Cloning, Expression, and Substrate Specificity of MeCPA, a Zinc Carboxypeptidase That Is Secreted into Infected Tissues by the Fungal Entomopathogen *Metarhizium anisopliae*

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To date zinc carboxypeptidases have only been found in animals and actinomycete bacteria. A cDNA clone (MeCPA) for a novel fungal (*Metarhizium anisopliae*) carboxypeptidase (MeCPA) was obtained by using reverse transcription differential display polymerase chain reaction to identify pathogenicity genes. MeCPA resembles pancreatic carboxypeptidases in being synthesized as a precursor species (418 amino acids) containing a large amino-terminal fragment (99 amino acids). The mature (secreted) form of MeCPA shows closest amino acid identity to human carboxypeptidases A1 (35%) and A2 (37%). MeCPA was expressed in an insect cell line yielding an enzyme with dual A1 + A2 specificity for branched aliphatic and aromatic COOH-terminal amino acids. However, in contrast to the very broad spectrum A + B-type bacterial enzymes, MeCPA lacks B-type activity against charged amino acids. This is predictable as key catalytic residues determining the specificity of MeCPA are conserved with those of mammalian A-type carboxypeptidases. Thus, in evolutionary terms the fungal enzyme is an intermediate between the divergence of A and B forms and the differentiation of the A form into A1 and A2 isoforms. Ultrastructural immunocytochemistry of infected host (*Manduca sexta*) cuticle demonstrated that MeCPA participates with the concurrently produced endoproteases in procuring nutrients; an equivalent function to digestive pancreatic enzymes.

The vertebrate pancreas synthesizes and secretes a subset of zinc carboxypeptidases that hydrolyze alimentary proteins and peptides from their COOH-terminal ends. These are traditionally classified into the A types (with a preference for apolar COOH-terminal residues) and the B types (with a preference for basic COOH-terminal residues) (1, 2). In contrast, actinomycetes (bacteria) produce carboxypeptidases with a dual A + B specificity toward both neutral and basic substrates and the distinct vertebrate A and B types presumably arose from such a precursor (3, 4). Why or when in the course of evolution a single carboxypeptidase with both A and B specificities was abandoned in favor of multiple enzymes with more limited specificities is not known. The classification of carboxypeptidases into the A and B forms has been further expanded with the identification of the A1 and A2 isoforms in rat and humans. Carboxypeptidase A1 preferentially catalyzes aliphatic COOH-terminal residues of peptide substrates, while the A2-type selectively acts on the bulkier aromatic COOH-terminal residues, being the only isoform that shows specificity toward tryptophan (2, 5, 6). Carboxypeptidase A2 is apparently absent from bovine pancreas, so in contrast the single carboxypeptidase gene has a relatively broad substrate specificity (2). A number of non-digestive zinc carboxypeptidases involved in hormone and neuropeptide processing, bioactive peptide activation or inactivation, or functional modulation of regulatory proteins have also been reported in the literature expanding the field of interest in metallocarboxypeptidases (1). Aside the actinomycete enzymes (7) there is very limited information on the categories and genealogies of carboxypeptidases present in species other than a few animals. To date among fungi, almost all studies have focused on serine carboxypeptidases and enzymes from *Aspergillus* spp. (8), *Penicillium* (3), *Mucor racemosus* (9) and yeasts (4) have been thoroughly characterized with regard to physiochemical properties and specificity. However, an open reading frame predicted to encode a metallocarboxypeptidase (YHT2) was identified as part of the *Saccharomyces cerevisiae* genome project (10) suggesting that these enzymes may also play a role in protein processing in fungi. Given the limited information about metallocarboxypeptidases, isolation and characterization of carboxypeptidases from species intermediate between bacteria and vertebrates might help to confirm their differential character and evolutionary pathways and to understand the molecular reasons for their specific functional properties. We report here the cDNA cloning of a carboxypeptidase A (MeCPA) that is secreted by an insect pathogenic fungus *Metarhizium anisopliae* into infected tissues. *M. anisopliae* produces a very large number of isoforms of different peptidases that often hinders the rapid purification of the correct protein (11). Consequently, Pro-MeCPA was over-expressed in an insect cell line to produce soluble catalytically active protein in quantities amenable to functional analysis and free of any possible fungal contaminants. Similarly human carboxypeptidase A2 was expressed in *Pichia pastoris* in order to characterize its activation pathway and facilitate its potential for biotechnology (12).

