquin‡, Hidemitsu Kobayashi‖, and Jean-Paul Latgé‡

From the ‡Laboratoire des Aspergillus, Institut Pasteur, Paris, France, ¶Service de Dermatologie, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland, and the ‖Department of Hygienic Chemistry, Tohoku College of Pharmacy, Aoba-ku, Sendai, Japan

A novel dipeptidyl-peptidase (DPP V) was purified from the culture medium of *Aspergillus fumigatus*. This is the first report of a secreted dipeptidyl-peptidase. The enzyme had a molecular mass of 88 kDa and contained approximately 9 kDa of *N*-linked carbohydrate. The expression and secretion of dipeptidyl-peptidase varied with the growth conditions; maximal intra- and extracellular levels were detected when the culture medium contained only proteins or protein hydrolysates in the absence of sugars. The gene of DPP V was cloned and showed significant sequence homology to other eukaryotic dipeptidyl-peptidase genes. Unlike the other dipeptidyl-peptidases, which are all intracellular, DPP V contained a signal peptide. Like the genes of other dipeptidyl-peptidases, that of DPP V displayed the consensus sequences of the catalytic site of the nonclassical serine proteases. The biochemical properties of native and recombinant DPP V obtained in *Pichia pastoris* were unique and were characterized by a substrate specificity limited to the hydrolysis of *X*-Ala, His-Ser, and Ser-Tyr dipeptides at a neutral pH optimum. In addition, we showed that DPP V is identical to one of the two major antigens used for the diagnosis of aspergillosis.

Aspergillus fumigatus causes severe pulmonary mycosis in immunocompetent as well as immunosuppressed patients (1). Diagnosis of aspergillosis is based on the detection of antigen or, depending on the immunological status of the host, of antibodies. Consequently, knowledge of the nature of the antigens secreted by the fungus is a prerequisite for the development of efficient methods for diagnosis of this disease. SDS-polyacrylamide gel electrophoresis/Western blot experiments have shown that crude extract of *A. fumigatus* contains more than 100 antigenic molecules (2). However, only a dozen of these antigens have been purified to homogeneity (3–6). Most of them exhibit an enzymatic activity and have been identified as ribonucleases, proteases, and oxidases (5–10). In addition, catalase and chymotrypsin activities displayed by *A. fumigatus* precipitins are currently used for the differential serodiagnosis of aspergilloma patients (11). The antigenic protein displaying the catalase activity has recently been isolated (12), whereas the chymotryptic antigen had not been characterized until now. The chymotrypsin activity of the precipitin had been defined only on the basis of a colorimetric reaction resulting from the release of naphthol radicals from the hydrolysis of the substrate *N*-acetylphenylalanine naphthyl ester (NAPNE)[1] (13). Purification of the chymotryptic antigen has been attempted by affinity chromatography on ϵ-aminocaproyltryptophan methyl-ester-agarose (14) or by immunoaffinity using rabbit antiserum directed against a precipitin band recovered from a two-dimensional immunoelectrophoresis gel (15). Preliminary gel filtration chromatography coupled to rocket immunoelectrophoresis experiments identified a fraction reactive to the specific antibody, allowing purification of this "chymotryptic" antigen to homogeneity for the first time.

This paper demonstrates that the 88-kDa antigen, which has recently been purified and shown to be specific for antibody detection in aspergillosis (3), is indeed the so-called chymotryptic antigen of *A. fumigatus* used for the detection of specific anti-*Aspergillus* antibodies by immunodiffusion or counterimmunoelectrophoresis (15). The gene coding for the 88-kDa antigen was cloned and shown to contain homologies with dipeptidyl-peptidase genes. The biochemical characterization of the 88-kDa antigen isolated from *A. fumigatus* or produced as a recombinant protein in *Pichia pastoris* has shown that this antigen is indeed a new dipeptidyl-peptidase that was previously unknown in the fungal kingdom.

MATERIALS AND METHODS

Organism and Culture Conditions—A. fumigatus strain CBS 144.89 was maintained on 2% malt extract agar slants. Mycelia were obtained in fermenters after 40–48 h of culture at 25 °C in three different liquid media: (*a*) 2% (w/v) glucose + 1% (w/v) mycopeptone (Biokar) (SAB); (*b*) 1% (w/v) yeast extract (Difco) (EXL); and (*c*) 0.2% (w/v) collagen (Serva) (COLL). Preculture and culture conditions were as described previously (4). Conidia were produced on 2% malt agar in Petri dishes.

Chromatographic Purification of Dipeptidyl-Peptidase V (DPP V)—DPP V was purified as described previously (3) (Table I). An ethanol precipitate of a 44-h culture of *A. fumigatus* in 1% yeast extract medium was dissolved in 50 mM Tris-HCl, pH 8.8. Insoluble material was discarded after centrifugation (15 min, 10,000 rpm), and the supernatant was dialyzed against the same buffer at 20 mM (48 h, 4 °C). After filtration through 0.2-μm membranes (Sartorius) the extract was loaded onto a Mono Q column (Pharmacia Biotech Inc.) and was eluted in the Tris buffer with a sodium acetate gradient (0–350 mM) at a flow rate of 0.8 ml/min. DPP V active fractions (see below) were vacuum-concentrated, centrifuged (1 min, 13,000 rpm), and developed in a

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) L48074.

§ To whom correspondence should be addressed: Laboratoire des Aspergillus, Institut Pasteur, 25 Rue du Docteur Roux, 75724 Paris, Cedex 15, France. Tel.: 33-1-45-68-82-25; Fax: 33-1-45-68-84-20.

[1] The abbreviations used are: NAPNE, *N*-acetylphenylalanine naphthyl ester; DPP, dipeptidyl-peptidase; HPLC, high performance liquid chromatography; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; ZPCK, *N*-CBZ-L-phenylalanine chloromethyl ketone; pNA, paranitroanilide; NA, β-naphthylamide; MNA, methoxy-β-naphthylamide; PCR, polymerase chain reaction; kb, kilobase pair(s); Z(NO_2), 4-nitrobenzylocarbonyl.

