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Expression and Characterization of ECM14, a Metallocarboxypeptidase from Saccharomyces Cerevisiae

Matthew James Schott
Andrews University, schottm@andrews.edu

ABSTRACT

EXPRESSION AND CHARACTERIZATION OF ECM14,
A METALLOCARBOXYPEPTIDASE FROM
SACCHAROMYCES CEREVISIAE

by

Matthew James Schott

Chair: Peter J. Lyons

ABSTRACT OF GRADUATE STUDENT RESEARCH

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Title: EXPRESSION AND CHARACTERIZATION OF ECM14, A
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Name of researcher: Matthew James Schott

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Metallocarboxypeptidases are found in most organisms and function in the
digestion and maturation of proteins. Ecm14 is a putative metallocarboxypeptidase found
in the yeast *Saccharomyces cerevisiae* vacuole. The function of Ecm14 as an enzyme has
been unclear due to the presence of active site amino acids not typically found in
metallocarboxypeptidases, suggesting either no enzymatic activity or a unique
mechanism. In order to investigate the enzymatic mechanism of Ecm14, expression of
histidine-tagged Ecm14 protein was attempted in human HEK293T cell culture, *S.
cerevisiae*, and baculovirus expression systems. No expression was detected in HEK293T
cells in preliminary experiments. Expression in the yeast system resulted in insolubility
of Ecm14, regardless of induction time, temperature, or inducer concentration.

In contrast, following expression of Ecm14 in Sf9 cells using the baculovirus system, approximately 31% of Ecm14 was soluble and detected as a 40 kDa histidine-tagged protein by western blotting. Sf9-expressed Ecm14 was purified using metal affinity chromatography. No enzymatic activity could be detected for purified Ecm14 in the presence of substrate consisting of chromogenic 3-(2-furyl) acryloyl conjugated to a C-terminal dipeptide, PhePhe. Activation of Ecm14 by enzymatic removal of the prodomain was successful with the addition of chymotrypsin. Ecm14 cleaved of its prodomain showed activity in the presence of the above substrate with a preference for substrate at a pH of 6.0, similar to the known pH of the yeast vacuole. Ecm14 showed activity towards substrate proportional to the amount of enzyme present, suggesting that Ecm14 is non-catalytic in activity. Ecm14 can bind to substrate and limit the activity of a carboxypeptidase with known activity, CPA1, indicating that Ecm14 binds and cleaves but does not release the cleavage products.

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A METALLOCARBOXYPEPTIDASE FROM
SACCHAROMYCES CEREVISIAE

A Thesis

Presented in Partial Fulfillment

of the Requirements for the Degree

Master of Science

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APPROVAL BY THE COMMITTEE:

Peter J. Lyons, Ph.D., Chair

Robert Zdor, Ph.D.

Marlene Murray, Ph.D.   Date approved

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CHAPTER 1

INTRODUCTION

Peptidases, also termed proteases, proteolytic enzymes or proteinases, are enzymes that are responsible for performing proteolysis, the catabolism of peptide bonds linking amino acids in a protein polypeptide chain. This catabolism functions to digest long protein chains into smaller fragments. Proteolytic enzyme activity can serve a multitude of functions, such as food digestion or the regulation of catalytic cascades. The enzymes can break single, specific peptide bonds or break down an entire peptide into individual amino acids. Peptidases can function to activate processes, break down proteins for digestion or to render the protein nonfunctional, and act as a signal in a more complex signaling pathway. There is evidence that peptidases are present in animals, plants, bacteria, archaea, and viruses, illustrating their essential nature to all types of life.

Rawlings and Barrett founded the MEROPS classification system for peptidases in 1993 and introduced the MEROPS database for peptidases in 1996 (N. D. Rawlings & Barrett, 1993; Neil D. Rawlings, Morton, & Barrett, 2006). MEROPS provides classification and nomenclature for all known proteolytic enzymes and their inhibitors (Neil D. Rawlings et al., 2006). As recently as 1999, only 600 distinct peptidases could be recognized (Barrett, 1999) but by 2009 nearly 2,500 peptidases had been identified (Neil D. Rawlings, 2009). Roughly 13,000 physiological cleavages have been described, representing 45% of all peptidases known (Neil D. Rawlings, 2009). With so many

peptidases performing a wide range of functions existing in nature, and even more when considering synthetics, the MEROPS system is an essential tool for researchers.

Classifications under MEROPS are hierarchical and derived from a single, structural peptidase domain where activity is present in the multi-domain protein (Neil D. Rawlings, Barrett, & Bateman, 2012). The highest level of the hierarchy is the clan and represents proteins grouped by similar tertiary structures (Neil D. Rawlings et al., 2012) and by the residue present in the active site. The active site is the location on the enzyme where the substrate, in this case another protein, binds and proteolysis occurs. Peptidases may use aspartate, cysteine, glutamic acid, asparagine, serine, threonine, a metal ion, or a mixed combination of cysteine/serine/threonine in the active site. These active site residues determine which clan each peptidase belongs to, with each clan representing a type that arose from a single evolutionary origin (Neil D. Rawlings et al., 2012). Within each clan are one or more families that show evolutionary relationship to one another based on similar tertiary structures, active site residues and common active site sequence motifs (Neil D. Rawlings et al., 2006).

MEROPS classification also indicates various data about each family, including whether the proteins are endo- or exopeptidases, history, activities and specificities, molecular structure, distribution, and biological significance (Neil D. Rawlings et al., 2006). Every family may contain hundreds of related peptidases. At the bottom of the hierarchy is the peptidase unit. Genetic sequences that represent the same protein, even if they are from different organisms, are categorized as a single protein species (Neil D. Rawlings et al., 2012). A peptidase must demonstrate a completely different specificity from the other members of its family to be classified as unique, or a new "holotype" (Neil

D. Rawlings et al., 2012). Essentially it must cleave different substrates, cleave the same substrates in different places, or interact with a different set of inhibitors to warrant its own holotype (Neil D. Rawlings et al., 2012).

Metallopeptidases, or M-type peptidases, represent the most diverse clan, with over 50 unique families. First grouped in 1993, metallopeptidases require a metal ion to perform their catalytic activity. The largest group of metallopeptidases belongs to the MA clan and represents a wide range of functions, including aminopeptidase, metallo-exopeptidase, peptidyl-dipeptidase, oligopeptidase and endopeptidase activity. Clan MC contains primarily the metallocarboxypeptidases and clan MD represents a small number of peptidases that facilitate bacterial cell wall biosynthesis and lysis. All members of the M-type peptidases use water nucleophiles, and the majority of metallopeptidases require a zinc ion in the active site, but also can utilize ions such as nickel, cobalt, or manganese.

One particular type of metallopeptidases is grouped into the M14 subfamily and includes a number of proteins from several species. Members of this subfamily hydrolyze single, C-terminal amino acids from polypeptide chains and share the zinc-dependency common to most metallopeptidases. However, a distinctive characteristic of the M14 subfamily exists in the sequence motif that is responsible for binding a tetrahedrally coordinated zinc ion (Jongeneel, Bouvier, & Bairoch, 1989).. Rather than the typical HEXXH motif, M14 peptidases present a HXXE motif, or His-Xaa-Xaa-Glu (Neil D. Rawlings, 2009). A second histidine is typically found between 103 and 143 amino acids C-terminal to the HXXE motif (Becker & Roth, 1992). It was confirmed that the HXXE sequence was indeed the active site for catalytic activity when the histidines were mutated to arginine and the glutamate to glutamine in *Drosophila melanogaster* Protease

III (Becker & Roth, 1992). The histidine mutants possessed no proteolytic activity and showed negligible zinc signals, confirming their role in coordinating the zinc ion (Becker & Roth, 1992).