The expressed MeCPA resembled mammalian enzymes in demonstrating an A-type specificity toward aromatic and bulky aliphatic P1 side chains, and contrasted with bacteria in possessing only marginal B-type activity against charged residues. This substrate specificity was associated with hydrophobic amino acids in the S1 subsite that are mostly either the same, or conserved substitutions with those in A-type carboxypeptidases. Thus, the separation into distinct A- and B-types ap-
pears to have occurred early in the evolution of the eukaryotes, and despite distant phylogenetic relationships, hypotheses concerning structural determinants contributing to the A-type specificity of mammalian enzymes (2) are backed in the fungal counterpart.

**Experimental Procedures**

**Organism and Growth**—M. anisopliae strain ARSEF 2575 (formerly ME-1) was maintained on potato dextrose agar.

**Preparation and Analysis of Culture Filtrates of M. anisopliae**—Standardized mycelial inocula (5 g wet weight) from 48-h Sabouraud dextrose broth cultures were incubated with shaking (100 rpm) for 14 h in 100 ml of minimal media (0.05% KH₂PO₄, 0.01% MgSO₄; pH 6) supplemented with insect cuticle or alternate carbon and/or nitrogen sources at 1% (w/v) (13). Clean samples of cuticle from 3-day-old fifth instar *Manduca sexta* larvae were prepared as described previously (14). Cuticle was obtained from the giant cockroach (*Blaberus giganteus*) by extracting soft tissue from homogenized insects with sodium tetraborate (15). Samples of culture for enzyme assay were clarified by filtration through Whatman 1 filter paper and centrifugation (1,800 g for 10 min at 4 °C).

**Enzymatic Reactions**—The rate of hydrolysis against a range of carboxypeptidase substrates was continuously monitored spectrophotometrically in 50 ml Tris-HCl, 0.5% NaCl, pH 7.5, at 25 °C. The wavelengths used to monitor the various reactions were as follows: Bz-Gly-Arg, 254 nm; Cbz1-Gly-Gly-Phe, Cbz-Gly-Gly-Leu, Cbz-Gly-Gly-Ala, and Cbz-Gly-Phe, 225 nm; Cbz-Gly-Trp and Cbz-Gly-Tyr, 235 nm (2). Initial rates were obtained at substrate concentrations bracketing the Km value whenever possible. The values of the kinetic parameters, kcat and Km, were obtained by direct fit of 5 points using a nonlinear least squares regression analysis (16). Bovine carboxypeptidase (Type 1) and enzyme substrates were supplied by Sigma.

**Cloning and DNA Sequencing of MeCPA**—Differential display was used to identify genes that are induced when *M. anisopliae* grows on insect cuticle (17). Total RNA from *M. anisopliae* cultures grown in the presence or absence of insect cuticle was isolated with Tri-Reagent (Molecular Research Center) from homogenized fungal mycelia and analyzed using the RNaseq DNA microarray analysis was performed (17). The 454-base pair MeCPA PCR band differentially expressed in cuticle grown cultures (Fig. 1) was isolated, re-amplified, and ligated into pCRII (Invitrogen) and transformed into One Shot competent cells (Invitrogen). The plasmid DNA was purified using the Plasmid Mini Kit (Qiagen). Purified plasmid DNA fragments were sequenced using a 373 Stretch DNA Sequencer (Applied Biosystems) on both strands.

The PCR fragment was 32P-labeled by random primer and used to screen a λ ZAP II cDNA library constructed from mycelia grown on insect cuticle (17). Total RNA from *M. anisopliae* cultures grown in the presence or absence of insect cuticle was isolated with Tri-Reagent (Molecular Research Center) from homogenized fungal mycelia and analyzed using the RNAseq DNA microarray analysis was performed (17). The 454-base pair MeCPA PCR band differentially expressed in cuticle grown cultures (Fig. 1) was isolated, re-amplified, and ligated into pCRII (Invitrogen) and transformed into One Shot competent cells (Invitrogen). The plasmid DNA was purified using the Plasmid Mini Kit (Qiagen). Purified plasmid DNA fragments were sequenced using a 373 Stretch DNA Sequencer (Applied Biosystems) on both strands.

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**Construction and Purification of a Baculovirus Expression Vector (BEV) for MeCPA (BEV-MeCPA)**—The MeCPA gene was isolated as a 1.5-kb EcoRI DNA fragment from a Bluescript/MeCPA and ligated into the pBacPAK8 transfer vector plasmid (CLONTECH Laboratories, Inc., Palo Alto, CA). Spodoptera frugiperda cells (SF-21) were co-transfected with AcMNVP DNA (1.5 μg) and the pBacPAK8/MeCPA plasmid DNA (3 μg) using Lipofectin (Life Technologies, Inc., Gaithersburg, MD) as described (18). Recombinant virus (AcMNVP/MeCPA) in which the polyhedrin gene was replaced with the MeCPA gene was isolated by the absence of polyhedral in infection loci of tissue culture plaques using SF-21 cells (19). The progeny recombinant virus was purified with four sequential plaque isolations.

