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Purification and Characterization of a Developmentally Regulated Carboxypeptidase from *Mucor racemosus*

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A developmentally regulated carboxypeptidase was purified from hyphae of the dimorphic fungus *Mucor racemosus*. The enzyme, designated carboxypeptidase 3 (CP3), has been purified greater than 900-fold to homogeneity and characterized. The carboxypeptidase migrated as a single electrophoretic band in isoelectric focusing polyacrylamide gel electrophoresis (PAGE), with an isoelectric point of pH 4.4. The apparent molecular mass of the native enzyme was estimated by gel filtration to be 52 kDa. Sodium dodecyl sulfate (SDS)-PAGE under nonreducing conditions revealed the presence of a single polypeptide of 51 kDa. SDS-PAGE of CP3 reacted with 2-mercaptoethanol revealed the presence of two polypeptides of 31 and 18 kDa, indicating a dimer structure ($\alpha_1\beta_1$) of the enzyme with disulfide-linked subunits. By using [1,3-^{3}H]diisopropylfluorophosphate as an active-site labeling reagent, it was determined that the catalytic site resides on the small subunit of the carboxypeptidase. With *N*-carboben zoxy-L-phenylalanyl-L-leucine (N-CBZ-Phe-Leu) as the substrate, the K_m, k_{cat}, and V_{max} values were 1.7×10^{-4} M, 490 s^{-1}, and 588 μmol of Leu released per min per mg of protein, respectively. CP3 was determined to be a serine protease, since its catalytic activity was blocked by the serine protease inhibitors diisopropylfluorophosphate, phenylmethylsulfonyl fluoride, and 3,4-dichloroi Socoumarin (DCI). The enzyme was strongly inhibited by the mercurial compound *p*-chloromercuribenzoate. The carboxypeptidase readily hydrolyzed peptides with aliphatic or aromatic side chains, whereas most of the peptides which contained glycine in the penultimate position did not serve as substrates for the enzyme. Although CP3 activity was undetectable in *Mucor* yeast cells, antisera revealed the presence of the enzyme in the yeast form of the fungus. The partial amino acid sequence of the carboxypeptidase was determined.

Mucor racemosus is a dimorphic fungus that can be induced to grow either as a multipolar budding yeast under carbon dioxide in the presence of a hexose sugar or as branching hyphae upon transfer to air (3). Because of its bidirectional life cycle, *M. racemosus* is a good model eukaryote with which to investigate the role of proteolysis in the control of cell metabolism. In recent years, it has become increasingly apparent that proteolytic enzymes play important roles in numerous cellular regulatory events. Proteases have been associated with the metabolic processes of protein maturation, inactivation or modification of active proteins, zymogen conversion, turnover to regulate protein levels, elimination of defective polypeptides, degradation of proteins to provide amino acids and energy during starvation or differentiation, and hydrolysis of exogenous peptides to amino acids for nutrition. (for review, see reference 28). In the course of our investigations on the proteases of *M. racemosus* during morphogenesis, it was shown that multiple peptidases are produced and that the activities of aminopeptidase and carboxypeptidase (CP) achieve a maximum level of expression prior to the period of rapid germ tube formation (6). Upon examination of the spore, yeast, and hyphal forms of the fungus, we found that two carboxypeptidases, CP1 and CP3, are expressed solely in the hyphal form of the organism. CP3 activity became detectable prior to germ tube emergence and remained at a high level in stationary-phase hyphae, whereas CP1 activity was apparent only in hyphae entering stationary growth. A third carboxypeptidase, CP2, and three aminopeptidases are expressed in all cell forms. Together, these findings suggest that peptidase expression is a biochemical correlate of morphogenesis.

Peptidase production probably has at least a temporal relationship with the cellular development of *M. racemosus*. Our 1989 study (see above) was the first detailed analysis of the peptidases of *M. racemosus*, in addition to being the first investigation of the relationship between protease expression and morphogenesis in this fungus. Considerable work, however, has been conducted on other biochemical aspects of *Mucor* dimorphism, including macromolecule synthesis and enzyme regulation (for review, see reference 25).

As a preliminary approach to elucidating the role of CP3 in cellular metabolism, we undertook to isolate this developmentally regulated protease and study its biochemical properties. The carboxypeptidase was purified to homogeneity and identified as a serine protease of apparent M_r 50,000. Polyclonal antisera against the carboxypeptidase revealed the presence of CP3 in the yeast and hyphal phases of this dimorphic fungus, whereas enzyme activity was only detected in the hyphal phase. We also report on the partial primary structure of the enzyme, information which should lead to the isolation of the carboxypeptidase gene.

MATERIALS AND METHODS

Materials. All peptides, L-amino acid oxidase (crude type I), horseradish peroxidase (type II), *O*-dianisidine dihydrochloride, protease inhibitors, Freund's complete and incomplete adjuvants, substrate for enzyme-linked immunosorbent assay (ELISA), and protein standards for polyacrylamide gel electrophoresis (PAGE) and gel chromatography were from

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Sigma Chemical Co. (St. Louis, Mo.). The pI marker protein kit was from U.S. Biochemical Corp. (Cleveland, Ohio). Reagents for PAGE, goat anti-mouse immunoglobulin G (IgG), alkaline phosphatase color reagent, and nitrocellulose membranes were from Bio-Rad Laboratories (Richmond, Calif.). The DEAE-Sepharose CL-6B resin was from Pharmacia/LKB (Piscataway, N.J.). The quaternary ammonium cellulose (QA52) resin was from Whatman, Inc. (Hillsboro, Ore.). Protein assay reagents were from Pierce Chemical Co. (Rockford, Ill.). [1,3-^{3}H]diisopropylfluorophosphate ([1,3-^{3}H]DFP) and autoradiography enhancer (En^{3}Hance) were from Du Pont NEN Research Laboratories (Boston, Mass.).