TABLE I
Purification of the DPP V

Step	Total protein	Total enzyme activity	Specific activity	Yield	-Fold purification
	mg	*units*[a]	*units/mg*	%	
EtOH precipitate	55,626	567	0.010	100	1.0
Water-soluble fraction	33,450	383	0.011	67	0.89
Mono Q	14,563	256	0.018	45	1.73
Gel filtration (Superdex)	1,026	215	0.210	37.9	20.56
Propac PA1	447	175	0.392	30.9	38.5

[a] One unit of enzyme activity is defined as the amount of the enzyme required to hydrolyze 400 μmol of pNA in 22 °C in 1 h.

Superdex 75 HR 10/30 gel filtration column (Pharmacia) in the same buffer supplemented with 150 mM sodium acetate. Collected fractions, corresponding to the 80–95-kDa size range, were dialyzed and then loaded onto a Propac PA1 anion-exchange HPLC column (Dionex) and eluted in Tris buffer with a sodium acetate gradient (0–500 mM). DPP V activity of the fractions was monitored between each purification step using Ala-Ala paranitroanilide (pNA) as substrate in conditions described below. Purified active fractions were stored at −20 °C.

Electrophoresis—SDS-polyacrylamide gel electrophoresis on 7.5% acrylamide was done as described previously (3) after boiling the samples in a buffer containing 0.5% (w/v) SDS and 1.25% (v/v) mercaptoethanol. Deglycosylation of DPP V was performed using pNGase F (Oxford Glycosystems) as described previously (3). Two-dimensional electrophoresis was performed on the horizontal system Multiphor II (Pharmacia) according to the manufacturer's instructions using Immobilon pH 3–10 Dry Strip for the first dimension and an Excel Gel SDS 8–18% gradient in the second dimension. 7.5% acrylamide nondenaturing gels were prepared as described previously (16). Proteins were stained with Coomassie Blue or silver nitrate (3).

Immunoassays—Human anti-*Aspergillus* antisera from patients with aspergilloma (provided by J. P. Bouchara, CHR, Angers, France) were pooled. Monospecific mouse anti-DPP V antiserum was obtained from mice after 15 days of repeated inhalation of *A. fumigatus* conidia suspension.[2] Preimmune mouse serum and human sera from *Candida* patients were used as control sera.

Antigens used for Western blot experiments were the following: (*a*) culture filtrates of the EXL, COLL, and SAB media; (*b*) supernatant of intracellular mycelial or conidial extracts obtained after centrifugation at 12,000 × *g* of mycelium (grown in EXL and SAB) or conidia (produced on malt agar) disrupted in a MSK Braun cell homogenizer in a 50 mM Tris buffer, pH 7.5, under CO_2 cooling; (*c*) a supernatant of an aqueous conidial suspension ultrasonicated in a bath (Branson 2200 at 40 watts) for 1 h; and (*d*) purified DPP V. After electrophoresis, samples were electrotransferred onto nitrocellulose membranes and immunoblotted as described previously (3) using a 1:1000 dilution of antisera and their respective peroxidase-conjugated anti-IgG (H+L) antibodies (Sigma).

Counterimmunoelectrophoresis on cellulose acetate membrane (Sartorius) was done as described previously (11) using 15-μl aliquots of undiluted patient serum and antigen extracts (culture filtrate of a 1-month static culture of *A. fumigatus* in SAB medium concentrated under vaccuum). Membranes were washed with 0.9% NaCl before staining with the NAPNE reagent or mouse antiserum followed by anti-mouse IgG peroxidase conjugate.

Enzymatic Reactions—All enzymatic reactions used 2–3 μg of DPP V. NAPNE hydrolysis was visualized by incubating enzymatic fractions in a fresh mixture of 0.1 ml of dimethylformamide containing 250 μg of NAPNE with 1 ml of 50 mM Tris-HCl, pH 7.5, containing 500 μg of *O*-dianisidine at 25 °C. This method was used in both solutions and gels. NAPNE hydrolysis can also be quantified at 545 nm after addition of 250 μl of dimethyl sulfoxide to the reaction mixture. Using this substrate, the following inhibitors were tested: antipain (86 μM, Boehringer Mannheim); bestatin (136 μM, Boehringer Mannheim); chymostatin (0.14 μM, Boehringer Mannheim); E-64 (117 μM, Boehringer Mannheim); leupeptin (84 μM, Boehringer Mannheim); pepstatin (60 μM, Boehringer Mannheim); EDTA (5.6 mM, Sigma); aprotinin (6.3 μM, Boehringer Mannheim); TLCK (46 nM, Sigma); TPCK (48 nM, Sigma); ZPCK (51 nM, Sigma); and diethyl *p*-nitrophenyl phosphate (2–20 mM, Sigma).

Dipeptidyl-peptidase activity was estimated using different pNA derivatives of peptides at 0.4 mM concentration in 100 μl of 50 mM Tris-HCl, pH 7.5, at 25 °C: Gly-Pro pNA (Sigma); Ala-Pro pNA (Saxon Biochemicals GmbH); Ala-Ala pNA (Saxon Biochemicals GmbH); Lys-Ala pNA (Sigma); Gly-Arg pNA (Sigma); Arg-Pro pNA (Sigma); Gly-Phe pNA (Sigma); Lys-Ala pNA (Sigma); *N*-acetyl-Ala-Ala-Ala pNA (Sigma); *N*-succinyl-Gly-Gly-Phe pNA (Sigma); Ala-Ala-Phe pNA (Sigma); *N*-acetyl-Ala pNA (Sigma); L-Lys pNA (Sigma); L-Phe pNA (Sigma); and *N*-succinyl-Ala-Ala-Pro-Leu pNA (Sigma). The coloration was measured at 405 nm after 15 min to 1 h. β-Naphthylamide (NA)- or methoxy-β-naphthylamide (MNA)-conjugated dipeptides were also used at 0.4 mM: Lys-Pro MNA (Sigma); Arg-Arg NA (Sigma); Ser-Tyr NA (Sigma); His-Ser MNA (Sigma); and Leu-Gly NA (Sigma). For both NA and MNA derivatives, substrate hydrolysis was quantified by fluorimetry at 335-nm excitation and 405-nm emission wavelengths.