To express MeCPA in insect cells (designated BEV-MeCPA), Hi-5 cells (Invitrogen) were infected with AcMNVP/MeCPA at a multiplicity of infection of 10 plaque forming units per cell. MeCPA activity versus Bz-Gly-Arg, 254 nm; Cbz1-Gly-Gly-Phe, Cbz-Gly-Gly-Leu, Cbz-Gly-Gly-Ala, and Cbz-Gly-Phe, 225 nm; Cbz-Gly-Trp and Cbz-Gly-Tyr, 235 nm (2). Initial rates were obtained at substrate concentrations bracketing the Km value whenever possible. The values of the kinetic parameters, kcat and Km, were obtained by direct fit of 5 points using a nonlinear least squares regression analysis (16). Bovine carboxypeptidase (Type 1) and enzyme substrates were supplied by Sigma.

**Preparation of Antibodies and Immunoblotting**—MeCPA expressed in insect cells and purified by affinity chromatography was analyzed by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was stained with 0.5% Coomasie Blue, and the single 35-kDa protein band was excised with a scalpel. The gel slices were lyophilized, ground into a powder, and resuspended in a volume of water equal to one-half of the original volume. The suspension was divided into three aliquots, mixed with Freund’s complete adjuvant, and injected at 14-day intervals into New Zealand White rabbits. Antibodies were affinity purified on a Affi-Gel column (Bio-Rad) and used to detect MeCPA protein by Western blot.

For Western blot analysis, proteins were transferred from gels to nitrocellulose and immunoblotted as described previously (14). Blots were developed with the ProtoBlot Western blot AP system (Promega). NH₂-terminal sequence analysis of proteins blotted on nylon membranes followed previously used procedures (15).

**Immunogold Labeling**—Cuticles from fifth-instar *M. sexta* larvae were excised, soaked in a saturated solution of phenylthiourea (to inhibit phenoloxidase) inoculated with conidia, and following incubation for up to 48 h, processed for electron microscopy as described previously (15) using antibodies to MeCPA. Ultrathin sections of LR White-embedded tissue were placed in blocking solution (15) for 1.5 h. The sections were then washed in distilled water and stained for contrast in 4% (w/v) uranyl acetate in 50% (v/v) ethanol for 20 min. Observations were made with a Zeiss EM10 transmission electron microscope.

Specificity of the labeling was determined by: (i) incubation of the sections with serum obtained before the rabbits were immunized, (ii) omission of primary antisera, and (iii) treatment with Protein A prior to treatment with Protein A-gold.

**RESULTS**

**cDNA Cloning and Sequence Analysis**—A PCR product of a *M. anisopliae* transcript specifically expressed during growth on insect host cuticle was isolated using differential display (Fig. 1). Approximately 0.1% of the clones in a λ ZAP II cDNA library constructed from mycelia grown on insect cuticle hybridized to the radiolabeled PCR product. Complementary DNA from 10 hybridization positive clones was isolated at the preparative level and the longest clone, called MeCPA, was entirely sequenced in both directions. The nucleotide sequence
### FIG. 2. The nucleotide sequence of MeCPA and deduced amino acid sequence.

An in-frame start codon and stop codon (TAA) are shown by underlines. Cysteine residues with the potential of forming two disulfide bonds as in pancreatic carboxypeptidases are indicated by arrows. Potential cleavage sites for the signal and pro-sequence sites of MeCPA are shown. The nucleotide sequence was submitted to GenBank database (accession number U76003).
and the corresponding amino acid sequence of the protein (MeCPA) are shown in Fig. 2.

The analyzed full-length cDNA insert contained standard 5'- and 3'-flanking regions and an open reading frame of 1254 base pairs coding for 418 amino acids. Analysis of the NH2-terminal amino acid sequence of the primary translation product suggests the existence of a hydrophobic 23-amino acid signal peptide which is presumably cleaved during expression of the inactivezymogen. The format of the signal includes a charged residue (Arg), a core of hydrophobic residues and a helix breaking residue (Pro), five residues before a peptidase cleavage site (SPV). This is consistent with the empirical rules of fungal presecretory sequences and is similar to the NH2-terminal sequence of M. anisopliae subtilisin proteinase (Pr1) (13). The NH2-terminal of the BEV-expressed protein was determined by microsequencing to be GTIQAYAA. This sequence was located 99 residues from the NH2 terminus of the 418 amino acid sequence (Fig. 2). The predicted Mw of the mature protein is 35,151.