Organism and culture conditions. *M. racemosus* (formerly *M. lusitanicus*) ATCC 1216B was used in all experiments. The growth medium (YPG) contained (wt/vol): yeast extract, 0.3%; Bactopeptone, 1%; and glucose, 2%. Sulfuric acid was added to achieve pH 4.5. *Mucor* yeast was obtained by inoculating YPG medium to a final density of 2×10^5 sporangiospores per ml. The culture was shaken at 125 rpm and maintained in an atmosphere of reduced oxygen by bubbling CO_2 gas at a flow rate of approximately 0.1 volume of gas per volume of culture per min. Sporangiospores were produced by inoculating YPG agar plates with approximately 200 yeast cells and incubating for 4 to 6 days at ambient temperature. Spores were harvested from plates into sterile water, washed once by centrifugation, and stored in 20% (vol/vol) glycerol at −20°C. Fresh spores were produced at weekly intervals. For spore to hyphae conversion, cells were inoculated to a final density of 10^6 spores per ml and incubated on a rotary shaker (200 rpm) at 28°C.

Protein determination and carboxypeptidase assay. Protein was determined by the method of Lowry et al. (18) or Bradford (4). The latter method was used for samples containing low concentrations of protein. The standard for both assays was bovine albumin fraction V.

Carboxypeptidase activity was measured by using an enzyme-coupled colorimetric assay of N-CBZ-peptide hydrolysis as described by Fujita et al. (10) and modified by us (6). One unit of enzyme activity was defined as the amount of carboxypeptidase which released 1 μmol of amino acid per min. Specific activity was expressed as units per milligram of protein.

Purification of CP3. For the purification of CP3 from *M. racemosus*, spores were harvested and inoculated into six 2-liter flasks containing 600 ml of YPG at a density of 10^6 spores per ml. Since CP3 is expressed maximally in stationary-phase hyphae, the cultures were allowed to grow aerobically (200 rpm) at 28°C for 3 days.

(i) Cell extract preparation. The hyphae from a 3-day culture (80 to 120 g wet weight) were harvested by vacuum filtration onto Whatman no. 1 filter paper. The hyphae were divided into 9-g portions and combined with 25 g of glass beads (0.4 to 0.5 mm in diameter) and 14 ml of 0.1 M Tris-HCl (pH 7), chilled on ice, and homogenized for 30 s in a Braun homogenizer. After an incubation on ice for 5 min, the preparation was homogenized for an additional 15 s. The percentage of cell disruption, as determined by light microscopy, ranged from 90 to 95%. The glass beads, unbroken cells, and cell debris were removed by centrifugation for 30 min at 20,000 × *g*. The supernatant fraction, designated the crude extract (S-20), was analyzed for protein content and carboxypeptidase activity as indicated above. From this point, all preparations were maintained at 4 to 10°C.

(ii) Ammonium sulfate precipitation. The supernatant was clarified by filtering through Whatman no. 1 paper. Solid ammonium sulfate was slowly added to the supernatant to achieve 50% saturation, and mixing was continued for 20 min after complete dissolution of the salt. After centrifugation for 15 min at 20,000 × *g*, the supernatant was removed and brought to 90% saturation with ammonium sulfate as above. After centrifugation, the pellet was resuspended in a small volume of 0.1 M Tris-HCl (pH 7) and dialyzed overnight against 200 volumes of the same buffer.

(iii) Heat denaturation. The cell extract was heated to 50°C for 10 min, rapidly cooled, and clarified by centrifugation for 10 min at 20,000 × *g*. The pellet was discarded, and the supernatant was dialyzed against 0.1 M imidazole-HCl (pH 5.5). For the following purification steps (iv and v), the ionic strength of the medium was monitored with a conductivity meter (Fisher Scientific Co., Pittsburgh, Pa.).

(iv) Chromatography on DEAE-Sepharose CL-6B. The protein (52 mg) solution was passed through a DEAE-Sepharose CL-6B anion-exchange column (1.6 by 15 cm) which was preequilibrated with 0.1 M imidazole-HCl (pH 5.5). The column was washed with 50 ml of the same buffer, and carboxypeptidase was then eluted by washing the column with 0.1 M imidazole-HCl (pH 5.5) and a linear gradient of NaCl (0 to 0.35 M). Carboxypeptidase was released from the column at about 0.15 M NaCl. Fractions containing high carboxypeptidase activity were collected and dialyzed against 0.1 M imidazole-HCl (pH 5.5) to remove the sodium chloride.

(v) Chromatography on quaternary ammonium cellulose (QA52). The protein (5 mg) was passed through a quaternary ammonium cellulose QA52 anion-exchange column (1.6 by 15 cm) preequilibrated with 0.1 M imidazole-HCl (pH 5.5). The column was washed with 50 ml of the equilibration buffer, and carboxypeptidase was then eluted with a linear gradient of NaCl (0 to 0.35 M)–0.1 M imidazole-HCl (pH 5.5). The peak of carboxypeptidase corresponded to fractions containing 0.15 M salt. Fractions containing the highest carboxypeptidase activity were collected and dialyzed against 0.1 M Tris-HCl (pH 7.0).

(vi) Chromatography on Sephadex G-75. The protein (2.5 mg) was passed through a column (2.6 by 88 cm) of Sephadex G-75, and fractions with the highest carboxypeptidase activity were pooled and concentrated by dialysis against 0.1 M Tris (pH 7)–polyethylene glycol 20,000 (15%, wt/vol). Glycerol was added to a final concentration of 20% (vol/vol), and the carboxypeptidase was stored at −20°C. These conditions resulted in a negligible loss of enzyme activity during up to 6 months of storage. The enzyme was also shown to be stable for at least 6 months of storage at 10°C.

