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The substrate specificity of *Metarhizium anisopliae* and *Bos taurus* carboxypeptidases A: Insights into their use as tools for the removal of affinity tags

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ABSTRACT

Carboxypeptidases may serve as tools for removal of C-terminal affinity tags. In the present study, we describe the expression and purification of an A-type carboxypeptidase from the fungal pathogen *Metarhizium anisopliae* (MeCPA) that has been genetically engineered to facilitate the removal of polyhistidine tags from the C-termini of recombinant proteins. A complete, systematic analysis of the specificity of MeCPA in comparison with that of bovine carboxypeptidase A (BoCPA) was carried out. Our results indicate that the specificity of the two enzymes is similar but not identical. Histidine residues are removed more efficiently by MeCPA. The very inefficient digestion of peptides with C-terminal lysine or arginine residues, along with the complete inability of the enzyme to remove a C-terminal proline, suggests a strategy for designing C-terminal affinity tags that can be trimmed by MeCPA (or BoCPA) to produce a digestion product with a homogeneous endpoint.

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Introduction

Metallocarboxypeptidases are enzymes that have evolved to remove C-terminal amino acid residues of proteins or peptides with the aid of a Zn^{2+} ion. They are commonly regarded as being involved in the degradation of proteins and peptides, yet recent findings suggest a wide range of physiological roles for such enzymes [1]. Carboxypeptidases are classified on the basis of their specificity. For example, type A carboxypeptidases preferentially remove C-terminal amino acid residues having aromatic or branched aliphatic side chains whereas type B carboxypeptidases exhibit a strong preference for basic amino acids. The A-type carboxypeptidases are further sub classified into type A1 and type A2 isoforms in rodents and humans. Carboxypeptidase A1 preferentially catalyzes the removal of aliphatic residues from peptide substrates, while the A2 isoforms show higher specificity toward aromatic residues such as phenylalanine and tryptophan. Carboxypeptidase A2 is not present in the bovine pancreas. Instead the single bovine carboxypeptidase A (BoCPA)[1] has relatively broad substrate specificity [2].

The purification of a novel, type A carboxypeptidase (MeCPA) from the fungal entomopathogen *Metarhizium anisopliae* has been reported, and its specificity was compared to that of BoCPA using a limited set of substrates [3,4]. The catalytic mechanism of MeCPA was originally uncertain because its activity was inhibited by both di-isopropyl fluorophosphates (DIFP) and 1,10-phenanthroline [4], but later it was established to be a zinc carboxypeptidase [3]. Like animal type A carboxypeptidases and in contrast to bacterial carboxypeptidases, MeCPA lacks type B specificity.

Current strategies for recombinant protein expression frequently involve the use of affinity tags, often joined to the N-terminus of the protein being expressed. The hexahistidine tag (His-tag) is far and away the most commonly used affinity tag [5–7] and one of the few tags that is frequently fused to the C-termini of recombinant proteins. Endoproteolytic removal of C-terminal His-tags is complicated by the fact that the principal specificity determinants of endoproteolytic enzymes (*e.g.*, Factor Xa, thrombin, enteropeptidase/enterokinase, tobacco etch virus protease) are located on the N-terminal side of the scissile bond. Consequently, the removal of a C-terminal tag by any of them would leave behind a number of non-native residues (six in the case of tobacco etch virus protease). One could argue, therefore, that any gain achieved by endoproteolytic removal of a C-terminal hexahistidine tag would be offset by the presence of the residual protease recognition site.

A promising alternative is exoproteolytic removal of a C-terminal His-tag by a carboxypeptidase, as demonstrated previously with BoCPA (*e.g.*, [8,9]). To refine this method, we engineered a recombinant form of MeCPA with a C-terminal hexahistidine tag followed by two arginine residues. We chose MeCPA because it

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[1] *Abbreviations used:* BoCPA, bovine carboxypeptidase A; MeCPA, *Metarhizium anisopliae* carboxypeptidase A; DIFP, di-isopropyl fluorophosphates; ORF, open reading frame; PCR, polymerase chain reaction.

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can be produced in baculovirus infected insect cells and because its amino acid sequence suggested that it might have even broader specificity than BoCPA [3,4]. The arginine residues were intended to prevent the enzyme from digesting its own C-terminal His-tag. The polyhistidine tag facilitates the purification of recombinant pro-MeCPA, which is secreted from insect cells, and also assists in its separation from the products of a carboxypeptidase digest. Here, we describe the cloning, expression, and purification of the active enzyme using the baculovirus expression system. We also compare the specificity of recombinant MeCPA to that of BoCPA, the most commonly used carboxypeptidase for research and biotechnological purposes, using an oligopeptide-based HPLC assay. Finally, we show that recombinant MeCPA is readily able to remove polyhistidine tags from the C-termini of globular proteins.

Materials and methods

Molecular modeling of MeCPA

A molecular model of MeCPA was built by Modeller [8] based on the structure of BoCPA (PDB code: 3CPA) [9]. A sequence alignment of MeCPA and BoCPA, performed with the ClustalW program [10], is presented in Fig. 1. The alignment was verified by comparison of carboxypeptidases with deposited structural coordinates (data not shown). Structures were examined on a Silicon Graphics Fuel workstation using Sybyl (Tripos, St. Louis, MO, USA).

BoCPA

BoCPA (Type II-PMSF, C-9268) was purchased from Sigma–Aldrich (St. Louis, MO, USA).

Cloning of the MeCPA gene

M. anisopliae (Metschnikoff) Sorokin mycelium was a gift from Dr. Richard A. Humber of the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) in Ithaca, NY, USA. Genomic DNA was isolated from the mycelium using a kit from Invitrogen (Carlsbad, CA, USA).