Southern analysis with MeCPA produced a single approxi-
mately 2-kilobase fragment (Fig. 3), indicating that it is a single copy gene as this band is too small to contain more than one gene copy.

Sequence Comparisons—That MeCPA belongs to the A group of digestive metalloendopeptidases is suggested by the comparison of its overall amino acid sequence with proteins of other organisms. The most closely related proteins are human carboxypeptidase A2 (37% identity) and carboxypeptidase A1 (35% identity), rat carboxypeptidase A2 (35% identity), bovine carboxypeptidase A (34% identity), and the hypothetical yeast carboxypeptidase YHT2 (33% identity). YHT2 showed 31% identity to human carboxypeptidase A2 (Fig. 4). By contrast, bacterial carboxypeptidases show about 25% identity to the pancreatic enzymes (22). The fungal carboxypeptidases show much less overall sequence similarities to the regulatory enzymes (Fig. 5). In fact MeCPA and human carboxypeptidases share only a 15% amino acid identity with the non-digestive mammalian carboxypeptidases.

The fungal carboxypeptidases (MeCPA and YHT2) show highest local identity with the domains of zinc carboxypeptidases that contain residues important for catalysis and for the delineation of the active site (22–24). These include residues involved in zinc binding (His69, Glu72, and His196), catalysis (Glu270 and Arg287), and substrate anchoring and positioning (Arg71, Asn144, Arg145, Pro250, Ile247, Tyr248, and Asp256). However, Asn144 and Arg145 are replaced by Asp and His in YHT2 so these particular residues can no longer be regarded as strictly invariant in zinc carboxypeptidases. The S2 (Arg71, Ser197, Tyr198, and Ser199) and S3 (Phe279) subsites involved in binding and torsion of peptide substrates are the same in pancreatic enzymes, MeCPA and YHT2, although they are variable among the regulatory zinc carboxypeptidases (22).

Variations between carboxypeptidases occur at residues that form the S1 specificity pocket (positions 194, 203, 243, 250, 253, 255, and 268) and are responsible for the observed differences in primary specificity (2, 22, 24). In Table I substitutions in these positions are compared for MeCPA, YHT2, and zinc carboxypeptidases with different specificity types. The B-type specificity for positively charged residues is determined by a negatively charged residue (Asp) in positions 253 or 255, i.e. at the center of the binding pocket of the catalytic domain (22, 25). MeCPA and A-type carboxypeptidases have no equivalent Asp residue; they possess a hydrophobic Ile residue at position 255 (Leu in the yeast enzyme) that is believed to be a critical parameter in determining a specificity for hydrophobic substrates (25). Conversely, the presence of an Asp residue at position 194 in MeCPA and YHT2, replacing a hydrophobic residue in A-types, will decrease the hydrophobic character of the active site. MeCPA resembles the human A-type enzymes in possessing the flexible Met at position 203, and also resembles the A2 isoform and bovine carboxypeptidase in possessing small Gly and Ser residues at positions 253 and 254 (Fig. 4). These substitutions should provide a stronger hydrophobic character and favor the enlargement of the specificity cavity to facilitate the recognition of substrates with bulkier aromatic residues (23). However, while also adding to the pockets hydrophobic character, the Val250 in MeCPA will reduce the pocket size versus the A2 form. MeCPA resembles most other carboxypeptidases in possessing a Thr residue at 268, in place of Ala in carboxypeptidase A2.

Kinetic Properties of Bovine Carboxypeptidase and MeCPA—To confirm that the protein encoded by MeCPA is a carboxypeptidase, we expressed MeCPA cDNA in insect cells and compared the kinetic properties of affinity purified enzyme (Fig. 6) with bovine carboxypeptidase against typical substrates for carboxypeptidases (Table II). For both enzymes the most effectively hydrolyzed substrates contained P1 phenylalanine, and substituting with leucine, tyrosine, or tryptophan reduced activity. The catalytic efficiencies (kcat/Km) of MeCPA toward Cbz-Gly-Phe and Cbz-Gly-Trp substrates is higher than bovine carboxypeptidase by 1.8- and 2.0-fold, respectively, reflected primarily in increased Km values exhibited by MeCPA. The catalytic efficiency of bovine carboxypeptidase toward the Tyr substrate is 3.3-fold higher than MeCPA reflected in both Km and Kcat values. The Kcat values of MeCPA and bovine carboxypeptidase are approximately the same for Cbz-Gly-Gly-Phe and Cbz-Gly-Gly-Leu, although the Km values of MeCPA are lower than bovine carboxypeptidase by 1.7- and 4.5-fold, respectively. MeCPA showed 100-fold less activity against typical substrates for carboxypeptidase B (Hippuryl-Arg, Cbz-Gly-Arg) compared with Cbz-Gly-Phe, and no activity against amionopeptidase (p-nitroanilide derivatives of Ala, Leu, and Pro) or endopeptidase (Hide protein azure, casein) substrates.