Molecular mass determination by gel filtration. The molecular mass of the native enzyme was estimated by performing gel filtration through a column of Sephadex G-75 (2.6 by 88 cm) which was previously calibrated with standard proteins. Molecular mass standards were bovine albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa), and aprotinin (6.5 kDa). The void volume of the column was determined by measuring the elution volume of blue dextran.

IEF and SDS-PAGE. The isoelectric pH for the purified carboxypeptidase was determined by isoelectric focusing (IEF)-PAGE. Migrations of the IEF standards (acetylated cytochromes *c*) were recorded, the gel rods (0.5 by 10 cm) were sliced into 3-mm segments, and carboxypeptidase activity was determined after eluting the enzyme from the gel.

Sodium dodecyl sulfate (SDS)-PAGE was performed in a stacking gel of 5% (wt/vol) acrylamide and a separating gel consisting of a gradient (5 to 20%, wt/vol) of acrylamide as described by Laemmli (16). The molecular weight and sub-

TABLE 1. Purification of CP3 from *M. racemosus*

Step	Vol (ml)	Protein (mg)	Activity (U)	Sp act (U/mg)	Purification (fold)	Recovery (%)
Crude extract (S-20)	178	1,837.0	1,102.2	0.6	1	100
Ammonium sulfate (50%–90%)	40	590.0	1,416.0	2.4	4	128
Heat (50°C)	60	52.0	707.2	13.6	23	64
DEAE-Sepharose CL-6B	35	5.1	583.3	114.9	192	53
Quaternary ammonium cellulose (QA52)	15	2.5	443.3	174.8	291	40
Sephadex G-75	20	0.4	243.8	555.0	925	22

unit composition of CP3 were determined by electrophoresis under nonreducing and reducing conditions, respectively. The standard proteins were bovine albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa).

Amino acid analysis and partial amino acid sequence determination of CP3. The purified carboxypeptidase was extensively dialyzed against distilled water to remove the salts and sent to William Lane of the Microchemistry Facility at Harvard University (Cambridge, Mass.) for total amino acid analysis and partial amino acid sequence determination. For the determination of the partial amino acid sequence, Lane digested CP3 with protease, purified the fragments by high-pressure liquid chromatography, and sequenced two of the peptides. We used the fast pairwise comparison of sequences (FastDB; IntelliGenetics, Inc.) to determine whether CP3 has homology with other proteases.

Active-site labeling of CP3. [1,3-^{3}H]DFP was used to determine which of the subunits contains the catalytic center of CP3. The method we used to label specifically the active site of the carboxypeptidase was modified from that of MacGregor et al. (19). Purified CP3 (6 μg) was mixed with [1,3-^{3}H]DFP (8 μM; 22 μCi) and 0.1 M Tris-HCl (pH 7) in a volume of 1 ml. The reaction mixture was incubated for 3 h at 30°C, the reaction was terminated by the addition of 2× SDS-PAGE sample buffer (with or without 2-mercaptoethanol), and the reaction mix was heated (100°C) for 3 min. SDS-PAGE was carried out in a stacking gel of 5% (wt/vol) acrylamide and a separating gel of 10% (wt/vol) acrylamide as described by Laemmli (16). The radiolabeled proteins were detected by impregnating the gels with autoradiography enhancer (En^{3}Hance); the gels were dried under vacuum and exposed to X-Omat X-ray film (Eastman Kodak Co., Rochester, N.Y.) with a Lightning-Plus intensifying screen at −70°C for 8 days.

Production of polyclonal antibodies to CP3. Purified CP3 (50 μg) was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into 6-week-old female BALB/c mice. Fourteen days later, the mice were injected with a mixture containing 50 μg of the immunogen and an equal volume of Freund's incomplete adjuvant. By day 28, ELISA indicated that the mouse serum had an antibody titer of 1:20,000 with 100 ng of purified CP3 (9). A booster of purified immunogen (35 μg) alone was administered intraperitoneally, and on day 35 the mice were killed. Serum was collected and stored at −80°C. The antibody titer as determined by ELISA was found to be 1:80,000.

Western immunoblot analysis. Proteins were separated by SDS-PAGE in slab gels with a gradient of 5 to 20% acrylamide (see above). After electrophoresis, the proteins were transferred to a nitrocellulose membrane (0.45 μm) with a Hoefer (San Francisco, Calif.) TE 50 Transphor unit (26). Transfer was toward the anode at 4°C for 1 h at 110 V in a transfer buffer which consisted of 20 mM Tris, 150 mM glycine, and 20% (vol/vol) methanol. Following transfer, half of the nitrocellulose was stained with amido black (0.1%, wt/vol) to ensure that efficient transfer had occurred, and the remaining half was blocked for 1 h with 10% (wt/vol) bovine serum albumin. Primary antibody (CP3 antiserum) was added at a dilution of 1:10,000 containing 1% (wt/vol) bovine serum albumin and incubated for 1 h. After the membrane was washed five times for 5 min each, the second antibody (goat anti-mouse IgG) was added at a dilution of 1:3,000 containing 1% (wt/vol) bovine serum albumin for 1 h. The membrane was again washed as above and developed with a Bio-Rad alkaline phosphatase conjugate substrate kit. Blocking, antibody reactions, and washing were all performed at 37°C with gentle agitation in 0.01 M phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20.

RESULTS

Purification of CP3. Previous studies in our laboratory have shown that CP3 is expressed to maximum levels in stationary-phase hyphae of *M. racemosus* (6). The specific activity of carboxypeptidase in crude extracts of logarithmic-phase hyphae was about threefold less than that in stationary-phase cells. Therefore, to increase the amount of CP3 for purification, spores were germinated in YPG medium under air and allowed to grow (28°C, 200 rpm) for 72 h. The typical yield of hyphae from a 3.6-liter culture was 80 to 120 g (wet weight), a quantity of cell material which yielded 1.8 to 2.0 g of protein upon cell disruption and centrifugation at 20,000 × *g* (S-20; crude extract).