The first of the M14 proteins was identified over 80 years ago in bovine pancreatic extracts. Given the name carboxypeptidase (CP), it was found to be produced by the pancreas before secretion into the intestine for digestion of proteins (Waldschmidt-Leitz & Purr, 1929). A second metallocarboxypeptidase was discovered in 1956 and given the name basic carboxypeptidase (CPB), owing to the fact that it hydrolyzes basic C-terminal amino acids (Folk, 1956). The original carboxypeptidase, CP, was renamed CPA because its own cleavage of aliphatic/aromatic amino acids. CPA underwent a further name change in 1988 to CPA1 when a second pancreatic enzyme was discovered and named CPA2 (Gardell et al., 1988). Many other metallocarboxypeptidases have since been discovered, with dozens identified and spread across four M-subfamilies in plants, animals, bacteria, fungi, protozoa and archaea (Rodriguez de la Vega et al., 2007). These metallocarboxypeptidases share an affinity for hydrolyzing C-terminal amino acids and contain a metal in the active site. All of the metallocarboxypeptidases that share homologies with the original CPA are currently grouped into the M14A subfamily. This group contains the original two CPAs expressed in the human pancreas, as well as four other human CPAs that are not expressed in the pancreas but share CPA1/2-like function and the characteristic active site metal and affinity for aliphatic/aromatic amino acids (Huang et al., 1999; Reynolds, Gurley, & Austen, 1992; Wei et al., 2002). The M14A subfamily also includes CPB as well as CPO, the first metallocarboxypeptidase identified to cleave acidic C-terminal amino acids (Lyons & Fricker, 2011).

Unique among the metallocarboxypeptidases, but still a member of the M14 subfamily, is Ecm14, a putative enzyme found in the yeast *Saccharomyces cerevisiae*. Ecm14 possesses a lysine at position 270 in its putative active site, while all known CPA/CPB-type metallocarboxypeptidases have a glutamate at position 270 in the active site. Wu, Zhang, Xu, and Guo (2010) showed that along with amino acid residues Arg127, Tyr248, Arg71, Asn144, and Arg145, the Glu270 forms a shell around the active site and contributes to the catalytic function of CPA through the stabilization of substrate molecules. The Glu270 functions as a general base to activate the water molecule, which functions as a nucleophile. After substrate cleavage, it is hypothesized that the Glu270 functions as a general acid giving up the proton to the amide group of the cleaved amino acid, releasing the product. The Lys270 present in Ecm14 is an important variation because while glutamate is negatively charged, lysine is positively charged. This change may alter the ability of the Lys270 to perform the actions of a Glu270. Although the lysine at amino acid site 270 is unique to Ecm14 among the M14 subfamily, there are many fungal orthologs of Ecm14 that present a lysine in an identical manner, suggesting that this is an evolutionarily conserved portion of the gene and not a single mutation distinctive only in *S. cerevisiae* (Fig. 1).

Ecm14 consists of two domains, a prodomain and a catalytic domain (Fig. 2). This is typical for most related carboxypeptidases. A prodomain commonly functions to promote folding (Baker, Shiau, & Agard, 1993) and secretion (Vernet et al., 1990), and its presence maintains the peptidase in its inactive state (Segundo, Martinez, Vilanova, Cuchillo, & Aviles, 1982). Proteolytic cleavage of the prodomain catalyzes activation of

the peptidase; its removal is required to generate an active enzyme. The prodomain of carboxypeptidases is usually cleaved by an enzyme such as trypsin, which will preferentially cleave at arginine. However, Ecm14 does not contain a conserved arginine at this cleavage site; rather, a conserved Phe-Phe motif is present in most Ecm14 orthologs (Fig 1). With a PhePhe sequence present at the end of its prodomain, Ecm14 is likely to be cleaved by an enzyme that preferentially cleaves at phenylalanine. One potential such enzyme is proteinase A, which has preference for hydrolyzing hydrophobic residues with a PhePhe sequence (Kondo et al., 1998) (Fig. 2).

Localization data for Ecm14 indicate that it is located in the yeast vacuole (Huh et al., 2003) and has been quantified at 468 protein molecules per cell (Ghaemmaghami et al., 2003). Previous studies have proposed that Ecm14 is required for normal cell wall assembly (Jonson, Rehfeld, & Johnsen, 2004; Lussier et al., 1997) but its presence in yeast cell vacuoles suggests that there are perhaps other functions, as yeast cell vacuoles are often involved in many processes including homeostasis and osmoregulation.

In order to characterize the potential enzymatic activity of Ecm14, expression of histidine-tagged Ecm14 protein was attempted in three expression systems, human HEK293T cell culture, *S. cerevisiae*, and baculovirus. Ecm14 was successfully expressed in Sf9 cells using the baculovirus system in a soluble form. This Ecm14 was detectable by western blotting and was purified using Cobalt-metal affinity chromatography. Purified Ecm14 was activated by the enzymatic removal of the prodomain using chymotrypsin.

CHAPTER 2

MATERIALS AND METHODS

Saccharomyces cerevisiae Cell Culture and Expression System

Y1239 yeast cells with an Ecm14 deletion had previously been transformed with a

pEMBL plasmid (Dente, Cesareni, & Cortese, 1983) expressing histidine-tagged Ecm14

under the control of a galactose inducible promoter. The cells were retrieved from cold

storage (-80°C) and grown on SC-Ura (6.8 g/L yeast nitrogen base, 0.79 g/L dropout mix,

15 g/L agarose in H_2O). Individual colonies were selected from the plates and grown in

SC-Ura w/ dextrose nutrient broth overnight at 30°C. The cells were washed in ddH_2O

and induced for 16 hours in SC-Ura w/ 2.0% galactose at room temperature. In alternate

trials the cells were induced at 17°C, for 1, 2, 4 and 8 hours, and in the presence of 0.2%

galactose.

Saccharomyces cerevisiae Cell Lysis

After Ecm14 induction, the cells underwent an alkaline lysis in 0.1 N NaOH

(Kushnirov, 2000) to loosen the cell walls, followed by the addition of 1% NP-40 buffer

(150 mM NaCl, 1% NP-40, 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM PMSF) to

weaken the cell walls without denaturing the proteins. The cells were spun down in a

centrifuge at 14,000 rpm for 1 minute. The insoluble portion was dissolved in 1% SDS.

Both the insoluble and soluble portions were stored in SDS-PAGE sample buffer (62.5

mM Tris-HCl pH 6.8, 2.5% sodium dodecyl sulfate, .002% bromophenol blue, 5% β-

mercaptoethanol, 10% glycerol) for detection by western blotting. For guanidine and urea treatments, alkaline lysis was performed but treatment with NP-40 buffer was replaced with a guanidine treatment. Cells were introduced into guanidine buffers of varying concentration (6M, 3M or 1.5M guanidine, 50 mM Tris-HCl pH 7.5), and heated at 35°C for 15 minutes. The cells were spun down in a centrifuge at 14,000 RPM for 1 minute. The insoluble portion was dissolved in 1% SDS. Both the insoluble and soluble portions were stored in SDS-PAGE sample buffer for detection by western blotting. Denaturation in urea followed a similar protocol. Yeast cells were grown as above but introduced into urea (8M, 6M, 4M, 2M, 1M, 0.75M, 0.5M or 0.25M urea, 50 mM Tris-HCl pH 7.5) at room temperature for 2 hours. The cells were spun down, separated into insoluble and soluble portions and stored as above.