Production of MeCPA in Culture—Antibodies to MeCPA were raised in rabbit, affinity purified, and found to recognize recombinant 35-kDa MeCPA and a 35-kDa band in culture filtrates of M. anisopliae grown on proteins (Fig. 7). Passing culture filtrates through a carboxypeptidase affinity column eliminated the immunoreactive band confirming that it is MeCPA. The immunoreactive band and CPA activity versus CBZ-Gly-Gly-Phe were produced in cultures in unsupplemented minimal medium indicating that limitation of carbon...
and nitrogen source derepressed CPA secretion, but the presence of host cuticle or proteins was required for secretion of high levels of MeCPA. Secretion was repressed when NH₄Cl was added to media containing protein showing that nitrogen repression overrides the enhancing effect of polymeric substrates. Consistent with regulation at the level of transcription, a 1.2-kilobase MeCPA transcript is present in very low abundance in a peptone medium but is highly expressed during growth on insect cuticle (Fig. 8).

**Localization of the MeCPA in Vivo**—Conidia of *M. anisopliae* germinated to form appressoria on the surface of *M. sexta* and penetrated cuticles within 40 h of inoculation as described previously (15, 14). Using ultrathin sections and immunogold cytchemistry we explored the ultrastructural localization of MeCPA during these infection processes. The localization of sections from cuticles at 24 and 48 h post-infection with antibodies to MeCPA revealed labeling in and over the appressorial wall confirming production by prepenetration fungal structures (Fig. 9). Production continued during penetration with the growth of hyphae through the cuticle (Fig. 10). The enzyme remained localized in the cell wall and the immediate vicinity of the fungal structures. Very few gold particles were found intracellularly.

Control sections (see “Experimental Procedures”) showed only a very low level of nonspecific binding of gold particles (0.08 ± 0.03 gold particles per mm²), with the results for all

**Fig. 4.** Amino acid alignment of MeCPA with those of bovine procarboxypeptidase A (accession number EMBL Z33996), human procarboxypeptidase A1 (accession number SwissProt P15085), human procarboxypeptidase A2 (accession number SwissProt P48052), and yeast putative carboxypeptidase (accession number SwissProt P38836). The numbering system is made according to the bovine A enzyme (17). Identical amino acids are boxed together. Asterisks are placed over the functionally important residues, which are discussed in the text. Open triangles above the alignments identify the cysteine residues that form disulfide bonds in pancreatic enzymes.
controls being negative (data not shown), confirming that use of antisera to the overexpressed carboxypeptidase in conjunction with the Protein A-gold complex is a valid labeling method for detecting the enzyme in insect cuticle.

**DISCUSSION**

The significant number of partially or completely known sequences of mammalian and bacterial procarboxypeptidases has permitted the formulation of some evolutionary hyptohe-

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**FIG. 5.** **Position of MeCPA in a phylogenetic tree of the metalloproteinase superfamily.** The nodes have been placed according to the distance between sequence pairs. The units on the bottom scale indicate the number of substitution events. Accession number: rat CPB1 (rat carboxypeptidase A1), SwissProt P00731; human CPA1 (human pancreatic carboxypeptidase A1), SwissProt P15085; BosCPBA (bovine carboxypeptidase A), EMBL Z33906; human CPA2 (human carboxypeptidase A2), SwissProt P48052; ratCPB2 (rat carboxypeptidase A2), SwissProt P19222; yeast YHT2 (putative carboxypeptidase-nucleotide sequence in S. cerevisiae chromosome VIII), SwissProt P39586; ThermCPT (Thermoplasma acidophilum carboxypeptidase T), SwissProt P29068; StreptCPA (Streptomyces griseus carboxypeptidase), SwissProt P39041; human CPN (human carboxypeptidase N), SwissProt P15169; human CPM (human carboxypeptidase M), SwissProt P14384.