The specific activity of total carboxypeptidase in the crude extract (S-20 fraction) as measured by N-CBZ-Phe-Leu hydrolysis was 0.6 to 0.8 U/mg of protein (Table 1). Each of the five subsequent procedures in our purification protocol yielded increasingly pure CP3 (Fig. 1 and Table 1). The final step resulted in a homogeneous product that was purified 925-fold and 22% recovery of the enzyme (Table 1).

Properties of CP3. The purity of CP3 was assessed and its subunit structure was examined by gel electrophoresis. Carboxypeptidase migrated as a single protein-stainable band with an estimated molecular mass of 51 kDa in SDS-PAGE under nonreducing conditions (Fig. 2). Sephadex G-75 gel filtration column chromatography allowed us to estimate the molecular mass of the native enzyme, since the column was calibrated with standard proteins. As shown in Fig. 1C, CP3 eluted from the sizing column as a single symmetrical peak with a molecular mass of 52 kDa. Taken together, these results show that our preparation of CP3 was purified to homogeneity. When CP3 was reacted with 2-mercaptoethanol, the enzyme dissociated into two protein bands with molecular weights of 31,000 and 18,000 (Fig. 2).

CP3 migrated as a single protein band in IEF-PAGE, with

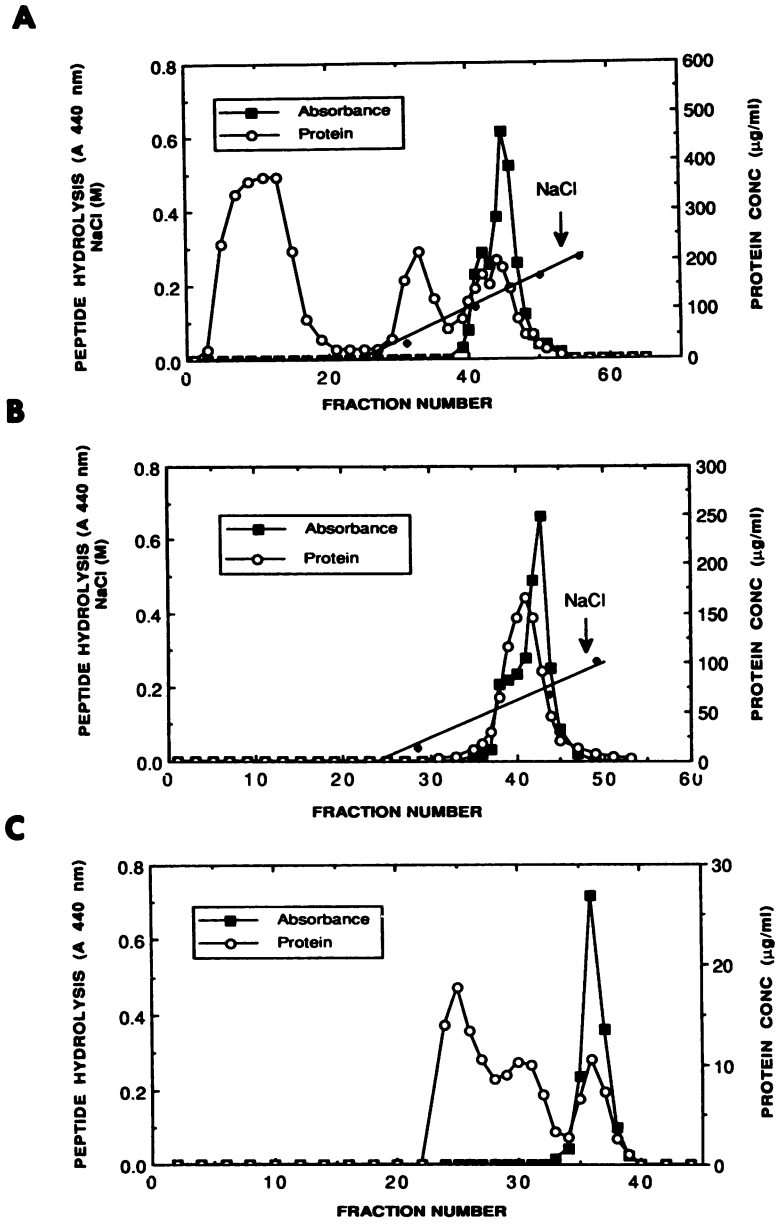

FIG. 1. Chromatography of *M. racemosus* carboxypeptidase on anion-exchange and gel filtration columns. (A) 52 mg of protein from ammonium sulfate precipitation was chromatographed on a column of DEAE-Sepharose CL-6B. The column was washed with buffer, and carboxypeptidase was eluted from the column with a linear gradient of NaCl. The fractions (41 to 47) containing high carboxypeptidase activity were pooled and dialyzed against buffer to remove the sodium chloride. (B) 5 mg of protein was chromatographed on a column of quaternary ammonium cellulose (QA52). The column was washed with buffer, and carboxypeptidase was eluted from the column with a linear gradient of NaCl. The fractions (40 to 42) containing the highest carboxypeptidase activity were pooled and dialyzed against buffer to remove the salt. (C) 2.5 mg of protein was chromatographed on a column of Sephadex G-75 resin. Fractions (35 to 38) containing the highest carboxypeptidase activity were collected. The column was calibrated with molecular weight protein standards as indicated in the text. Five-milliliter fractions were collected in all of the above chromatographic separations.

an isoelectric point at pH 4.4 (Fig. 3). When CP3 was extracted from a duplicate IEF acrylamide gel and tested for peptide hydrolase activity with N-CBZ-Phe-Leu as the substrate, we found that catalytic activity corresponded well with the Coomassie-stained protein band in the gel.