The influence of pH on DPP activity was evaluated in 50 mM Tris-HCl buffer from pH 6.5 to 9 and in 50 mM sodium acetate buffer from pH 3.5 to 6 using the dipeptides Ala-Ala pNA, Lys-Ala pNA, His-Ser MNA, and Ser-Tyr NA.

K_m values were determined in 50 mM Tris-HCl, pH 7.5, with several dipeptides (Ala-Ala pNA, Lys-Ala pNA, His-Ser MNA, and Ser-Tyr NA) at concentrations ranging from 0.0125 to 1.6 mM.

For inhibition studies, different dilutions of inhibitors were added to 50 mM Tris-HCl buffer, pH 7.5. Enzyme and 0.4 mM Ala-Ala pNA were then added. The inhibitors tested on the pure DPP V were: phenylmethylsulfonyl fluoride (0.2 and 2 mM); diisopropyl fluorophosphate (0.2 and 2 mM, Sigma); Pefabloc (16 and 32 mM, Boehringer Mannheim); phosphoramidon (0.7 and 1.4 mM, Boehringer Mannheim); Lys-[$Z(NO_2)$]-pyrolidide (10 and 80 μM); and Lys-[$Z(NO_2)$]-thiozolidide (10 and 80 μM).

Chymotrypsin from porcine pancreas (1291 units/mg, U. S. Biochemical Corp.) was used as a control enzyme at 0.5 μg in 50 mM Tris-HCl, pH 7.5. Inhibition experiments using the same molecules as for DPP V were performed with 0.4 mM *N*-succinyl-Ala-Ala-Pro-Leu pNA, which is a specific substrate for chymotrypsin.

Amino Acid Sequence Determination of Peptide Fragments of DPP V—To obtain a peptide sequence of DPP V, the protein was excised from a 7.5% SDS-polyacrylamide gel electrophoresis (16 cm) preparative gel or from an Immobilon polyvinylidene difluoride or Problott (Applied Biosystems) membrane after blotting. Sequencing of internal peptides obtained by endolysin and trypsin digestion was performed as described previously (17, 18) with the following modifications: the peptides were injected into a DEAE HPLC column linked to a C_{18} reversed-phase HPLC column and eluted with an acetonitrile, 0.1% trifluoroacetic acid gradient of 2–45%. The NH_2-terminal peptide sequencing was performed as described previously (19). Sequencing was performed using an Applied Biosystems 470 gas phase sequencer. Spectra were recorded with an Applied Biosystems 1000S detector.

Cloning and DNA Sequencing of DPP V—A degenerate oligonucleotide, 5′ ACN GAR GAR CTY TGG TTY ATG CA 3′, defined by the internal amino acid sequence TEELWFMG, was synthesized with a DNA synthesizer (Millipore), labeled with ^{32}P, and used to screen a bacteriophage λ EMBL 3A *Sau*3A genomic library of *A. fumigatus* (20) as described previously (21). The cloning vector was Bluescript SK^+ plasmid (Stratagene). A ^{32}P-labeled *Sal*I genomic DNA fragment was used as a hybridization probe to screen a cDNA library of *A. fumigatus* constructed in λgt 11 from RNA of *A. fumigatus* grown in COLL medium (5). Labeling of DNA was performed using a random primed DNA labeling kit (Boehringer Mannheim) and [α-^{32}P]dCTP. The transfer of the phage plate on the nylon Hybond N^+ and the hybridization conditions were according to the manufacturer's instructions (Amersham Corp.). The cloning vector was also Bluescript SK^+ plasmid (Stratagene).

Double-stranded DNA was sequenced using the Sequenase version 2.0 DNA sequencing kit (U. S. Biochemical Corp.) and [α-^{35}S]dATP according to the manufacturer's instructions. DNA sequence data were analyzed using the University of Wisconsin Genetics Computer Group program (22). The sequences of the genomic DNA of DPP V will appear in the GenBank™/EMBL Sequence Data Bank under accession number L48074.

[2] A. Beauvais, M. Monod, J.-P. Debeaupuis, M. Diaquin, H. Kobayashi, and J.-P. Latgé, manuscript in preparation.

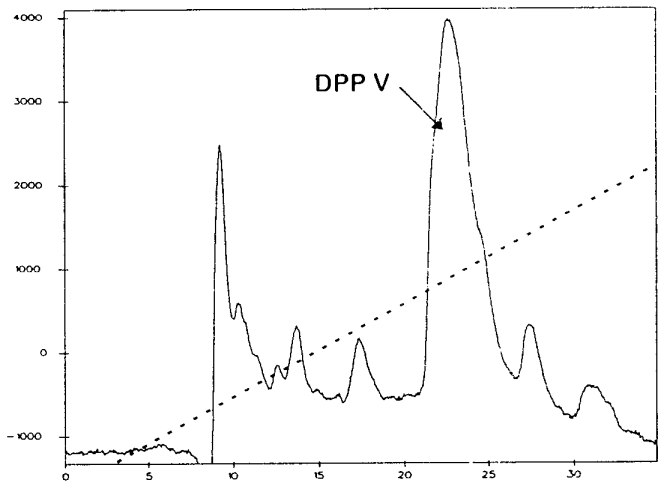

FIG. 1. **Separation of DPP V by ion-exchange chromatography (Propac PA1, Dionex) with a sodium acetate linear gradient (0–350 mM in 27 min).**

To obtain the first bases at the 5′ end of DPP V, PCR was performed using two homologous primers based on the genomic DNA sequence: 5′ TC ATG GGA GCT TTC CGC TGG 3′ (bases 324–342) and 5′ TC GGA CAA CCA GAC AAT 3′ (antisense, bases 697–713). The total cDNA from the library was used as template. Thirty cycles were run, consisting of a 1-min 95 °C melting step, a 1-min 60 °C annealing step, and 1-min 70 °C extension. The PCR product was cloned into the *Eco*RI site of the cloning vector pCR™II provided by Invitrogen following the manufacturer's instructions (TA cloning kit, Invitrogen).