**FIG. 6.** **Affinity chromatography of M. anisopliae carboxypeptidase expressed in an insect cell line.** The Gly-Gly-L-tyrosyl-azo-benzylsuccinate affinity column was equilibrated and washed with 20 mM MES, 0.1 M NaCl (pH 6.0) until the A₂₈₀ had receded to base line. Enzyme was eluted with a linear gradient of Tris-HCl (20 mM, pH 7.5)-buffered NaCl (0.1–0.4 M). The flow rate was 15 ml h⁻¹ and 5-ml fractions were collected. Symbols are: square, carboxypeptidase activity versus Cbz-Gly-Gly-Phe; ○, absorbance at 280 nm; - - -, NaCl concentration.

**TABLE I**

| Specificity | Enzymes         | Positions |
|-------------|-----------------|----------|
|             | Type            | 194      | 203      | 243      | 250      | 253      | 254      | 255      | 268      |
| A           | Human Cp1       | Ser      | Met      | Ile      | Ala      | Ser      | Thr      | Ile      | Thr      |
|             | Human Cp       | Ile      | Met      | Ile      | Ala      | Gly      | Ser      | Ile      | Ala      |
|             | Bos CpBA       | Ser      | Met      | Val      | Val      | Gly      | Ser      | Ile      | Thr      |
|             | McCPA           | Asp      | Met      | Ala      | Gly      | Ser      | Ala      | Leu      | Q        |
|             | YHT2            | Asp      | Leu      | Ala      | Gly      | Ser      | Ala      | Leu      | Q        |
| B           | Bos CPBB       | Thr      | Leu      | Val      | Thr      | Asp      | Thr      | Thr      | Thr      |
| A + B       | Therm CPT      | Thr      | Leu      | Ala      | Thr      | Asp      | Met      | Thr      | Thr      |
|             | Strept CPA     | Asp      | Leu      | Ser      | Thr      | Ser      | Ile      | Asp      | Thr      |
ses. The calculated time of divergence between bovine carboxypeptidase A and *Thermoactinomyces* carboxypeptidase (about 1300 millions years ago) led Rawlings and Barrett (7) to suggest an endosymbiont origin for the family in eukaryotes. The discovery of a metallocarboxypeptidase from a "primitive" (fungal) eukaryote opens up new perspectives in this field by providing insights into when in the course of evolution the different forms of carboxypeptidase diverged.

A detailed evolutionary tree has not yet been drawn for the metallocarboxypeptidases, and we do not assume that the tree of contemporary sequences we present (Fig. 5) is an evolutionary one. However, it confirms the structural classification of the fungal enzymes inside the digestive group rather than the regulatory group. The bacterial enzymes are also in the digestive group. However, the degree of similarity between bacterial and animal digestive enzymes (25%) is less than that between fungal and animal enzymes consistent with their greater phylogenetic distance. The bacterial enzymes also differ from the eukaryote digestive carboxypeptidases in their dual A and B specificity. It is proposed that an ancestor of bovine carboxypeptidases gave rise to separate A and B forms before the radiation of mammals (1). The restricted A-type specificity of MeCPA for neutral residues suggests that this specialization occurred during the early development of eukaryotes and perhaps was already present in the originating endosymbiont. The A-type specificity of mammalian enzymes is associated with Ile or Leu in positions 243 and 255 (22). MeCPA possesses the equally hydrophobic Val243 and Ile255, whereas in the A + B bacterial enzymes the same positions are alternatively occupied by Ala-Ser243 and Thr/Asp. The substrate specificity of the yeast enzyme (YHT2) has not been determined but with Ala243 and Leu255 it may represent an intermediate stage. The ability

| Substrate       | MeCPA   | Bovine CPBA |
|-----------------|---------|-------------|
|                 | $K_M$  | $k_{cat}$  | $(k_{cat}/K_M) \times 10^{-5}$ | $K_M$  | $k_{cat}$  | $(k_{cat}/K_M) \times 10^{-5}$ |
|                 | $\mu M$| s$^{-1}$   | $s^{-1}$ |                          | $\mu M$| s$^{-1}$   | $s^{-1}$ |                          |
| Cbz-Gly-Phe     | 373 ± 11| 27.8 ± 1.2 | 0.74 |                          | 846 ± 48| 34.5 ± 1.6 | 0.41 |                          |
| Cbz-Gly-Tyr     | 615 ± 8 | 7.6 ± 0.3  | 0.12 |                          | 300 ± 16| 12.0 ± 0.6 | 0.40 |                          |
| Cbz-Gly-Trp     | 454 ± 22| 12.6 ± 0.4 | 0.27 |                          | 2320 ± 25| 31.6 ± 2.2 | 0.13 |                          |
| Cbz-Gly-Gly-Phe | 96 ± 6 | 128 ± 4   | 13.33 |                          | 166 ± 11| 156 ± 4.3 | 9.40 |                          |
| Cbz-Gly-Gly-Leu | 115 ± 19| 64 ± 3    | 2.97 |                          | 974 ± 61| 67.6 ± 2.5 | 0.69 |                          |
| Cbz-Gly-Gly-Ala | Tr$^a$ |          |      |                          |        |            |      |                          |