To determine which subunit of CP3 contains the catalytic center, we radiolabeled the carboxypeptidase specifically by using the active-site covalent inhibitor [1,3-^{3}H]DFP. Unreduced CP3 produced a strong signal with mobility that corresponded to 51 kDa (Fig. 4). However, when CP3 was reacted with 2-mercaptoethanol, the radioactivity comigrated with the 18-kDa polypeptide. These results imply that the active site resides on the small subunit of CP3. But when the two subunits of the carboxypeptidase were separated by treatment with SDS and 2-mercaptoethanol, catalytic activity was inhibited. The peptidase activity of the enzyme was not recovered when the denatured protein was dialyzed or fractionated by column chromatography.

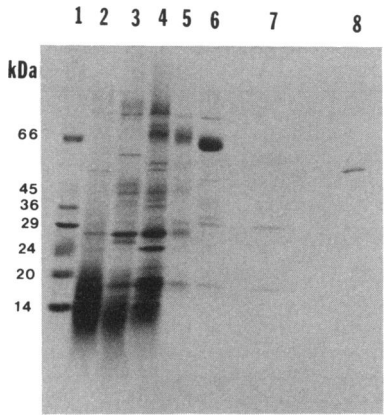

FIG. 2. SDS-PAGE of purified *M. racemosus* carboxypeptidase. Samples from all purification steps were denatured in SDS-PAGE sample buffer with (lanes 1 to 7) or without (lane 8) 2-mercaptoethanol and electrophoresed in a stacking gel of 5% (wt/vol) acrylamide and a separating gel consisting of a gradient (5 to 20%, wt/vol) of acrylamide. Lane 1, molecular size markers: bovine albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.2 kDa. Lane 2, crude extract, 100 μg of protein; lane 3, 50 to 90% ammonium sulfate precipitation, 50 μg of protein; lane 4, heat precipitation supernatant, 50 μg of protein; lane 5, DEAE-Sepharose column fractions, 8 μg of protein; lane 6, quaternary ammonium (QA52) column fractions, 8 μg of protein; lane 7, G-75 column fractions, 1 μg of protein (heated with 2-mercaptoethanol); lane 8, G-75 column fractions, 1 μg of protein (heated without 2-mercaptoethanol).

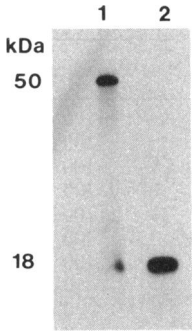

FIG. 4. Autoradiograph illustrating the active-site labeling of the purified *M. racemosus* carboxypeptidase. Purified carboxypeptidase from Sephadex G-75 column chromatography was incubated with [1,3-^{3}H]DFP, separated by SDS-PAGE, and exposed to X-ray film. Lane 1, 1 μg of carboxypeptidase heated without mercaptoethanol; lane 2, 1 μg of carboxypeptidase heated with mercaptoethanol. The molecular masses of the signals are indicated at the left.

The results of the amino acid analysis of carboxypeptidase are shown in Table 2. The amino acids in greatest abundance were aspartic acid and glutamic acid. Since the protein was not reduced and alkylated prior to acid hydrolysis, cysteine was not found in CP3. This amino acid was found, however, when a partial sequence determination was performed on the enzyme (Fig. 5). A computer-generated comparison of the partial amino acid sequences of CP3 with those of other proteins revealed that CP3 has homology with CPY of *Saccharomyces cerevisiae* (Fig. 5). Based on our computer search of protein sequences, the peptide fragments (F) of CP3, CP3FA and CP3FB, showed 42 and 52% homology to CPY, respectively. The information from the amino acid sequence is currently being incorporated into the design of synthetic oligonucleotides that will be used as probes to isolate the carboxypeptidase gene.

Enzyme kinetics. The carboxypeptidase had a temperature optimum for N-CBZ-Phe-Leu hydrolysis of 45°C. Above 55°C, the enzyme became rapidly inactivated. To examine the thermostability of CP3, the enzyme was incubated at various temperatures and then assayed to determine how much activity remained. During a 10-min incubation, the enzyme lost 38% of its activity at 45°C and 100% of the activity at 55°C. The K_m, V_{max}, and k_{cat} for N-CBZ-Phe-Leu hydrolysis (35°C) were 1.7×10^{-4} M, 490 s^{-1}, and 588 μmol

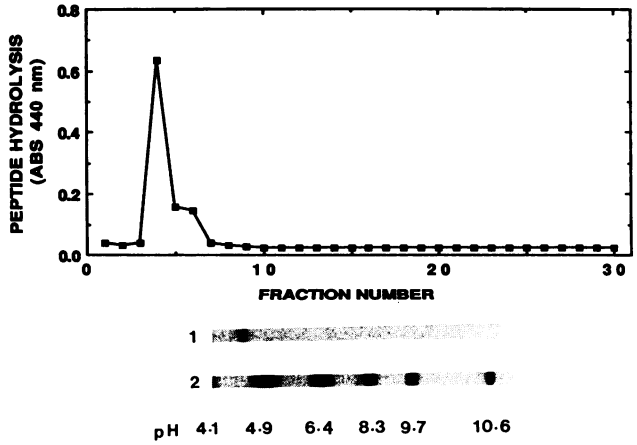

FIG. 3. IEF-PAGE of purified *M. racemosus* carboxypeptidase. Gel 1, 1 μg of purified carboxypeptidase from G-75 column chromatography stained with Coomassie blue; gel 2, acetylated cytochromes *c* markers stained with Coomassie blue. An additional gel containing carboxypeptidase was sliced into 3-mm segments, protein was eluted, and carboxypeptidase activity was determined by assaying peptide hydrolysis as indicated in the top graph.