SDS-PAGE

Polyacrylamide gels were cast at the desired percentage, typically 10%. Samples were loaded along with Precision Plus Dual Color Standard molecular weight marker (BioRad). The gels were run at 140V in 1X running buffer (25 mM Tris-Hcl, 192 mM glycine, 0.1% SDS).

Coomassie Blue Staining

Polyacrylamide gels were washed three times for 5 minutes in H_2O to remove excess SDS. The gel was then incubated in 50 mL of Bio-Safe Coomassie stain (BioRad) on a rocking table for 1 hour. The gel was washed in 200 mL of H_2O for 30 minutes. An image of the stained gel was documented.

Western blotting

Transfer of Proteins to Nitrocellulose

Proteins resolved on the SDS-PAGE gel were transferred to nitrocellulose in transfer buffer (25 mM Tris-Hcl, 192 mM glycine, 0.1% SDS, 10% MeOH) at 100V for 1 hour.

Antibody Staining

Following protein transfer, the nitrocellulose membrane was placed in a container with 25 mL of blocking solution (5% skim milk in TBST (137 mM NaCl, 2.7 mM KCl, 19 mM Tris-HCl pH 7.5, 0.1% Tween-20)) for 30 minutes at room temperature on a rocking table. The blocking solution was discarded and membrane was incubated with 10 mL of primary antibody (mouse anti-His6 antibody (Thermo Scientific Cat#MA1-21315)), diluted 1:2000 in 5% skim milk/TBST for 1 hour on a rocking table. The nitrocellulose membrane was washed three times for thirty minutes total in TBST. After the third wash, the membrane was incubated with 10 mL of secondary antibody (α-mouse IgG, HRP-linked antibody (Cell Signaling Technology Cat #7076S)), diluted 1:2000 in 5% skim milk/TBST for 1 hour on a rocking table. The nitrocellulose membrane was washed three times for 30 minutes again in TBST. After the final wash, the TBST was discarded and the membrane was incubated for 1 minute with 10 mL of chemiluminescent substrate (0.5 mL 20X LumiGLO, 0.5 mL 20X peroxide (Cell Signaling Technology) diluted in 9 mL of H_2O). In a dark room, visualization of proteins bound to the secondary antibody was performed using x-ray film.

Ponceau S Staining

The nitrocellulose membrane with bound proteins was incubated in Ponceau S stain (0.1% Ponceau S in 1% acetic acid) for 5 minutes. The membrane was then washed in 5% acetic acid for 5 minutes and twice in H_2O for 5 minutes. Bound proteins were visualized as they were stained red by the Ponceau S.

Plasmid Preparation

DH5α competent cells (New England BioLabs) transformed with pRS316 plasmids expressing C-terminal histidine-tagged Ecm14 (Ecm14-His6) were prepared using the NID method (Lezin, Kosaka, Yost, Kuehn, & Brunelli, 2011). The NID method allows for isolation using non-ionic detergents in a one-tube application that results in sufficiently pure plasmid DNA without the need for harsh alkaline or SDS treatments.

Plasmid Construction

In order to amplify the Ecm14 DNA insert, PCR was performed on 10 ng pRS316 plasmid with primers purchased from Invitrogen and PfuUltra DNA polymerase (Agilent). Proteinase K (Carolina) treatment as described by Crowe et al. (1991) was performed to improve cloning efficiency. DNA amplicons were purified using the QIAquick Gel Extraction Kit (Qiagen) and diluted in 30 µL of water. Purified amplicons were digested with restriction enzymes NotI and BamHI (Invitrogen).

Ligation of Ecm14-His6 insert into pVL1392
Plasmid and Transformation Into DH5α Cells

Recipient plasmid pVL1392 was digested with restriction enzymes NotI and BamHI. The ligation of the Ecm14-His6 insert into the pVL1392 recipient plasmid was performed using T4 DNA Ligase (Promega). Ligated pVL1392 plasmid was transformed

into competent DH5α cells and plated on LB/amp plates at 37°C overnight. Restriction digest of the newly transformed cells was performed with NotI and BamHI to confirm that the insert was properly transformed into the DH5α cells. Five samples of pVL1392 plasmid with the Ecm14-His6 insert were sent for sequencing (GenScript).

Ligation of Ecm14-His6 Insert Into pcDNA3.1+ Plasmid
and Transformation Into DH5α Cells

Sequencing results confirmed that the pVL1392 plasmid transformation contained the correct Ecm14-His6 insert. Plasmid preparation was performed as before on the DH5α cells containing the pVL1392 plasmid with the Ecm14-His6 insert. The plasmid was digested with NotI and BamHI as above. The Ecm14-His6 insert was then ligated into a new plasmid, pcDNA3.1+. The plasmid was transformed into competent DH5α cells which were plated on LB/amp plates at 37°C overnight. Restriction digest of these cells with NotI and BamHI was performed to confirm that the Ecm14-His6 inserts were properly transformed into the DH5α cells.

HEK293T Cell Culture, Transfection and Cell Lysis

HEK293T cells were retrieved from liquid nitrogen storage, were thawed and grown in a monolayer in Dulbecco's Modified Eagle Medium (Sigma) supplemented with 10% FBS and penicillin/streptomycin in 75 cm^2 culture flasks in a 5% CO_2 incubator at 37°C. pcDNA3.1+ plasmids containing the Ecm14-His6 insert were transfected into the HEK293T cells using polyethylenimine (PEI) (Ehrhardt et al., 2006). Cells were lysed 48 hours post-transfection in NP-40 buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl pH 8.0, 0.2 mM PMSF, 1% P8849 protease inhibitor cocktail purchased

from Sigma). Samples of media and soluble and insoluble cell lysate fractions were collected and added to SDS-PAGE sample buffer for detection by western blotting.

Sf9 Cell Culture, Transfection and Cell Lysis

Sf9 cells were retrieved from liquid nitrogen storage, were thawed and grown in suspension culture in Sf9 media (Gibco) in 250 mL polycarbonate Erlenmeyer shaker flasks at 28°C. In order to produce recombinant baculovirus expressing Ecm14, 0.1 μg of pVL1392 plasmid containing the Ecm14 insert was mixed with 2.5 μL of Progreen baculovirus vector DNA and brought to a total volume of 25 μL in H_2O. Twenty-five μL of Profectin transfection agent diluted to 10% in water was added dropwise to the baculovirus DNA and plasmid mixture (AB Vector). The mixture prepared above was added dropwise to 1 mL of Sf9 cells in adherent culture at $5x10^5$ cells/mL. The virus was harvested from this culture after 60 hours and saved as a P0 stock. The P0 stock (0.5mL) was added to 15 mL of Sf9 cells at a density of $1x10^6$ cells/mL in order to create a P1 stock with a high titer of baculovirus expressing Ecm14. The P1 stock was grown for 7 days at room temperature. A P2 stock with an even more amplified virus titer was created by adding 1 mL of the P1 stock to 50 mL of Sf9 cells that had reached a density of $2x10^6$ cells/mL. The cells were placed on a shaking table at room temperature. Sixty hours post viral infection the Sf9 cells were spun down at 5000 RPM. The recovered Sf9 media was composed of a high-titer of Ecm14-expressing baculovirus and was saved at 4°C for future infections. The pelleted cells were lysed in NP-40 buffer for analysis.