Expression of the DPP V Gene in the Yeast P. pastoris—The expression vector used was pHIL-S1 provided by the *Pichia* expression kit (Invitrogen). The DPP V cDNA was obtained using the PCR technique and the same program described above. The conserved sequences encoded by the primers are based on the genomic sequence: o1, 5′ GC GAA TTC CTT ACA CCT GAG CAG CTA ATC 3′ (bases 380–390 and 461–470), corresponding to the NH_2-terminal peptide of the protein; o2, 5′ GC AGA TCT TGG TAG TTG CAA CCT GAA GTA 3′ (antisense, bases 2988–3008), corresponding to a fragment localized downstream from the C-terminal extremity of the protein.

The total cDNA from the library was used as template. The PCR product was digested by *Eco*RI and *Bgl*II and inserted into the *Eco*RI and *Bam*HI sites of pHIL-S1. For the transformation in *P. pastoris*, the expression plasmid was linearized at the *Bgl*II site of pHIL-S1.

P. pastoris strain GS 115 (his 4) (Invitrogen) was used in the expression study. Yeast transformation was performed according to the spheroplast method described in the manual for version E of the *Pichia* expression kit (Invitrogen).

RESULTS

The Major Antigen of A. fumigatus Has a Dipeptidyl-Peptidase Activity—A preliminary experiment showed that the antigenic 88-kDa protein previously purified by Kobayashi *et al.* (3) hydrolyzed NAPNE but not complex proteins such as azocasein (data not shown), suggesting a peptidase or esterase activity for this antigen. The use of several peptidase and esterase substrates indicated that the fraction obtained from the Propac column containing the 88-kDa antigen (Fig. 1) displayed a dipeptidyl-peptidase activity (EC 3.4.14) able to cleave Ala-Ala pNA substrates. The positive fraction contained a protein doublet with molecular sizes of 87 and 88 kDa (Fig. 2). Deglycosylation experiments resulted in the appearance of a single band at 79 kDa, suggesting that the protein doublet of 87–88 kDa corresponded to two forms of the same protein with different glycosylation levels (Fig. 2). This result was confirmed by two-dimensional analysis of the doublet (data not shown). This protein was named DPP V for dipeptidyl-peptidase V.

In Western blot experiments, this doublet was recognized by human anti-*Aspergillus* antibodies as well as by the sera from mice following conidia inhalation (Fig. 2). Immunoblotting experiments with mouse antiserum and extracts from disrupted

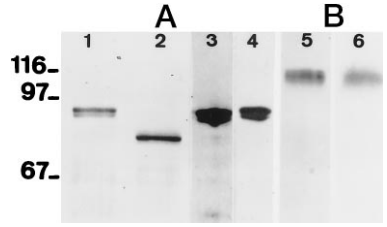

FIG. 2. **Proteic and antigenic characterization of DPP V.** *A*, 7.5% SDS-polyacrylamide gel electrophoresis gel stained with silver nitrate showing DPP V (*lane 1*) and its deglycosylated form (*lane 2*) using pNGase F. *Lane 3*, human serum test; immunoblot analysis of DPP V using a 0.1% dilution of a pool of sera from aspergilloma patients and anti-human peroxidase conjugate. *Lane 4*, mouse serum test; immunoblot analysis of DPP V using a 0.1% dilution of a mouse anti-DPP V antiserum and anti-mouse peroxidase conjugate. *B*, 7.5% acrylamide nondenaturing gel experiment showing that DPP V (stained with Coomassie Blue in *lane 1*) hydrolyzes *N*-acetylphenylalanine naphthyl ester in 2 ml of dimethylformamide and 20 ml of Tris-HCl, pH 7.5, containing 10 mg of *O*-dianisidine (*lane 2*).

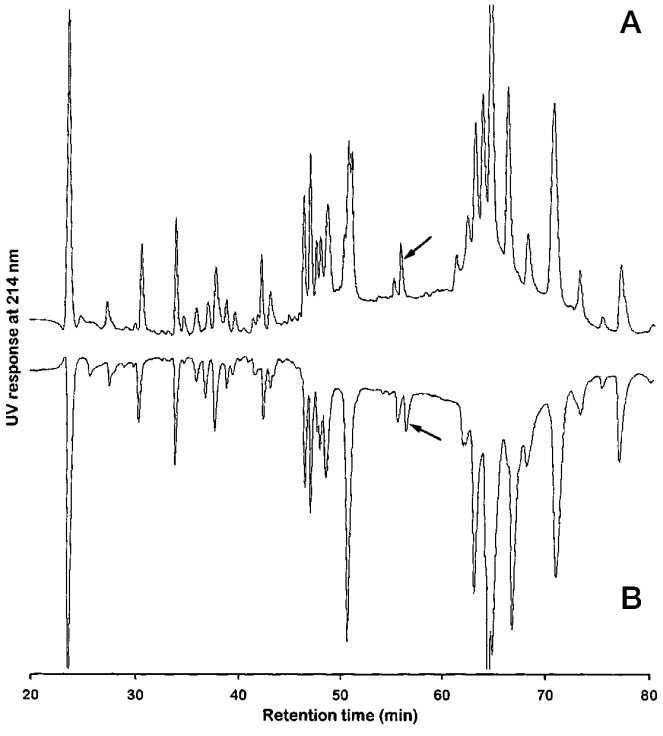

FIG. 3. **Chromatographic patterns of the endolysin digests of the 87- (*A*) and 88-kDa (*B*) DPP V on a reverse phase column (2–45% gradient of acetonitrile, 0.1% trifluoroacetic acid, 214 nm, 5 mm/min).** Note the position of the randomly selected peptide of 87 and 88 kDa on the chromatographic patterns (*arrow*) displaying the same sequence, KLAYF.