TABLE 2. Amino acid composition of CP3 of *M. racemosus*

Amino acid	No. of residues/ molecule
Aspartic acid	63
Glutamic acid	46
Serine	24
Glycine	44
Histidine	7
Arginine	14
Threonine	11
Alanine	33
Proline	26
Tyrosine	12
Valine	22
Methionine	9
Isoleucine	20
Leucine	34
Phenylalanine	22
Lysine	22
Tryptophan	ND[a]
Half-cystine	ND[b]
Total[c]	400

[a] Not detected.
[b] Not detected, residue unprotected; detected in sequence determination.
[c] Calculations are based on a molecular weight of 50,000 for CP3.

TABLE 3. Influence of protease inhibitors on CP3

Compound tested	Concn (μM)	CP3 activity[a] (% of control)
EDTA	100	98
1,10-Phenanthroline	100	97
TPCK	100	97
Leupeptin	100	95
TLCK	100	90
ZPCK	100	86
PMSF	100	28
DCI	10	4
DFP	0.6	8
PCMB	0.1	4

[a] CP3 (0.06 μg) was incubated with the compounds at the indicated concentrations for 10 min at 35°C prior to the addition of N-CBZ-Phe-Leu (1.1 mM). Measurements are the average of duplicate assays in a single experiment.

of leucine released per min per mg of protein, respectively. The turnover number and catalytic cycle at 35°C were 2.9×10^4 min^{-1} and 3.4×10^{-5} min, respectively. The optimum pH for the hydrolysis of N-CBZ-Phe-Leu was 6.5.

Influence of protease inhibitors. A number of potential inhibitors were incubated with CP3 to determine their influence on peptidase activity (Table 3). All of the compounds were incubated with the enzyme for 10 min at 35°C prior to the addition of substrate to start the assay. From previous studies in our laboratory with crude preparations of CP3, we expected the purified carboxypeptidase to be sensitive to serine protease inhibitors. The results here confirmed this expectation, as the serine protease inhibitors phenylmethylsulfonyl fluoride (PMSF), 3,4-dichloroisocoumarin (DCI), and diisopropylfluorophosphate (DFP) were very effective in blocking carboxypeptidase activity. PMSF probably interacts with an active-site serine, since it blocked peptide hydrolysis in a competitive way, with a K_i of 100 μM (data not shown). The mercurial compound *p*-chloromercuribenzoate (PCMB) had a strong influence on CP3 in that it blocked enzyme activity by 96% at 0.1 μM.

The trypsin and papain inhibitor tosyl-L-lysine chloromethyl ketone (TLCK) and the histidine-alkylating reagent benzyloxy-carbonyl-L-phenylalanine chloromethyl ketone (ZPCK) were only slightly inhibitory, in that both of these compounds only blocked peptide hydrolysis by 10 to 14% at 100 μM. The thiol proteinase inhibitor leupeptin, chymotrypsin inhibitor tosyl-L-phenylalanine chloromethyl ketone (TPCK), and the metal chelators EDTA and *O*-phenanthroline, each at 100 μM, were virtually ineffective at blocking the enzymatic reaction.

Substrate specificity. A number of N-CBZ-peptides and unblocked peptides were incubated with the purified carboxypeptidase to ascertain the substrate specificity of the enzyme. As shown in Table 4, CP3 preferentially hydrolyzed peptides containing amino acids with aliphatic side chains in the terminal or penultimate position. For example, for the most part, N-CBZ-Ala-X peptides proved to be excellent substrates for the enzyme. Peptides with glycine in the penultimate position, however, were generally not hydrolyzed by the carboxypeptidase. The exceptions were N-CBZ-Gly-Met and N-CBZ-Gly-Phe, which were hydrolyzed at a low rate by the enzyme. Since CP3 is a carboxypeptidase, it was not expected to hydrolyze dipeptides, and it can be seen that the unblocked dipeptide Ala-Phe was not a substrate for the enzyme, whereas the tetrapeptide Val-Ala-Ala-Phe was readily hydrolyzed by the enzyme.

TABLE 4. Substrate specificity of CP3

Peptide tested	Rate (μmol/min/mg of protein)
N-CBZ peptides	
Ala-Leu	1,025
Ala-Met	892
Ala-Phe	750
Phe-Leu	541
Phe-Met	517
Ala-Ile	383
Glu-Phe	113
Ile-Phe	71
Ala-Val	63
Glu-Tyr	42
Gly-Met	13
Gly-Phe	13
Gly-Leu	0
Gly-Tyr	0
Gly-Ile	0
Gly-Val	0
Pro-Phe	0
Unblocked peptides	
Val-Ala-Ala-Phe	388
Phe-Leu-Glu-Glu-Ile	46
Ala-Phe	0

Immunochemical detection of CP3 in yeast and hyphal forms of *M. racemosus*. To determine whether CP3 is related to other carboxypeptidases of *M. racemosus* and to gather information relating to the regulation of CP3 expression, purified carboxypeptidase and crude cell extracts were analyzed by immunoblotting with polyclonal antiserum against the purified carboxypeptidase. It can be seen in Fig. 6 that CP3-specific antiserum did not cross-react with partially purified CP1. As previously stated, CP1 is a hypha-specific carboxypeptidase with a molecular mass of 250 kDa and is expressed at maximum levels in stationary-phase hyphae (6).

The antiserum reacted with CP3 in crude extracts of hyphae but showed little cross-reactivity to other cell proteins (Fig. 6 and 7). Although CP3 activity was not detected in spore and yeast forms of *M. racemosus*, the antiserum revealed cross-reactive material in the yeast cell extract (Fig. 7). The signals produced by the spore extract may have been caused by reaction either to the fragments of the degraded carboxypeptidase or to spore-specific polypeptides.

DISCUSSION

M. racemosus ATCC 1216B expresses at least three distinct carboxypeptidases, two of which are developmentally regulated (6). We undertook to purify and characterize the carboxypeptidase (CP3) which is expressed during the period of rapid fungal morphogenesis instead of CP1, since it is expressed long after the cells have undergone morphological conversion from yeast or spore to hyphae.