Purification of Ecm14

Ecm14 that had been denatured in urea or successfully expressed by the baculovirus expression system was purified using a modified version of the TALON IMAC (immobilized metal affinity chromatography) protocol (Clontech). One mL of metal affinity resin (0.5 mL bed volume) was resuspended in a 15 mL conical tube. The resin was spun down at 1500 RPM for 2 minutes and the supernatant was discarded. The resin was equilibrated in 10 mL of denaturing wash buffer for Ecm14 denatured in urea (50 mM Sodium Phosphate, 8 M urea, 300 mM NaCl) or native wash buffer (50 mM Sodium Phosphate, 300 mM NaCl) at pH 7.0 for 10 minutes and spun down at 1500 RPM for 2 minutes. The supernatant was discarded. The wash was repeated twice. After discarding the supernatant from the second wash, 10 mL of crude Ecm14 was added to the resin and shaken on a rocking table for 1 hour. After 1 hour, the resin bound with Ecm14 was spun down at 1500 RPM for 3 minutes. Two more washes as above were performed. After the supernatant from the second wash was discarded, 1 mL of wash buffer was added to the resin and was transferred to a 2 mL disposable gravity column (Clontech). Ten mL of wash buffer was allowed to run through the column for a final wash. The Ecm14 was eluted from the column using either a denaturing elution buffer (45 mM Sodium Phosphate, 7.2M urea, 270 mM NaCl, 150 mM imidazole) or native elution buffer (50mM Sodium Phosphate, 300 mM NaCl, 150 mM imidazole) at pH 7.0. Purified Ecm14 was collected in 0.5 mL fractions.

Enzyme Assay

Carboxypeptidase substrates in which chromogenic 3-(2-furyl) acryloyl is conjugated to a C-terminal dipeptide (either phenylalanine-alanine or phenylalanine-

phenylalanine) were purchased from Bachem. The substrates were dissolved in a buffer

(50 mM Sodium Acetate, 50 mM Tris, 150 mM NaCl) at the appropriate concentration

and pH. Proteins were incubated with substrate at room temperature. Cleavage of the

substrate was recorded as a decrease in absorbance at 340 nm. All trials were repeated at

least three times. Statistical calculations were performed using a paired Student's t-test.

CHAPTER 3

RESULTS

Ecm14 Expressed in Yeast

The first obstacle in studying Ecm14, from the yeast *Saccharomyces cerevisiae,*

was obtaining soluble protein, a requirement in order to further study its unique

characteristics. As Ecm14 is a protein native to yeast, the most sensible system to use to

overexpress and produce sufficient amounts of protein for study was the yeast cell itself.

In addition, expressing proteins in yeast is a relatively simple methodology that requires

simple media that is easily prepared in the lab and is much less expensive and time

consuming than other expression systems. In order to investigate the function of the

putative Ecm14 enzyme from *Saccharomyces cerevisiae*, Y1239 yeast cells with an

Ecm14 gene deletion were transformed with a plasmid expressing histidine-tagged

Ecm14 under the control of a galactose-inducible promoter (pEMBL).

To purify and analyze Ecm14, NP-40 detergent was added to the induced cells in

order to solubilize the cell membranes without denaturing the proteins. Centrifugation at

14,000 rpm separated the soluble fraction from the insoluble which was subsequently

dissolved in 1% SDS. Prominent immunoreactive bands were seen at roughly 40 kDa,

intermediate between the known molecular weights for proEcm14 before proteolytic

cleavage of the prodomain (50 kDa) and mature, cleaved Ecm14 (35 kDa). The molecular

weights for both the pro and mature forms of Ecm14 were derived from their respective

amino acid compositions and length. However, Ecm14 was insoluble. The length of

induction time can affect protein expression and solubility. To test if decreasing the

induction time would increase solubility, cells were induced for 1, 2, 4, and 8 hours

instead of 16 and while all showed immunoreactive bands at 40 kDa via western blotting,

all Ecm14 protein was insoluble (Fig. 3A). With 8 hours of induction there appeared to

be a small amount of soluble expressed protein but this result was not reproducible. In

order to investigate whether growing the yeast at a higher or lower temperature would

alter expression and potentially increase solubility of Ecm14, Y1239 cells were also

induced at both 30°C and 17°C instead of at room temperature. Histidine-tagged Ecm14

was detected via western blotting at roughly 40 kDa but again only in the insoluble

fraction (Fig. 3B). In order to test the potential of lower concentrations of galactose

during induction, limiting the expression of Ecm14 and increasing the probability that

Ecm14 was properly folded, cells were induced in SC-Ura media with 0.2% galactose

instead of 2.0%. All immunoreactive bands, specifically the 40 kDa band, remained

insoluble (Fig. 3C). These experiments confirmed that Ecm14 can be overexpressed in *S.

cerevisiae*, but in an insoluble form. This result may be due to an inability for the yeast

cell to properly fold the Ecm14 protein when required to do so on such a large scale.

Ecm14 Protein Denatured in Urea

With the ability to faithfully overexpress Ecm14 in a yeast cell expression system,

it was believed that the improperly folded protein could be denatured with either

guanidine or urea, purified and refolded from its denatured state into a properly folded,

soluble protein. Denaturing of proteins in guanidine or urea and subsequent refolding is

possible, whereas refolding from an SDS-denatured state is uncommon. An additional

benefit is the ion metal affinity chromatography (IMAC) purification protocol, which

allows purification of denatured protein (if necessary) before refolding. The IMAC resin

consists of a chelator charged with cobalt and exhibits both affinity to and specificity for

histidine-tagged proteins (Porath, Carlsson, Olsson, & Belfrage, 1975), such as Ecm14.

Y1239 yeast cells were grown overnight and subsequently induced in 2.0%

galactose as before. Alkaline lysis was performed but extraction with NP-40 buffer was

replaced with a guanidine extraction. Proteins were extracted with guanidine buffers of

varying concentration (6M, 3M and 1.5M), and heated at 35°C for fifteen minutes to

increase solubility of guanidine and assist in the denaturing process. Western blotting

showed apparent immunoreactive bands at roughly 40 kDa in some fractions but repeated

tests showed inconsistencies in results, possibly caused by precipitation of SDS-PAGE

samples by guanidine and subsequent distortion of the bands seen by western blotting. It

was decided that the denaturing agent would be switched to urea. The efficacy of urea to

denature Ecm14 protein was initially tested with urea denaturation buffer (8M and 6M) at

room temperature for twenty minutes. Both concentrations of urea were effective in

denaturing and solubilizing Ecm14 (results not shown).

IMAC Purification of Denatured Ecm14 Expressed in Yeast

Purification of urea-denatured Ecm14 was attempted by IMAC. Although Ecm14

solubilized by urea was not present in the flow-through, suggesting it was able to bind to

the resin, eluting it with the addition of a competitive binding agent (imidazole) proved

unsuccessful (Fig. 4). Attempts to remove Ecm14 from the resin using higher

concentrations of imidazole and SDS-PAGE sample buffer also proved unsuccessful.

Refolding Ecm14 was not possible with the unsuccessful purification.

Ecm14 Expressed in HEK 293T Cells

Expression of Ecm14 in insect and/or mammalian cells required the construction of new expression plasmids. PCR was performed to amplify the histidine-tagged Ecm14 insert present in pRS316 plasmids stored in the lab. DNA sequencing (GenScript) confirmed that the amplified Ecm14 insert was successfully ligated into the pVL1392 plasmids to be used during the baculovirus transfection process. Once the pVL1392-Ecm14-His6 plasmid sequence was confirmed, the insert was cut out and inserted by ligation into another plasmid, pcDNA3.1+, designed for expression of proteins in mammalian cells.