A. fumigatus cells showed that DPP V was present in both conidia and mycelia. The intracellular amount of DPP V in the mycelia grown in 1% yeast extract was 20 times higher than that in conidia or in mycelia from a SAB culture medium. Secretion of DPP V was 50 times higher in a protein (COLL) medium than in a protein hydrolysate (EXL)-based medium, whereas DPP V was not detectable in the culture filtrate of a 2% glucose + 1% mycopeptone medium (SAB). DPP V could also be released from conidia after a 1-h bath ultrasonication of a conidial suspension (data not shown).

Precipitin bands formed between sera from aspergilloma patients and total *Aspergillus*-soluble extracts are able to cleave NAPNE to release naphthol, which can be visualized by *O*-dianisidine (12). Chymotryptic activity, which has been known for a long time as a characteristic criterion for the

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  1 MGAFRWLSIAAAASTALALTPEQLITAPRRSEAIPDPSGKVAVFSTSQYSPETHKRTSWWSLLDLKTGQTKVLTNDSSVS  80

 81 EIVWLSDDSILYVNSTNADIPGGVELWVTQASSFAKGYKAASLPASFSGLKAAKTKSGDIRFVAYGQQSYPNGTAYNEELA 160

161 TAPLSSARIYDSIYVRHWDYWLSTTFNAVFSGTLKKGHGKNGYSLDGELKNLVSPVKNAESPYPPFGGASDYDLSPDGKW 240

241 VAFKSKAPELPKANFTTSYIYLVPHDASETARPINGPDSPGTPKGIKGDSSSPVFSPNGDKLAYFQMRDETYESDRRVLY 320

321 VYSLGSKKTIPSVAGDWDRSPDSVKWTPDGKTLIVGSEDLGRTRLFSLPANAKDDYKPKNFTDGGSVSAYYFLPDSSLLV 400

401 TGSALWTNWNVYTAKPEKGVIKKIASANEIDPELKGLGPSDISEFYFQGNFTDIHAWVIYPENFDKSKKYPLIFFIHGGP 480

481 QGNWADGWSTRWNPKAWADQGYVVVAPNPTGSTGFGQALTDAIQNNWGGAPYDDLVKCWEYVHENLDYVDTDHGVAAGAS 560

561 YGGFMINWIQGSPLGRKFKALVSHDGTFVADAKVSTEELWFMQREFNGTFWDARDNYRRWDPSAPERILQFATPMLVIHS 640

641 DKDDYRLPVAEGLSLFNVLQERGVPSRFLNFPDENEWVVNPENSLVWHQQALGWINKYSGVEKSNPNAVSLEDTVVPVVNYN 721
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FIG. 4. **Predicted amino acid sequence of DPP V of *A. fumigatus*.** The signal sequence is *underlined*. *Double underlining* indicates the corresponding peptide sequences that have been determined with a protein sequence. *Double-thickness letters* indicate the Gly^{558}-*X*-Ser^{560}-*X*-Gly^{562} consensus and the catalytic triad Ser^{560}, Asp^{643}, His^{675}.

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mouse DPP IV        520 YQ...M......ILPPHFDK..SKKYPLLLDVYAGPCSQKADASFRLNWATYLASTENII
rat DPP IV          527 YQ...M......ILPPHFDK..SKKYPLLIDVYAGPCSQKADAAFRLNWATYLASTENII
human CD 26         526 YQ...M......ILPPHFDK..SKKYPLLLDVYAGPCSQKADTVFRLNWATYLASTENII
yeast DPAP B        567 KD...ILVNSYEILPNDFDETLSDHYPVFFFAYGGPNSQQVVKTFSVGFNEVVASQLNAL
A. fumigatus DPP V  447 FQGNFTDIHAWVIYPENFDK..SKKYPLIFFIHGGPQGNWAD.GWSTRWNPKAWADQGYV

mouse DPP IV        569 VASFDGRGSGYQGDKIMHAINRRLGTLEVEDQIEAARQFV..KMGFVDSKRVAIWGWSYG
rat DPP IV          576 VASFDGRGSGYQGDKIMHAINKRLGTLEVEDQIEAARQFL..KMGFVDSKRVAIWGWSYG
human CD 26         575 VASFDGRGSGYQGDKIMHAINRRLGTFEVEDQIEAARQFS..KMGFVDNKRIAIWGWSYG
yeast DPAP B        624 VVVVDGRGTGFKGQDFRSLVRDRLGDYEARDQISAASLYG..SLTFVDPQKISLFGWSYG
A. fumigatus DPP V  504 VVAPNPTGSTGFGQALTDAIQNNWGGAPYDDLVK.CWEYVHENLDYVDTDHGVAAGASYG

mouse DPP IV        627 GYVTSMVLGSGSG.VFKCGIAVAPVSRWEYYDSVYTERYMGLPIPE...DNLDHYRNSTV
rat DPP IV          634 GYVTSMVLGSGSG.VFKCGIAVAPVSRWEYYDSVYTERYMGLPTPE...DNLDHYRNSTV
human CD 26         633 GYVTSMVLGSGSG.VFKCGIAVAPVSRWEYYDSVYTERYMGLPTPE...DNLDHYRNSTV
yeast DPAP B        682 GYLTLKTLEKDGGRHFKYGMSVAPVTDWRFYDSVYTERYM..HTPQ...ENFDGYVESSV
A. fumigatus DPP V  563 GFMINWIQGSPLGRKFKALVSHDGTFVADAKVSTEELWFMQREFNGTFWDARDNYRRWDP