By using a scheme consisting of six steps, including differential centrifugation, salt and heat precipitation, and ion-exchange and gel filtration column chromatography, the carboxypeptidase was purified 925-fold, with 22% yield. Gel filtration chromatography produced a single peak of enzyme activity with no shoulder and with an apparent M_r of 52,000. Carboxypeptidase migrated as a single protein-stainable band with an apparent M_r of 51,000 in SDS-PAGE under nonreducing conditions. Finally, the purified CP3 migrated as a single protein band in IEF-PAGE, with a pI of 4.4, and peptidase activity corresponded with the protein band. Elec-

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CP3 FA    YYAQMACDNS   YGP-VLDRAT    CDNMEASFP
CPY       YYEPMACGEG   GEPSVLPSEE    CSAMEDSLE
          300          310           320

CP3 FB    AWTQLPWTG    HEEFSNA
CPY       AWTDVLPWKY   DEEFASQ
          460          470
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FIG. 5. Partial amino acid sequence of *M. racemosus* carboxypeptidase and comparison with carboxypeptidase Y from *S. cerevisiae*. Purified carboxypeptidase from G-75 column chromatography was processed for sequencing as indicated in the text. Carboxypeptidase 3 fragment A (CP3FA) and fragment B (CP3FB) were compared with known protein sequences by the fast pairwise comparison of sequences (FastDB; IntelliGenetics, Inc.). The numbers are for the numbered sequence of the carboxypeptidase Y precursor, which contains 532 amino acids. Identical residues are underlined.

trophoresis of the carboxypeptidase under reducing conditions caused the carboxypeptidase to dissociate into subunits of unequal size, with molecular weights of 31,000 and 18,000, indicating that the carboxypeptidase is a dimeric protein of the structure $\alpha_1\beta_1$, with its subunits linked through an interchain disulfide bridge. Taken together, these results imply that the carboxypeptidase has been purified to homogeneity.

Because CP3 is highly sensitive to the serine-modifying compound DFP, the active site of the enzyme could be labeled. Using the covalent inhibitor [1,3-^{3}H]DFP, we determined that the active site of CP3 is probably carried on the small subunit of the dimeric carboxypeptidase. We were unable, however, to test the catalytic activity of the individual subunits, since separation of the two subunits by SDS and 2-mercaptoethanol resulted in a total loss of activity with no recovery of hydrolytic activity following dialysis or gel filtration chromatography. The human plasma carboxypeptidase N is a tetrameric metalloprotease of M_r 280,000 and consists of two small subunits of M_r 50,000 and two large glycosylated subunits. Only the small subunits were shown to possess catalytic activity (11). The authors have proposed that the enzymatically inactive large subunits somehow stabilize the protease and/or prevent its premature degradation in plasma.

Analysis of the amino acid composition of CP3 from *M. racemosus* revealed that aspartic and glutamic acids are the most abundant amino acids. This might be predicted, because the carboxypeptidase has an isoelectric point of pH 4.4.

Earlier studies by Doi et al. (7) and Hata et al. (13) provided evidence that CPY is a glycoprotein of 61 kDa, whereas the enzyme synthesized in the presence of tunicamycin had a molecular mass of 51 kDa. A more recent study on the synthesis and maturation of CPY revealed that the mature but unmodified protein has a molecular mass of 59 kDa and that glycosylation results in an increase in mass to 69 kDa (5). In our study, CP3 failed to stain with periodic acid-Schiff reagent, but several proteins in the crude cell extract stained intensely, suggesting that CP3 is not glycosylated. These findings demonstrate that the molecular weight obtained for CP3 is close to the value reported for mature but nonglycosylated CPY. However, the carboxypeptidase of *M. racemosus* is a dimeric protein, whereas the CPY of *S. cerevisiae* has been reported to be a single-subunit protein (12). In addition, by using immunoblot analysis, we determined that CPY failed to cross-react with the CP3-specific antisera (data not shown). The determination of the

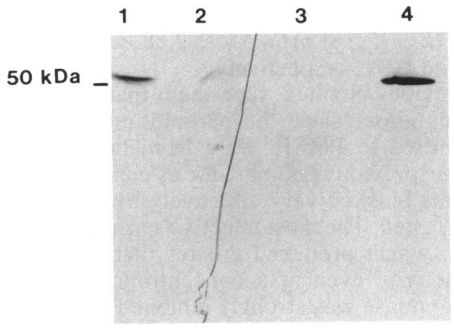

FIG. 6. Immunoblot analysis of purified *M. racemosus* carboxypeptidase and crude hyphal extracts. Purified protein and crude cell extracts were electrophoresed in SDS-PAGE (5 to 20% acrylamide). After electrophoresis, the protein was transferred to nitrocellulose by electroblotting; half of the nitrocellulose was stained with amido black, and the remaining portion was reacted with the mouse antiserum. Blots were incubated with a 1:10,000 dilution of antiserum, washed, incubated with goat anti-mouse IgG conjugated to alkaline phosphatase at a dilution of 1:3,000, washed, and developed with an alkaline phosphatase conjugate substrate kit. Lane 1, hyphal crude extract (S-50 fraction), 28 μg of protein; lane 2, hyphal crude extract (S-50 fraction), 14 μg of protein; lane 3, purified CP1, 2 μg of protein; lane 4, purified CP3, 1 μg of protein. The molecular mass marker is indicated at the left.

relatedness of CP3 and CPY awaits the isolation and characterization of the *Mucor* carboxypeptidase gene.