The first expression system attempted was a mammalian expression system using human HEK293T cells. The cells were grown in a 5% CO2 incubator at 37°C. The cells were transfected with the pcDNA3.1-Ecm14-His6 plasmid and proteins harvested after 48 hours in NP-40 buffer. Initial western blotting results showed an immunoreactive band at roughly 40 kDa in the secreted (media) sample and no bands in either the soluble or insoluble fractions of HEK293T cells (results not shown). However, these results were not consistent, overall suggesting weak or no expression of histidine-tagged Ecm14 in HEK293T cells.

Ecm14 Expressed in Sf9 Insect Cells

In order to examine the expression of Ecm14 in Sf9 cells, recombinant Ecm14-expressing baculovirus was made (see methods). Sf9 cells were infected with Ecm14-expressing baculovirus and collected 60 hours post infection. Western blotting of media and cell lysate showed an immunoreactive band at roughly 40 kDa in the soluble and insoluble portions of cell lysate and no bands in the media (Fig. 5). Processing of the

results using ImageJ showed the soluble portion to represent an average of 31% of the total protein present across three trials.

IMAC Purification of Histidine-tagged Ecm14 From Insect Cells

The next step was to purify the soluble Ecm14 using the TALON IMAC resin previously used in attempts to purify urea-denatured Ecm14. Purification using this method proved successful with Ecm14 from the baculovirus expression system. Ponceau S staining indicated that the very large number of proteins present in the crude Ecm14 extract was reduced to very few after purification (Fig 6A). Analysis of western blotting results (Fig. 6B) by ImageJ determined that approximately half of the soluble Ecm14 was recovered through the purification process. In addition, approximately half of the eluted proteins appear unrelated to Ecm14, as seen by Ponceau S staining.

Enzymatic Activity Assay of Purified Ecm14

With purified Ecm14 available, the next step was to determine if it was enzymatically active. Activity of purified Ecm14 could be measured as a decrease in the absorbance at 340nm of a synthetic 3-(2-furyl)acryloyl (FA) peptide carboxypeptidase substrate upon incubation with enzyme. Ten micrograms of Ecm14, as well as commercially available CPA1, were incubated with FA-PhePhe and FA-PheAla for 90 minutes at room temperature and at both pH 5.5 and 7.5. While the control enzyme, CPA1, showed robust activity upon incubation with both substrates and at both pH values, no activity was observed for purified Ecm14 (Fig. 7).

Enzymatic Removal of the Prodomain of Ecm14

It was hypothesized that Sf9 insect cells may not have a mechanism for removing the prodomain of Ecm14. In order to do so, 200 μg of purified Ecm14 was incubated with either 10 μg of trypsin or chymotrypsin. To stop the reaction, trypsin and chymotrypsin were inhibited at appropriate time points by the addition of 10 μg of PMSF, a serine protease inhibitor. Chymotrypsin digestion of Ecm14 resulted in the production of a new band at 35 kDa. This digestion appeared complete at 2 minutes, and the 35 kDa band remained stable over at least a 3-hour time period (Fig. 8A). Trypsin digestion of Ecm14 also resulted in a new 35 kDa band at 5 minutes that also remained stable over at least a 3-hour time period (Fig. 8B).

Activity Assay of Ecm14 Cleaved of Its Prodomain

Enzymatic assays previously done to test for Ecm14 activity were now performed with mature Ecm14, lacking the prodomain. Ecm14 (10 μg), previously cleaved with chymotrypsin, showed a small amount of activity after 2 hours of incubation with carboxypeptidase substrate FA-PhePhe (0.5 mM) at pH 7.5 (Fig. 9). Ecm14 samples tested were from multiple batches prepared on different days. Repeated assays confirmed that this level of activity was significantly greater than a no enzyme control ($p =$ 0.002886). CPA1 (10 μg) again showed much more robust activity against the substrate (results not shown). No activity was observed for purified Ecm14 (10 μg) not treated with endoprotease, confirming previous results, as well as for Ecm14 (10 ug) treated with trypsin. Chymotrypsin and trypsin were also incubated alone with substrate and showed no activity against this substrate.

Increasing the Purity of Ecm14

Previously enzymatic cleavage of the prodomain of Ecm14 was performed after

Ecm14 had been purified via metal affinity chromatography. Mature Ecm14 used in the

above enzymatic assays was complicated by the fact that it necessarily contained

chymotrypsin or trypsin, as well as the PMSF inhibitor. In order to eliminate these

contaminants, purification of Ecm14 from 10 ml crude Sf9 lysate ($2x10^7$ cells) was

performed after incubation for 10 minutes with 500 μg of either trypsin or chymotrypsin.

This resulted in purified mature Ecm14 (35 kDa) of much higher quality (Fig. 10).

Quantification of Coomassie stained gels using ImageJ shows that Ecm14 that was

trypsinized prior to purification results in an 80% pure sample. Ecm14 chymotrypsinized

prior to purification results in a sample that was > 95% pure. This is higher than seen in

previous purifications of Ecm14, which were limited to about 50% purity.

Activity Assay Using Highly Purified Ecm14

Ecm14 shows enzymatic activity against carboxypeptidase substrate FA-PhePhe

when the prodomain has been cleaved by chymotrypsin. Testing a range of pH values for

the substrate shows that Ecm14 has the greatest activity in the presence of substrate at a

pH of 6.0 (Fig. 11).

As a result of the minimal activity seen for chymotrypsinized Ecm14, it was

hypothesized that the chymotrypsinized Ecm14 was binding the FA-PhePhe substrate and

cleaving it but not releasing the product. To further test the theory that chymotrypsinized

Ecm14 is non-catalytic, Ecm14 (1 or 5 μg) was incubated with FA-PhePhe substrate at

pH 7.5, at a concentration of 10 μM. If Ecm14 activity was not catalytic it would be

presumed that roughly five times more activity would be seen in the presence of five

times more substrate. Ecm14 incubated in five times more substrate resulted in three to five times more activity (Fig. 12), suggesting that Ecm14 binds to and cleaves substrate but does not release the product.

Catalytic Activity of Ecm14

If Ecm14 was binding the substrate but not releasing any product, it was necessary to further investigate the binding properties of Ecm14 as they relate to function. The concentration of substrate was reduced significantly to compare activity against a CPA1 control. Decreasing the concentration of carboxypeptidase substrate FA-PhePhe to 1 μM at pH 7.5 revealed activity for chymotrypsinized Ecm14 (10 μg) similar to that seen for CPA1 (10 μg) upon 1 minute of incubation (Fig. 13). No additional activity was recorded for either enzyme after longer incubations, suggesting that all substrate had been cleaved. Ecm14 cleaved with trypsin (10 μg) showed no activity, confirming previous results. Trypsinized Ecm14 (10 μg) was incubated with substrate for 5 minutes prior to the addition of CPA1 (10 μg). This resulted in a decrease in activity for CPA1, indicating that trypsinized Ecm14 was binding to the substrate and inhibiting complete binding by CPA1; however, it was not enzymatically active. Bovine serum albumen was also incubated with carboxypeptidase substrate for 5 minutes prior to the addition of CPA1 as a control and did not inhibit CPA1 activity, as the cleaved Ecm14 did. These results show that both trypsinized and chymotrypsinized Ecm14 can bind substrate. The trypsinized Ecm14 binds the substrate but does not cleave it, while chymotrypsinized Ecm14 both binds and cleaves but appears to lack catalytic properties.