mouse DPP IV        683 MSRAEHFK.QVEYLLIHGTADDNVHFQQSAQISKVLVDAGVD.FQAMWYTDEDHGIASST
rat DPP IV          690 MSRAENFK.QVEYLLIHGTADDNVHFQQSAQISKALVDAGVD.FQAMWYTDEDHGIASST
human CD 26         689 MSRAENFK.QVEYLLIHGTADDNVHFQQSAQISKALVDVGVD.FQAMWYTDEDHGIASST
yeast DPAP B        737 HNVTALAQ.ANRFLLMHGTGDDNVHFQNSLKFLDLLDLNGVENYDVHVFPDSDHSIRYHN
A. fumigatus DPP V  623 SAPERILQFATPMLVIHSDKDYRLPVAEGLSLFNVLQERGVPS.RFLNFPDENHWVVNPE

mouse DPP IV        741 AHQHIYSHMSHFLQQCFSLH.....................
rat DPP IV          748 AHQHIYSHMSHFLQQCFSLR.....................
human CD 26         747 AHQHIYTHMSHFIKQCFSLP.....................
yeast DPAP B        796 ANVIVFDKLLDWAKRAFDGQFVK..................
A. fumigatus DPP V  682 NSLVWHQQALGWINKYSGVEKSNPNAVSLEDTVVPVVNYN
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FIG. 5. **Alignment of the predicted DPP V protein (amino acids 443–710) of *A. fumigatus* with the corresponding segment of rat and mouse DPP IV enzyme, human CD 26, and yeast dipeptidyl aminopeptidase B.** Sequences were from the Swiss-Prot data bank with accession numbers p27487 (human CD 26), p28843 (mouse DPP IV), p14740 (rat DPP IV), and p18962 (yeast dipeptidyl aminopeptidase B). The Gly^{558}-*X*-Ser^{560}-*X*-Gly^{562} consensus and the catalytic triad Ser^{560}, Asp^{643}, His^{675} are indicated by *arrows*.

serological diagnosis of aspergillosis, was ascribed to this enzymatic reaction. The purified DPP V degraded NAPNE either in gels or in solution (Fig. 2). Counterimmunoelectrophoresis experiments showed that the precipitin band that displayed the chromogenic reaction with NAPNE was also recognized by the monospecific mouse anti-DPP V antiserum (data not shown). These results indicated that the so-called chymotryptic antigen of *A. fumigatus* was identical to DPP V.

Molecular Characterization of DPP V—Chromatographic patterns of the endolysin digests of the 87- and 88-kDa DPP V isolated polypeptides were identical (Fig. 3). In addition, the sequences of two selected peptides of the 87- and 88-kDa species with the same position on the chromatogram were identical (Fig. 3). These results were in accordance with the *N*-deglycosylation experiments and showed that the two members of the protein doublet corresponded to the same protein with differently sized *N*-linked sugar moieties. The NH_2-terminal amino acid sequence of DPP V was LTPEQLITAPRRSEAIPDPSGKVA. One internal peptide generated after trypsin digestion of DPP V with the sequence KVSTEELWFMQ was used to design an oligonucleotide probe on the basis of the amino acid sequence TEELWFMG and the codon usage for the genes encoding alkaline protease (23), metalloprotease (24), and restrictocin (7) of *A. fumigatus*. This oligonucleotide probe was used to screen the *A. fumigatus* genomic library, and seven positive clones were identified. Restriction enzyme analysis of purified bacteriophage DNA revealed that the seven clones had a common 1.0-kb *Sal*I fragment that hybridized with the oligonucleotide probe. This fragment was subcloned and used for screening 100,000 plaques from the constructed λgt 11 *A. fumigatus* cDNA library. Four hybridizing clones were isolated. The longest cDNA, of 2.2 kb, was sequenced. In addition to the 1.0-kb *Sal*I fragment, a genomic sequence hybridizing with the whole 2.2-kb cDNA was located on another *Sal*I fragment of 4 kb. 2.0 kb of the nucleotide sequence of the latter fragment and the entire sequence of the 1.0-kb *Sal*I fragment were compared to the sequence of the cloned cDNA. The amino acid sequence deduced from the genomic nucleotide sequence suggested that the NH_2-terminal portion of the mature enzyme was preceded by a polypeptide signal of 18 amino acids. A short nucleotide sequence encoding amino acids four positions downstream from the initial Met were missing in the cloned cDNA. PCR experiments using two homologous primers (primer 1, bases 324–342; primer 2, bases 697–712; genomic sequence accession number L48074) and the total cDNA as template confirmed the presence of this 18-amino acid signal sequence.

The genomic sequence of DPP V contained 7 introns of 53–94 base pairs. They were located at the beginning (before 860 base

TABLE II
Substrate specificity of the DPP V and commercial chymotrypsin (Sigma)

Activity is expressed in μmol of substrate hydrolysed per μg of recombinant DPP V or μg of chymotrypsin. The reaction was performed at 22 °C for 1 h at pH 7.5.

Substrates	DPP V	Chymotrypsin
Gly-Pro pNA	0	0
Ala-Pro pNA	0	0
Gly-Phe pNA	21	55
Ala-Ala pNA	43	0
Lys-Pro MNA	0	ND[a]
Lys-Ala NA	53	0
Ser-Tyr NA	32	0
His-Ser MNA	48	0
Gly-Arg pNA	0	ND[a]
Leu-Gly NA	0	ND[a]
Arg-Arg NA	0	ND[a]
N-Acetyl-Ala pNA	0	0
Phe pNA	0	ND[a]
Ala-Ala-Phe pNA	0	ND[a]
N-acetyl-Ala-Ala-Ala pNA	0	0
Gly-Gly-Phe pNA	0	ND[a]
N-Succinyl-Ala-Ala-Pro-Leu pNA	0	440
NAPNE[b]	+	+

[a] ND, not done.
[b] Not compared because NAPNE is not a pNA or an arylamide derivative.