Our studies of the purified enzyme demonstrated that it has a temperature optimum of 45°C and a pH optimum of 6.5. Since the enzyme is relatively unstable at 45°C, all kinetic parameters were determined at 35°C. Under these conditions, the turnover number for the hydrolysis of N-CBZ-Phe-Leu was 29,000 molecules per min per molecule of enzyme. This is about four times greater than the value obtained for CPY (14). However, the apparent K_ms for CP3

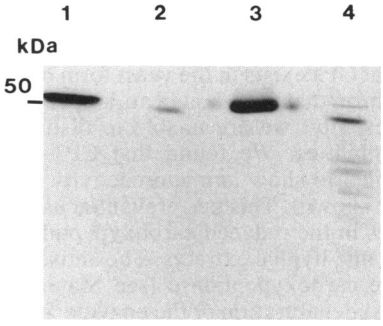

FIG. 7. Immunoblot analysis of carboxypeptidase from various morphological forms of *M. racemosus*. Purified protein and crude cell extracts of *Mucor* spore, yeast, and hyphae were electrophoresed in SDS-PAGE (5 to 20% acrylamide). After electrophoresis, the protein was transferred to nitrocellulose by electroblotting; half of the nitrocellulose was stained with amido black, and the remaining portion was reacted with the mouse antiserum. Blots were incubated with a 1:10,000 dilution of antiserum, washed, incubated with goat anti-mouse IgG conjugated to alkaline phosphatase at a dilution of 1:3,000, washed, and developed with an alkaline phosphatase conjugate substrate kit. Lane 1, hyphal crude extract (S-50 fraction), 28 μg of protein; lane 2, hyphal crude extract (S-20 fraction), 50 μg of protein; lane 3, yeast extract (S-50 fraction), 50 μg of protein; lane 4, spore extract (S-20 fraction), 55 μg of protein. The molecular mass marker is indicated at the left.

and CPY were 0.17 and 0.1 mM, respectively. The catalytic efficiency (k_{cat}/K_m) at pH 6.5 was 2,882 and 1,300 $mM^{-1}\ s^{-1}$ for CP3 and CPY, respectively.

Our previous studies suggested that CP3 is a serine carboxypeptidase, since the partially purified enzyme was quite sensitive to PMSF. Data from this study (Table 3) support our previous findings, for as can be seen, peptidase activity was blocked very effectively by all serine protease inhibitors tested. The most effective serine protease inhibitor was DFP, which produced greater than 90% inhibition of enzyme activity even at a concentration of 0.6 μM. The carboxypeptidase was strongly inhibited by PCMB, indicating either that the enzyme has a sulfhydryl group in the active site or that such a residue is important for the tertiary structure of the protein. Previous studies have provided evidence that CPY has a single sulfhydryl group that is located at the binding site of the penultimate amino acid residue of the substrate (2). Bai and Hayashi (2) reported that mercurial compounds did not cause complete inactivation with poor substrates but effectively inhibited peptide hydrolysis of good substrates. The authors suggested that when a mercurial compound occupies the subsite, it inhibits good substrate interaction with the active site due to steric hindrance.

The carboxypeptidase of *M. racemosus* exhibited a substrate specificity profile that is similar to that reported for yeast CPY (14). The following points were determined for CP3.

(i) The rate of peptide hydrolysis was high when an aromatic or aliphatic side chain was in the terminal and/or penultimate position of the substrate.

(ii) Placement of glycine in the penultimate position resulted in either a lack of hydrolysis altogether or a considerably slower rate of catalysis.

(iii) CP3 was unable to hydrolyze the unblocked dipeptide Ala-Phe, consistent with the fact that the enzyme is a carboxypeptidase.

The CP3-specific antiserum did not recognize *Mucor* CP1, which indicated that CP1 and CP3 are antigenically distinct enzymes. Although CP3 activity was not detected in the spore and yeast forms of *M. racemosus*, the antisera revealed CP3-cross-reactive material in yeast cell extract, suggesting that CP3 exists in the yeast form of *M. racemosus* as a zymogen or that it is bound to a specific endogenous inhibitor. Presently, we are unable to distinguish between the two possibilities. We found that CP3 under reducing conditions failed to show immunoreactivity with the antiserum (data not shown). This has prevented us from searching for differences in the reduced carboxypeptidase in immunoblots of yeast and hyphal extracts. The antiserum was raised against native carboxypeptidase (see Materials and Methods), and thus, denaturation of the enzyme may have caused a loss of immunoreactivity.

Endogenous polypeptide inhibitors which are specific for proteinase yscA (21), proteinase yscB (17), and protease yscC (17, 20) have been reported. It has been speculated that the purpose of the inhibitors, which are localized in the cytosol, is to prevent any unwanted proteolysis by proteinases A and B and protease C, all of which reside in the vacuole of *S. cerevisiae*.

Presently, we are uncertain of the cellular location of CP3. The fact that CP3 is nonglycosylated suggests that it is not localized in the vacuole. Studies with *S. cerevisiae* have revealed that the vacuole contains various hydrolases (27). The proteases shown to exist in the vacuole of bread yeast are the endopeptidases proteinase yscA and proteinase yscB and five exopeptidases: aminopeptidase yscI, aminopeptidase yscCo, dipeptidyl aminopeptidase yscV, carboxypeptidase yscY, and carboxypeptidase yscS (1, 22, 24). All of the vacuolar enzymes characterized so far are glycoproteins that are synthesized as inactive precursors of higher molecular weight (15). According to the findings of Stevens et al. (23), procarboxypeptidase Y is transported through the endoplasmic reticulum and the Golgi body. In the Golgi body, the vacuolar zymogen is sorted from secreted proteins and transported to the vacuole for proteolytic processing (23).

According to a study by Emter and Wolf in 1984 (8), the fungal vacuole is not the sole compartment of protease, since proteinases D and E and several other proteolytic enzymes were not enriched in the vacuolar fraction of *S. cerevisiae*. Unlike the vacuolar proteases of *S. cerevisiae*, which have a role in general protein degradation, the carboxypeptidase of *M. racemosus* probably exists outside of the vacuole and may carry out a specific function in the fungus. The fact that *Mucor* carboxypeptidase is not active in the yeast form and active in hyphae suggests that the serine protease may be an important regulatory factor of fungal morphogenesis.

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