CHAPTER 4

DISCUSSION

In this study of the function of Ecm14, three expression systems were employed with the goal of expressing and purifying Ecm14. The initial system chosen was a *Saccharomyces cerevisiae* yeast cell expression system. Ecm14 is a protein native to *S. cerevisiae*. Previous studies have shown success in overexpressing carboxypeptidase Y (Takegawa et al., 2003) and carboxypeptidase G2 from *Pseudomonas* (Clarke, Gibson, Sherwood, & Minton, 1985) in a similar expression system. Goda, Kyyaly, Al-Jabiry, Walls, and Al Sayrafi (2012) were able only to express human mast cell carboxypeptidase A in *E. coli* as inclusion bodies but found success producing soluble CPA using *S. cerevisiae*. However, a more commonly used system for expressing proteins is the yeast *Pichia pastoris*, first described by Ogata K (1969). Over 200 heterologous proteins have been successfully expressed in *P. pastoris* (Cregg, Cereghino, Shi, & Higgins, 2000), including human carboxypeptidase M (Craveiro et al., 2006) and human carboxypeptidase A4 (Tanco et al., 2010) among other M14 carboxypeptidases, the family Ecm14 belongs to. An inability to produce soluble Ecm14 using a *S. cerevisiae* expression system suggests issues in folding of this protein, a problem that might not be present in another yeast expression system such as *P. pastoris*.

Due to the inability to express soluble Ecm14 in a yeast cell expression system, a mammalian cell expression system was employed, human embryonic kidney or HEK293T cells (Graham, Smiley, Russell, & Nairn, 1977). Mammalian cells have been used for the expression of metallocarboxypeptidases, such as carboxypeptidase M (Zhang, Tan, Zhang, & Skidgel, 2008) and CPA6 (Lyons & Fricker, 2010), but these proteins are native to humans. There are examples of yeast proteins being successfully expressed in HEK293 cells (Yin et al., 2005) but these are not carboxypeptidases. There is no evidence in the literature of the successful expression of a fungal carboxypeptidase in a mammalian cell expression system, which may explain the difficulties in expressing Ecm14 in HEK293T cells.

Expression of proteins using baculovirus-infected insect cells has been for the past few decades a method of choice for heterologous protein expression. Thousands of recombinant proteins have been produced with this methodology, and the field continues to advance as insect cell lines are engineered to handle the specific molecular functions of the organisms from which the expressed proteins were originally derived (Kost, Condreay, & Jarvis, 2005). Carboxypeptidase E-1 from *Aplysia californica* was successfully expressed in baculovirus-infected Sf9 cells (Juvvadi, Fan, Nagle, & Fricker, 1997). MeCPA, a fungal, A-type carboxypeptidase from *Metarhizium anisopliae,* was successfully expressed using baculovirus-infected Sf9 and Sf21 cells (Brian P. Austin, Tözsér, Bagossi, Tropea, & Waugh, 2011; Joshi & Leger, 1999). Ecm14 expressed in baculovirus-infected Sf9 cells proved successful as well, suggesting that the production of soluble, fungal carboxypeptidases using a baculovirus expression system is an acceptable method.

There are many chromatographic methods for the purification of proteins. Immobilized metal ion affinity chromatography (IMAC) is one such method for isolating proteins (Porath, 1992). A metal ion such as cobalt binds the histidine-tagged protein, as all other untagged proteins pass through a column. Eluting the column with a competitive binding agent, such as imidazole, results in a sample of purified protein. There is ample evidence of the ability to successfully purify histidine-tagged metallocarboxypeptidases using ion metal affinity chromatography (Figueiredo & Duque-Magalhaes, 1994; Raksakulthai & Haard, 2001), including the purification of fungal carboxypeptidase MeCPA (B. P. Austin & Waugh, 2012). Ecm14 was successfully isolated to purity levels above 70% using this method. However, in order to increase the purity of the protein sample, secondary and even tertiary methods should be employed. Other typical methods of purification for carboxypeptidases include gel filtration and ion exchange chromatography (B. P. Austin & Waugh, 2012; Craveiro et al., 2006), as well as potato carboxypeptidase inhibitor (PCI) affinity chromatography (Goldstein, Kaempfer, Kealey, & Wintroub, 1989; Lyons & Fricker, 2010, 2011). Purification protocols combining at least two methods result in the greatest purity of final product. Employing a secondary purification method would likely increase the purity of Ecm14.

Metallocarboxypeptidases from the M14 family, such as Ecm14, that contain a prodomain, require its removal for activation of the enzyme (Gomis-Rüth, 2008; Ventura, Gomis-Ruth, Puigserver, Aviles, & Vendrell, 1997). The typical carboxypeptidase prodomain ends with a conserved arginine and can be proteolytically cleaved by an enzyme with a preference for arginine, such as trypsin (Tanco et al., 2010). Ecm14 is

unique in that the prodomain ends with a PhePhe sequence and therefore is unlikely to be cleaved by a tryspin-like enzyme. An enzyme more likely to cleave the prodomain from Ecm14 is proteinase A. Proteinase A shows substantial homology to another protease, pepsin, which itself preferentially cleaves at phenylalanine, and was determined to be the enzyme responsible for activating several yeast vacuolar zymogens (Ammerer et al., 1986). Other studies have shown that proteinase A is most effective at acidic pH (Sorensen, van den Hazel, Kielland-Brandt, & Winther, 1994) and preferentially cleaves between two hydrophobic amino acids (Dreyer, 1989), like the PhePhe sequence in Ecm14. To investigate the involvement of proteinase A in the cleavage of the prodomain, I co-expressed PEP4, the gene responsible for encoding proteinase A, with Ecm14 in yeast cells. This was an attempt to remedy the continual production of insoluble Ecm14. It was hypothesized that with sufficient proteinase A present, the overexpressed Ecm14 would be cleaved of its prodomain and matured into its active state. The addition of PEP4 did not change either the solubility or size of expressed Ecm14 (results not shown). It is likely that the improperly folded Ecm14 expressed in yeast cells did not allow proteinase A access to the PhePhe cleavage site and, therefore, was not able to cleave the prodomain at all. An additional enzyme that may be responsible for cleaving the prodomain from Ecm14 is Kex2. Kex2 is another yeast endoprotease but it cleaves preferentially at dibasic amino acids (Fuller, Brake, & Thorner, 1989), which do not exist in the Ecm14 prodomain. It is possible there is weak cleavage at a single arginine site in the prodomain but this gives further evidence towards proteinase A being the likely enzyme cleaving the prodomain.

Ecm14 expressed from the baculovirus expression system appeared to have no enzymatic activity towards a substrate consisting of chromogenic 3-(2-furyl) acryloyl conjugated to a C-terminal dipeptide (PhePhe). It was hypothesized that the prodomain of Ecm14 still remained after protein expression. Purified Ecm14 was incubated with trypsin, an enzyme known to cleave the prodomain of CPA/B-type carboxypeptidases (Tanco et al., 2010) and chymotrypsin, an enzyme shown to cleave the prodomain from MeCPA (Sangawa et al., 2013). Both trypsin and chymotrypsin appeared to successfully cleave the prodomain, resulting in a roughly 35 kDa mature protein. Trypsin, as stated above, preferentially cleaves arginine. There are several sites in the prodomain of Ecm14 where trypsin might cleave (see Fig. 1), releasing the prodomain. However, these sites are N-terminal to the PhePhe likely to be the prodomain cleavage site and may result in a fragment of the prodomain blocking the active site; this is consistent with the lack of enzymatic activity seen with trypsinized Ecm14. Chymotrypsinized Ecm14 does appear to have activity, suggesting that chymotrypsin may be cleaving at the PhePhe site *in vitro*.