Oligodeoxyribonucleotide primers complementary to the 5′ (5′-GGGG ACA ACT TTG TAC AAA AAA GTT GTG ATG AGA GTG GTT GCT TTC TTC GCC TG-3′) and 3′ (5′-GGGG ACA ACT TTG TAC AAG AAA GTT GCA CTC ATC TGC TGG AAG AGA TGC ATG G-3′) ends of the MeCPA cDNA sequence reported by Joshi and St Leger [4] with the addition of terminal attB1 and attB2 recombination sites were used to generate an amplicon by polymerase chain reaction (PCR) that was subsequently inserted by Gateway recombinational cloning into pDONR201 and sequenced in its entirety (Genebank accession code: EU919684). The genomic clone contained five exons, which were subsequently joined together by overlap extension PCR [11] to assemble the complete, uninterrupted pre-pro-MeCPA open reading frame (ORF) in the donor vector pDONR223 (Invitrogen). In the process, a DNA sequence encoding the residues His–His–His–His–His–His–Arg–Arg–STOP was added in-frame to the 3′ end of the final exon. The MeCPA ORF and the C-terminal His_6Arg_2 tag were subsequently inserted via the LR reaction into the insect cell transfer vector pDEST-8 (Invitrogen), which contains the baculovirus polyhedrin promoter, yielding baculovirus expression clone 1177-X1-8.

Construction of recombinant baculovirus and optimization of MeCPA production

The baculovirus expression clone 1177-X1-8 was introduced into DH10Bac cells (Invitrogen), and the transformants were selected on

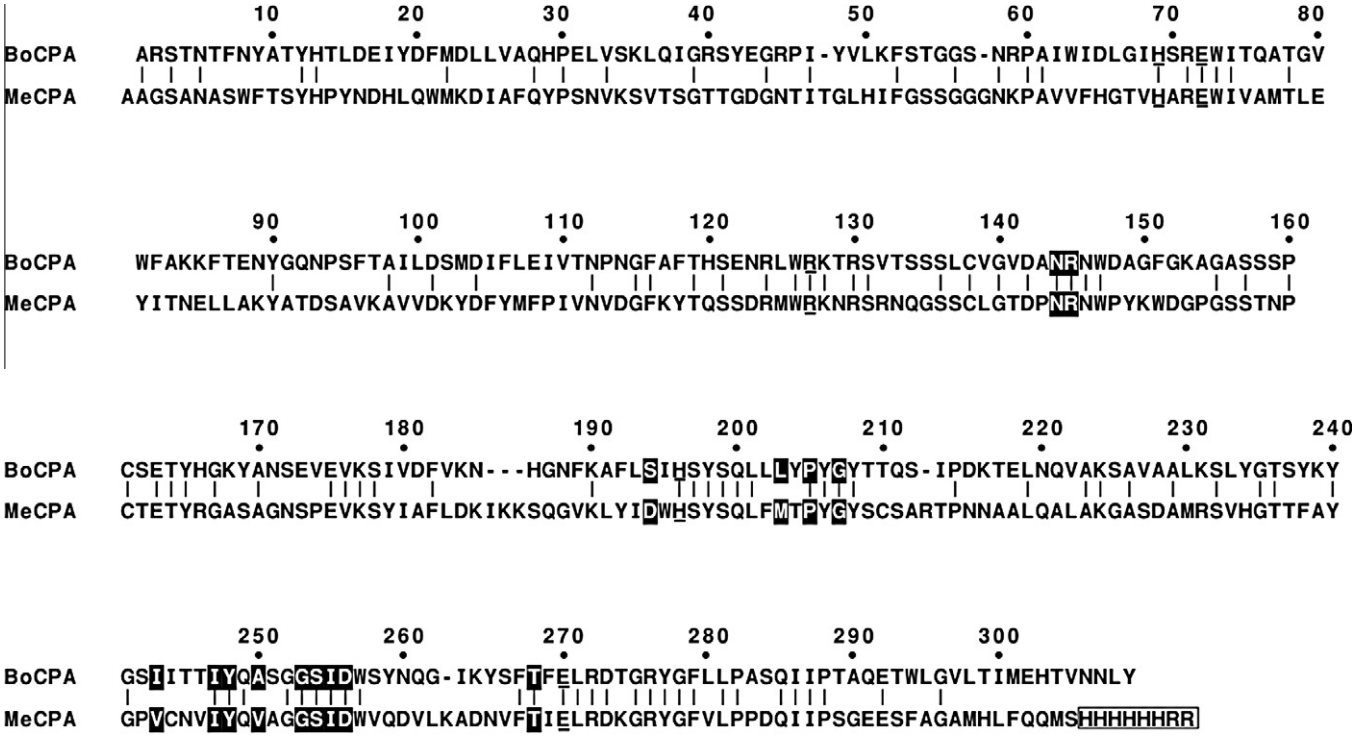

Fig. 1. Sequence alignment of bovine carboxypeptidase A (BoCPA) and *Metarhizium anisopliae* carboxypeptidase A (MeCPA). The sequence alignment is part of a multiple sequence alignment of carboxypeptidases made by ClustalW. Active site and Zn-coordinating residues are underlined. Residues forming the S1′ binding site are indicated in reverse-bold lettering. The recombinant form of MeCPA included an HHHHHHRR C-terminal sequence tag (boxed). The N-terminus of "mature" MeCPA shown here was generated by digestion of pro-MeCPA with thermolysin and verified by N-terminal amino acid sequencing (data not shown).