$^a$ Tr, low rates of hydrolysis of Cbz-Gly-Gly-Ala precluded determination of kinetic constants.

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**FIG. 7.** Examination of secreted MeCPA activity and demonstration by immunoblotting that antibody raised against MeCPA in a baculovirus expression vector reacts with a same sized protein in culture filtrates of *M. anisopliae*. Proteins in 0.5 ml of crude culture filtrates exhibiting MeCPA activity versus CBZ-Gly-Gly-Phe were concentrated 100-fold and separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and tested with antibodies raised against BEV-MeCPA (lane 1). Molecular weight markers are as indicated. MeCPA activity in culture filtrates is expressed as the amount of enzyme that released 1 μmol of CBZ-Gly-Gly-Phe/min at 25 °C.

**FIG. 8.** Detection of the MeCPA mRNA during growth of *M. anisopliae* on cockroach cuticle. Poly(A)$^+$ RNA isolated from mycelium of *M. anisopliae* grown for 32 h in SDB and transferred for 12 h to minimal media supplemented with 1% cockroach cuticle (+) or 1% peptone (−).

**FIG. 9.** Protein A-gold labeled MeCPA produced by a *M. anisopliae* appressorium on excised *M. sexta* cuticle (24 h after inoculation). AP, appressorium; Ep, epicuticle; Pc, procuticle. Bar, 0.5 μm.
Fungal Carboxypeptidase A

Fig. 10. Protein A-gold labeled MeCPA produced by M. anisopliae during penetration of excised M. sexta cuticle (48 h after inoculation). CW, cell wall; Pc, procuticle; PH, penetrant hyphae. Bar, 0.5 µm.

to cleave off positively charged residues always depends on the presence of an Asp residue, but as shown by the two related bacterial enzymes (Table I), its position is not exactly fixed (22). MeCPA showed only marginal activity against Arg residues so Asp cannot provide B-type specificity.

MeCPA shows similar overall amino acid identity to human carboxypeptidase A1 (35%) and A2 (37%). Even though there are no reports of A2 in bovine, and taking into account the broad specificity of the known enzyme, it has been proposed that mammalian carboxypeptidases A1 and A2 are derived from the duplication of an ancestor of the bovine carboxypeptidase gene before the radiation of mammals (23). MeCPA provides the first direct evidence for a common ancestral gene as it has a specificity that is intermediate between A1 and A2. Using primary specificity for aromatic (Phe) amino acids as a criteria the similarity is closer to A2, but MeCPA differs from A2 in also hydrolyzing a Leu-containing substrate for A1; a broader specificity more closely resembling bovine carboxypeptidase. The different preferences of human A1 and A2 forms for aliphatic or aromatic amino acids are a consequence of amino acid substitutions in positions 253 and 268 (2). MeCPA shows close local identity with regions of mammalian enzymes known to be critical for folding and delineation of the active site, indicative of similar three-dimensional structures. However, the role of position 268 in MeCPA and YHT2 specificity is ambiguous as the insertion of two residues between 266 and 267 (or their deletion in the mammalian enzymes), could displace 268 at the active site and influence specificity of these enzymes. In addition, the broader specificity demonstrated by MeCPA as compared with the mammalian enzymes could be a consequence of subtle substitutions at several positions. Thus MeCPA shares Gly354 with A2 and Thr268 with both A1 and bovine carboxypeptidase. The Ala → Thr substitution is linked to the broader substrate specificity of the bovine enzyme as compared with A2 carboxypeptidases (2). The pocket size in the bacterial (A + B) enzymes is reduced compared with B-type carboxypeptidases by large residues in positions 243, 250, 253, and 255. This facilitates A-type specificity by allowing tighter fixation and more appropriate positioning of hydrophobic P1 residues (22). Likewise, apparent pocket size reduction by Val250 in MeCPA might explain the dual specificity for branched aliphatic (Leu) residues and Phe. Carboxypeptidase A2 is 23-fold less active toward a leucine containing substrate than is bovine carboxypeptidase (2), while activity by MeCPA is 4-fold higher as compared with the bovine enzyme (Table I). Conversely, bovine carboxypeptidase is more active against Tyr, also implying that it possesses a larger active site pocket than MeCPA. However, any reduction of available space in MeCPA does not allow significant activity against an Ala-containing substrate for carboxypeptidase A1 (Table I). MeCPA is also approximately 3-fold more active than bovine carboxypeptidase against a Trp-containing substrate that is also hydrolyzed more efficiently by rat carboxypeptidase A2, but is not hydrolyzed by carboxypeptidase A1 (2).