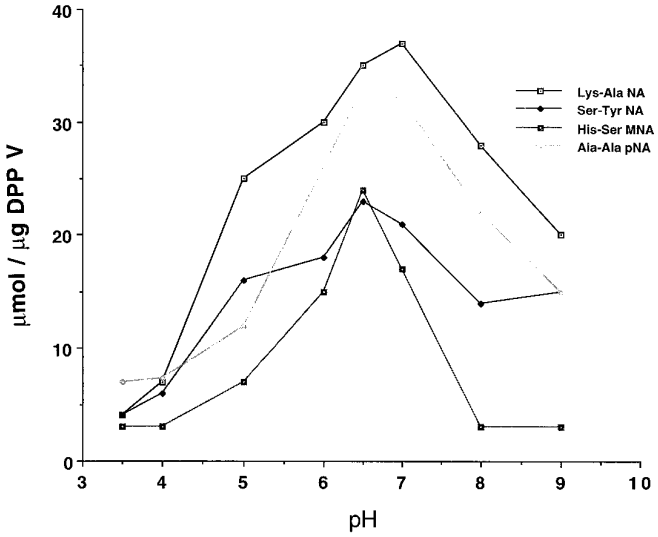

FIG. 6. **Influence of pH on the activity of DPP V.** Reactions were performed at 25 °C for 15 min using Ala-Ala pNA, Lys-Ala MNA, Ser-Tyr NA, and His-Ser NA as substrates. Activity is expressed in μmol of substrate hydrolyzed/μg of DPP V.

pairs from the 5′ end) and in the second half (after 1870 base pairs from the 5′ end) of the sequence. The open reading frame of DPP V contained 2163 base pairs that code for 721 amino acids, accounting for an estimated size of 79 kDa. The deduced amino acid sequence is shown in Fig. 3. The open reading frame started with a short signal sequence of 18 amino acids containing a hydrophobic stretch of 8 amino acids (WLSIAAAA) and the secretion consensus ALA just before the NH_2-terminal sequence (Fig. 4).

The amino acid sequence showed homology to DPP IV from rat, mouse, and human and to dipeptidyl aminopeptidase B from yeast (Fig. 5). This homology is located at the C-terminal end of the DPP V protein of *A. fumigatus* and the conserved structural domain of 200 amino acids from the C terminus of the DPP IV and dipeptidyl aminopeptidase B proteins. This stretch of DPP V also contained the putative catalytic triad of DPP IV arranged in the same topological order (Ser^{560}, Asp^{643}, His^{675}; Fig. 5). No homology was found with other serine proteases such as chymotrypsin or subtilisin. The dipeptide substrate specificity, the conserved structural domain, the same topological order of the catalytic triad, the absence of homology to chymotrypsin and subtilisin, and the presence of the Gly-*X*-Ser-*X*-Gly consensus motif (position 558–562; Fig. 5) of classical serine proteases or other enzymes with serine in their active site confirmed that DPP V (87–88 kDa) belongs to the nonclassical serine hydrolases of the subfamily of dipeptidyl-peptidases (EC 3.4.14).

The cDNA corresponding to the mature DPP V (without the 18 amino acids of the signal sequence) was cloned in the expression vector pHIL-S1. The DPP V expressed by *P. pastoris* GS 115 was secreted in the culture medium upon induction of expression with methanol at a rate of 0.15 mg/ml with maximum production after expression for 48 h. The molecular mass of the recombinant DPP V, which consisted of an 87–88-kDa doublet, was identical to that of chemically purified DPP V (data not shown). The deglycosylation of the doublet also resulted in the formation of a single band of 79 kDa, suggesting that the recombinant DPP V was glycosylated in *P. pastoris* in a similar way as in *A. fumigatus* (data not shown). The recombinant DPP V was also recognized by the specific mouse anti-DPP V antiserum.

Biochemical Characterization of the Native and Recombinant DPP V—The native and recombinant DPP V displayed the same substrate specificities; among the dipeptidyl-peptidase substrates tested, *X*-Ala dipeptides such as Ala-Ala pNA and Lys-Ala pNA were preferentially cleaved. The reaction was linear for 30 min. However, the hydrolytic specificity was not exclusively restricted to these dipeptides since His-Ser MNA and Ser-Tyr NA were also cleaved by DPP V (Table II). Yet, this peptidase did not cleave the *X*-Pro dipeptide, which is specifically hydrolyzed by the DPP IV class. Other substrates, such as mono- or tripeptides or the specific chymotryptic substrate Ala-Ala-Pro-Leu pNA, were not hydrolyzed by DPP V. Commercial chymotrypsin hydrolyzed only Ala-Ala-Pro-Leu pNA. Dipeptides having Phe in position 2, such as Gly-Phe pNA, were hydrolyzed in a nonspecific way by DPP V or commercial chymotrypsin and esterases (data not shown). The apparent K_m values, determined using Lineweaver-Burk plots, were 0.4 mM for Ala-Ala pNA, 0.26 mM for Lys-Ala pNA, 0.44 mM for His-Ser MNA, and 0.37 mM for Ser-Tyr NA (data not shown).

DPP V was active over a very large range of pH values (6–8), with a pH optimum at 6.5 regardless of the substrate used (Fig. 6).

No specific inhibitor of DPP V was found (Table III), although 100% inhibition by boiling confirmed the enzymatic activity of this protein. Classical inhibitors of the serine proteases such as diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, and Pefabloc blocked the activity of chymotrypsin but did not inhibit DPP V at the same concentrations. The low inhibition of DPP V obtained with Pefabloc did not seem specific since the decrease in activity was not concentration-dependent. Phosphoramidon, a specific inhibitor of neutral endopeptidases that was inactive on the chymotrypsin activity at 1.4 mM, reduced the velocity of DPP V activity. However, the amount of substrate hydrolyzed was the same after a 30-min reaction in the absence of inhibitor and after a 1-h incubation in the presence of the inhibitor. The specific inhibitors of dipeptidyl-peptidase IV (the Lys-[$Z(NO_2)$]-pyrolidide and the Lys-[$Z(NO_2)$]-thiozolidide) did not affect the cleavage of Ala-Ala pNA by DPP V. All the other proteolytic inhibitors tested (antipain, bestatin, chymostatin, E-64, leupeptin, pepstatin, EDTA, aprotinin, TLCK, TPCK, ZPCK, and diethyl *p*-nitrophenyl phosphate) were without any effect on DPP V.