Although enzymatic activity for Ecm14, activated by removal of the prodomain with chymotrypsin, was recorded, it was discovered that the amount of activity was directly proportional to the concentration of Ecm14 incubated with substrate. This suggests that Ecm14 is binding the substrate and cleaving but is not releasing the product, making Ecm14 enzymatically non-catalytic. One hypothesis for this phenomenon is directly related to the presence of a Lys270 in Ecm14 instead of the Glu270 typically seen in carboxypeptidases. Christianson and Lipscomb (1989) hypothesized that after cleavage of the substrate by typical carboxypeptidases, the negatively charged Glu270 repels the ionized product, facilitating its release. The Lys270 of Ecm14 might act as a

base and subsequent acid, similar to Glu270, in order to facilitate cleavage of the substrate but may not release the product when the Lys270 returns to its uncharged state.

Ecm14 from *S. cerevisiae* can be expressed in soluble form using a baculovirus-infected insect cell expression system and purified using ion metal affinity chromatography. It does not exhibit activity when expressed in this system but can be activated by proteolytic cleavage of the prodomain using chymotrypsin. Greatest activity for Ecm14 was observed when incubated with a FA-PhePhe substrate with pH 6.0. Although activity was seen, it was non-catalytic, which may be a result of the Lys270 atypical of M14 carboxypeptidases. Further research into this putative enzyme should investigate the activity of Ecm14 towards other substrates. Site-directed mutagenesis would provide confirmation of the activity of chymotrypsinized Ecm14. Edman degradation would provide insight into the differences in observed activity for Ecm14 cleaved of its prodomain by either trypsin or chymotrypsin. Using X-ray crystallography to determine the 3d structure of Ecm14 would provide a more detailed explanation of the mechanisms by which the prodomain is cleaved and the enzymatic activities of this unique protein.

Table 1. Plasmids Used in This Study

Plasmid name	Origin	Relevant characteristics	Reference
pEMBLyEX4-Ecm14-His6	Dr. Peter Lyons	Contains histidine-tagged Ecm14. Galactose inducible promoter.	(Cesareni & Murray, 1987)
pRS316-Ecm14-His6	Dr. Peter Lyons	Contains histidine-tagged Ecm14.	(Sikorski & Hieter, 1989)
pcDNA3.1(+)-Ecm14-His6	This study.	Contains histidine-tagged Ecm14. Mammalian expression.	
pVL1392-Ecm14-His6	This study.	Contains histidine-tagged Ecm14. Baculovirus expression.	

Figure 1. Amino acid sequence for *Saccharomyces cerevisiae* Ecm14 and four other related proteins.

Sequences for *Saccaharomyces cerevisiae* Ecm14 (50 kDa, prodomain: 13 kDa; NM_001179262.1), *Candida maltosa* Ecm14 (54 kDa, prodomain: 17 kDa; M3K6C1), *Wickerhamomyces ciferrii* carboxypeptidase (58 kDa, prodomain: 21 kDa; K0KG05), *Metarhizium anisopliae* zinc carboxypeptidase (61 kDa, prodomain: 20 kDa; E9EUG8) and *Aspergillus fumigatus* zinc carboxypeptidase (66 kDa, prodomain: 21 kDa; A0A084BIB7) reveal many similarities. These sequences were chosen as they illustrate conservation of amino acid sequences across a wide range of fungal organisms. The black and grey shading represents amino acid homologies between two or more species. The PhePhe sequence found at the end of the prodomain is present in all five species (*). The lysine found in the active site is also found in all five species (+).
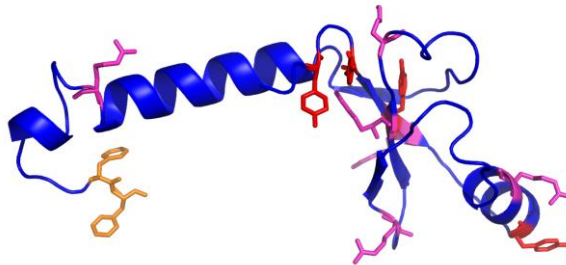
A.
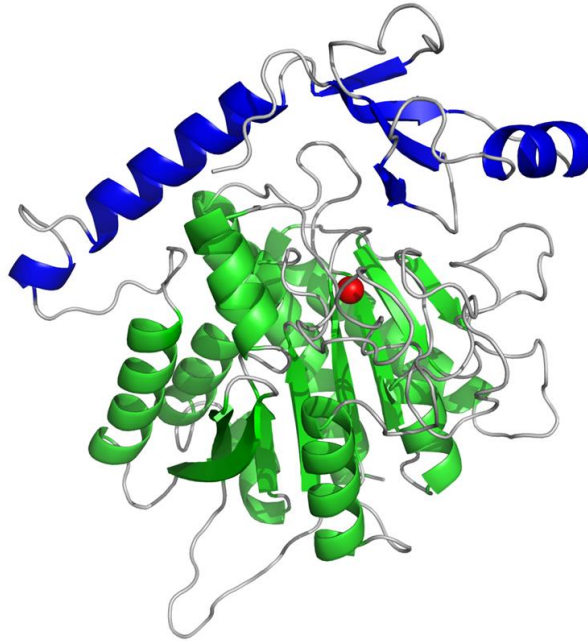
B.

Figure 2. Ecm14 is similar in structure to other metallocarboxypeptidases, such as CPA1.

A. The structure of Ecm14 was modeled with the homology modelling program SwissModeller using the X-ray crystal structure available for bovine CPA1 (PDB ID 1PYT) as a template. The prodomain of Ecm14 is shown in blue, while the mature form of Ecm14 (the catalytic domain only) is shown in green. Most carboxypeptidases contain a zinc in the active site, shown here as a red sphere. B. Potential cleavage sites in the prodomain (>90% probability as determined by the ExPASy PeptideCutter) for trypsin and chymotrypsin are shown in red and magenta, respectively. The predicted PhePhe cleavage site for the prodomain, a potential cleavage site for chymotrypsin, is shown in orange. Image created with Pymol.
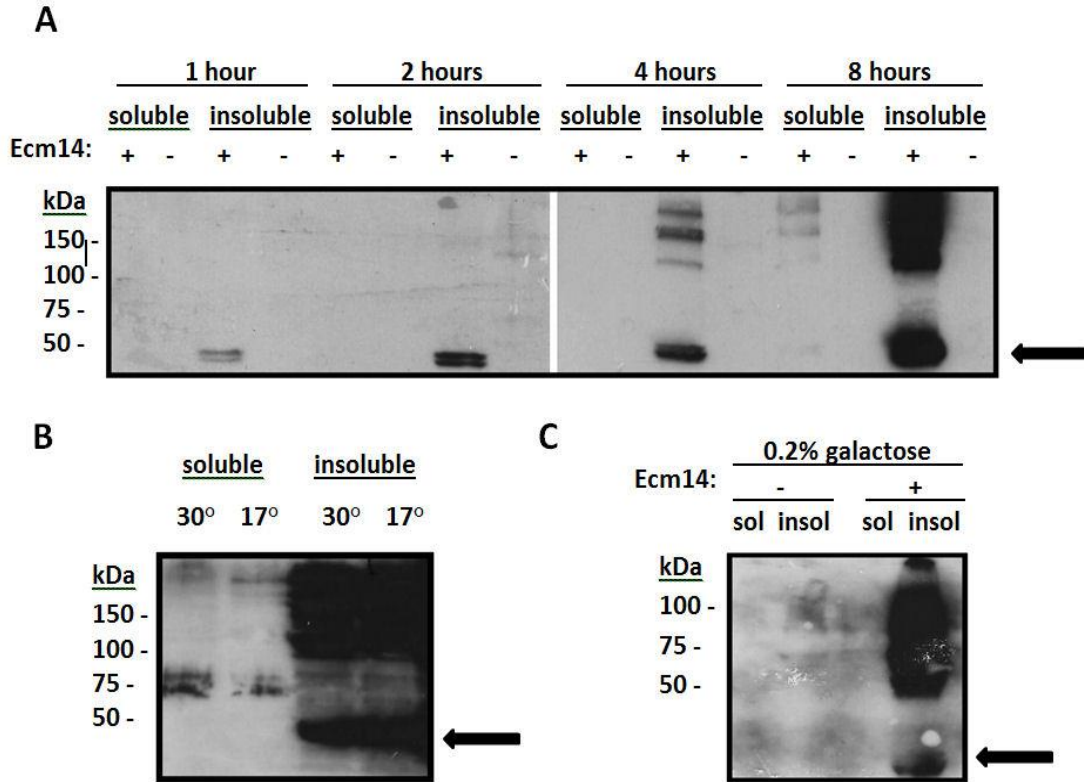
Figure 3. Ecm14 is expressed in yeast as an insoluble 40 kDa protein.