It has been suggested that as both the A1 and A2 types are expressed in the rat pancreas, these enzymes have been allowed to diverge from one another with respect to substrate specificity, resulting in complementary preferences (2). Genomic studies have revealed only a single gene for metallo-carboxypeptidase in yeast (10) and Southern analysis indicates the same in M. anisopliae, suggesting that these genes may have been under selection pressure to maintain a relatively broad substrate specificity. The fungal enzymes can be proposed as intermediates between the bacterial and mammalian enzymes being positioned after the origin of separate A- and B-types of carboxypeptidase, but before the A-type gene duplicated and differentiated into separate A1 and A2 isofoms.

Aside from the fungal enzymes, only the pancreatic carboxypeptidases, mast cell carboxypeptidase, human plasma carboxypeptidase B, and the bacterial enzymes are encoded and synthesized with an NH2-terminal activation segment, as procarboxypeptidases (1). These function by binding to the enzyme active site as an inhibitor and are required for proper folding and secretion of the enzyme (26). Although there is a coincidence in size, there are few sequence similarities between the fungal and mammalian activation segments which suggests that they may have evolved independently. This supports a previous hypothesis that the pro-regions appeared at a late stage of evolution of proteases (27). One aim of our study was the development of an efficient recombinant expression system for MeCPA. Pancreatic carboxypeptidases are difficult to express in native and soluble forms in E. coli, but the P. pastoris heterologous system has been used to study the proteolytic activation and maturation pathway of human Pro-CPA2 in vitro (12). The resulting recombinant human Pro-CPA2 required activation by treatment with trypsin which cleaves within the pro-region of the protein. No such trypsin-promoted maturation was required to obtain active MeCPA using an insect cell line. Correctly processed propeptides are commonly observed using baculovirus expression systems (28) and there was no evidence from protein microsequencing for heterogeneity at the NH2 terminus of the secreted MeCPA that would have resulted from different cleavage points in the processing of the Pro-MeCPA presequence. It remains to be determined whether this system would correctly cleave within the pro-region of mammalian enzymes. However, in view of their similarity with MeCPA we believe our results could facilitate efforts directed toward expressing active vertebrate carboxypeptidases.

Pancreatic carboxypeptidases exhibit roles in digestion. Since the MeCPA transcript is not detected when the fungus is grown on nutrient-rich conditions, the high expression of MeCPA with insect cuticle or other proteins as sole carbon and nitrogen source suggests a function in procuring nutrients during protein digestion; an equivalent function to digestive pancreatic enzymes. In previous studies we identified a subtilisin-like protease which rapidly degrades cuticle and can be used to genetically enhance virulence by transformation with multiple copies of the gene under altered regulation (29). The specificity of MeCPA appears to be adapted to complement that of the protease, as the protease shows a clear preference for cleavage of bonds COOH-terminal to aromatic residues, particularly Phe and to a lesser extent amino acids containing long hydrophobic
side chains (16). This action would release peptides terminating in Phe or other amino acids with hydrophobic side groups, that would then be further degraded by the carboxypeptidase to single amino acids. The protease is the major protein secreted into host cuticle during invasion (30). The secretion of carboxypeptidase into host cuticle as shown by the immunogold study indicates that MeCPA probably functions after the protease solubilizes cuticle proteins to peptides to supply amino acids for nutrition.

In conclusion, we report here the properties of MeCPA, the first fungal metallo-carboxypeptidase to be characterized. From data on primary structure and substrate specificity it is suggested that MeCPA is a prototype of the two mammalian enzymes carboxypeptidase 1 and 2. Development of the baculovirus expression system will further studies on the structural and functional determinants of its activation, behavior, and role in fungal pathogens. As baculoviruses are themselves insect pathogens we will also be able to determine by baculovirus bioassays the extent to which expression of the fungal protein enhances the speed of killing insect hosts. Finally, the expression system will facilitate determination of whether the microbial enzyme can supplement mammalian enzymes in their current and hypothesized future biotechnological applications.

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