TABLE III
Inhibition of the DPP V of A. fumigatus and the commercial chymotrypsin (Sigma)

Activity is expressed as percentage of inhibition obtained. Reactions were performed at 22 °C for 1 h at pH 7.5 for the case of phosphoramydon, in which the reaction was performed for 30 min.

	Inhibitors	DPP V	Chymotrypsin
		%	%
Diisopropyl fluorophosphate	0.2mM	0	95
	2 mM	0	100
Phenylmethylsulfonyl fluoride	0.2mM	0	98
	2 mM	0	100
Pefabloc	2 mM	ND[a]	ND[a]
	16 mM	20	100
	32 mM	30	100
Phosphoramydon	0.7mM	48	0
	1.4mM	60	0
Lys-[$Z(NO_2)$]-pyrrolidide	10 μM	0	
	80 μM	0	ND[a]
Lys-[$Z(NO_2)$]-thiozolidide	10 μM	0	
	80 μM	0	ND[a]

[a] ND, not done.

DISCUSSION

Several proteases have been isolated from *A. fumigatus*. They belong to the serine protease, aspartyl-protease, or metalloprotease families (5, 6, 10). Some of them are intracellular, whereas others are secreted in the culture medium. However, no dipeptidyl-peptidases have been described previously in *A. fumigatus*. In fungi only two dipeptidyl-peptidases have been purified previously, and only from *Saccharomyces cerevisiae*: dipeptidyl aminopeptidase A and B (25, 26). Both enzymes act on the same *X*-Pro dipeptides and are membrane-associated (25, 26).

The dipeptidyl-peptidase isolated from *A. fumigatus* does not belong to any of the four classes of dipeptidyl-peptidases reported in the literature. It is not a DPP IV because *X*-Pro dipeptides were not released and DPP V does not bind to collagen (data not shown) (27, 28). Nor is it a DPP II because DPP V was unable to release tripeptides such as Ala-Ala-Ala and *X*-Pro dipeptides, as do the DPP IIs from bovine anterior pituitary gland (29) and bovine dental pulp (30). DPP IIIs are characterized by the ability to release the dipeptide Arg-Arg (29), which is not cleaved by DPP V of *A. fumigatus*. However, DPP V appears to be more closely related to the DPP I isolated from human splenic lysozomes, which can release the dipeptides His-Ser, Ser-Tyr, and Ala-Ala but not *X*-Pro (31). Yet, in contrast to DPP I, which also uses Gly-Arg as substrate and is highly active at pH 5, the DPP V of *A. fumigatus* was mostly active between pH 6 and 8, with a maximum at pH 6.5. Moreover, it is the only DPP that is secreted and not membrane-associated, as are the other dipeptidyl-peptidases. For example, dipeptidyl aminopeptidase A and B of *S. cerevisiae* have been localized to the membranes of vacuoles and the Golgi apparatus, respectively (25, 26). Consequently, the DPP of *A. fumigatus* belongs to a new class of dipeptidyl-peptidases named DPP V. No homolog of DPP V had been found in *A. fumigatus* by Southern blot analysis under low stringency hybridization conditions (data not shown).

The function of DPP V of *A. fumigatus* is presently unknown. However, current information suggests that its role may be 2-fold. First, DPP V may play a nutritional role related to the metabolism of dipeptides. The expression and secretion of DPP V in *A. fumigatus* is dependent on the external environment, and the highest level of DPP V was observed in mycelium or culture filtrate when the medium contained only protein or protein hydrolysate. The same culture conditions favor the secretion of neutral proteases that are able to degrade the extracellular matrix of the fungus (23). The product of hydrolysis of the proteases could then be processed by a family of dipeptidases produced by *A. fumigatus*. A homolog of a DPP IV has also recently been identified in *A. fumigatus*.[2] It is possible that *in vivo* the dipeptides generated by the action of DPP V can then be used as a source of amino acids for fungal growth. In *Candida albicans*, multiple peptide permeases have been reported, and in *S. cerevisiae*, genetic experiments have demonstrated the presence of a di- and/or tripeptide transporter (32). Second, this molecule may affect host defense mechanisms and in particular trigger T-cell activation, which has recently been shown to be essential for the treatment of *Aspergillus* infection (33). Stimulation of T-cell populations by animal and human DPPs has already been demonstrated (34–36). A putative role of DPP V in the immune defense reaction against *A. fumigatus* is suggested by the identity between DPP V and the major chymotryptic antigen of *A. fumigatus*. In addition, recent unpublished studies have demonstrated a possible role of DPP V in protection against infection in a murine model of aspergillosis. Mice surviving infection with *A. fumigatus* have antibodies monospecifically recognizing DPP V.[2] As DPP V is present in the spores of *A. fumigatus* and is easily released after a short ultrasonication, a quick release of the enzyme from the spores at the beginning of infection is probable. The presence of this molecule may activate the T-cell population and trigger host defense mechanisms. The role of the 87–88-kDa DPP V in the activation of T-cells is presently under study. Such antigenic molecules produced by a fungus and triggering host defense reactions have been described in *Cryptococcus neoformans*, *Candida albicans*, and *Histoplasma capsulatum* (37–39). In *C. neoformans*, the glucuronoxylomannan constituent of the capsule can elicit protective antibodies (37). In *C. albicans*, a molecular complex of mannoproteins of 65 kDa stimulated the production of cytokines interleukin-2 and interferon-γ. This suggests activation of $CD4^+$ Th1 cells, which is considered of protective significance (38). In *H. capsulatum*, two antigens (62 and 80 kDa) can immunize mice by stimulating cell-mediated immune responses (39).

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