Ecm14 expression was induced for varying lengths of time (A), at varying temperatures (B), and in 0.2% galactose (C). Histidine-tagged Ecm14 was detected by western blotting. ProECM14 was detected as a 40 kDa histidine-tagged protein (arrow). Higher molecular weight bands are thought to be multimeric forms of Ecm14.
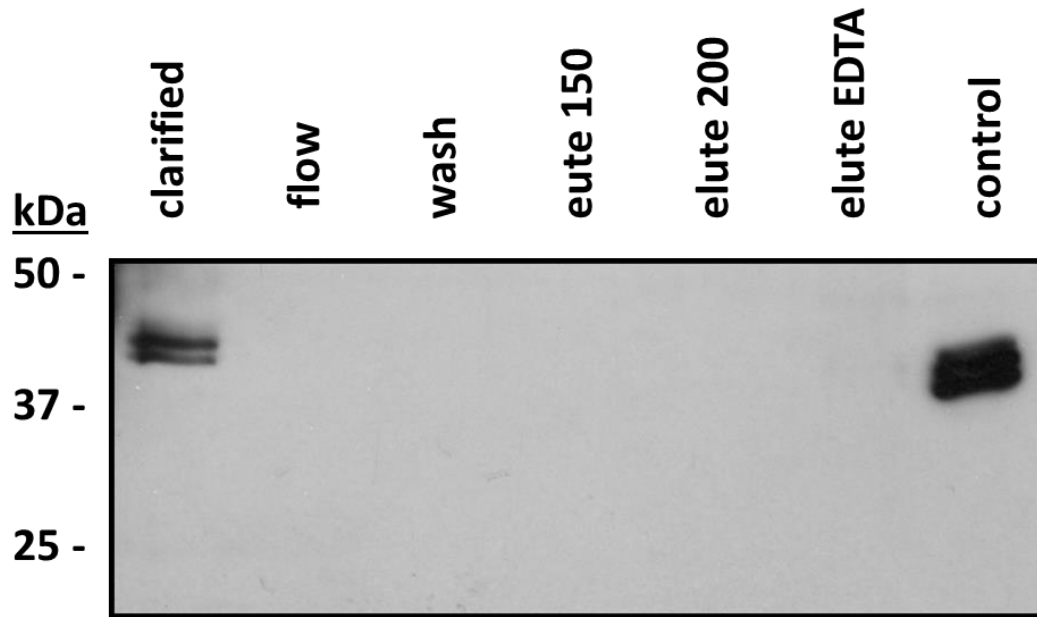
Figure 4. Denatured Ecm14 expressed in yeast cannot be purified using a Cobalt-metal affinity column.

Attempts to purify urea-denatured Ecm14 (clarified) failed to result in any eluted protein. Histidine-tagged Ecm14 was detected by western blotting. The lack of a band seen in the flowthough (flow) suggests that Ecm14 successfully bound the IMAC resin. No bands are seen in the wash which suggests Ecm14 did not wash away due to weak binding. Washing the column with either 150 mM (elute 150) or 200 mM (elute 200) imidazole resulted in no eluted protein. Washing with EDTA also failed to elute the column, suggesting that urea-denatured Ecm14 is bound too strongly to the resin to be removed. Insoluble Ecm14 expressed in yeast is seen in the final lane as a control.
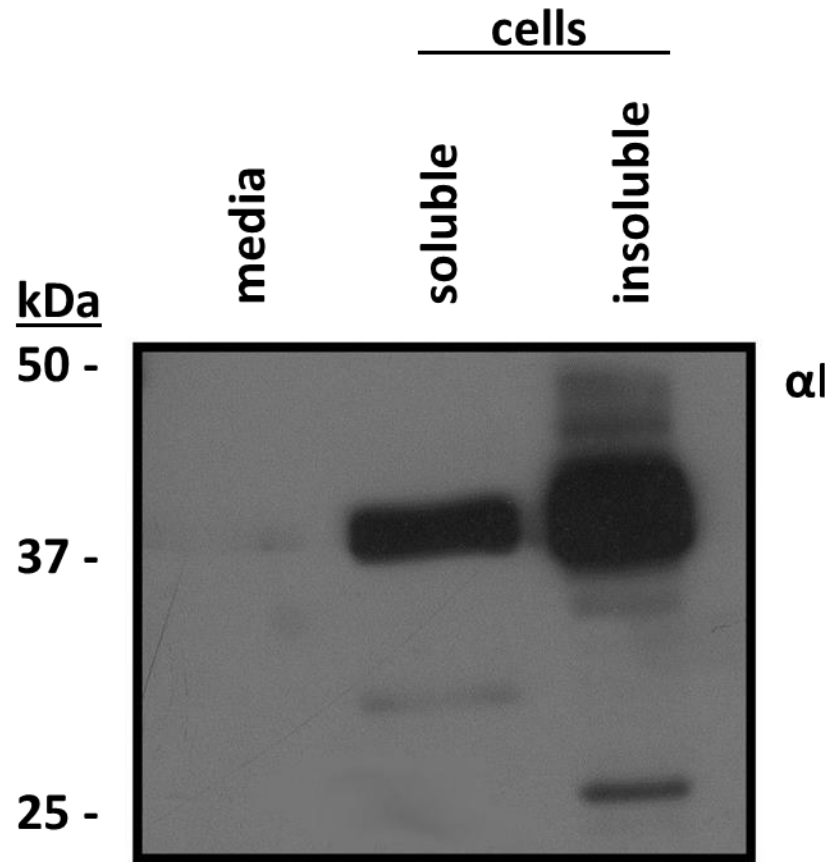
Figure 5. Ecm14 is expressed in Sf9 insect cells in soluble form.

Histidine-tagged Ecm14 expressed in Sf9 cells with a baculovirus system was detected by western blotting. Intracellular Ecm14 was detected in both soluble and insoluble forms as a 40 kDa protein.
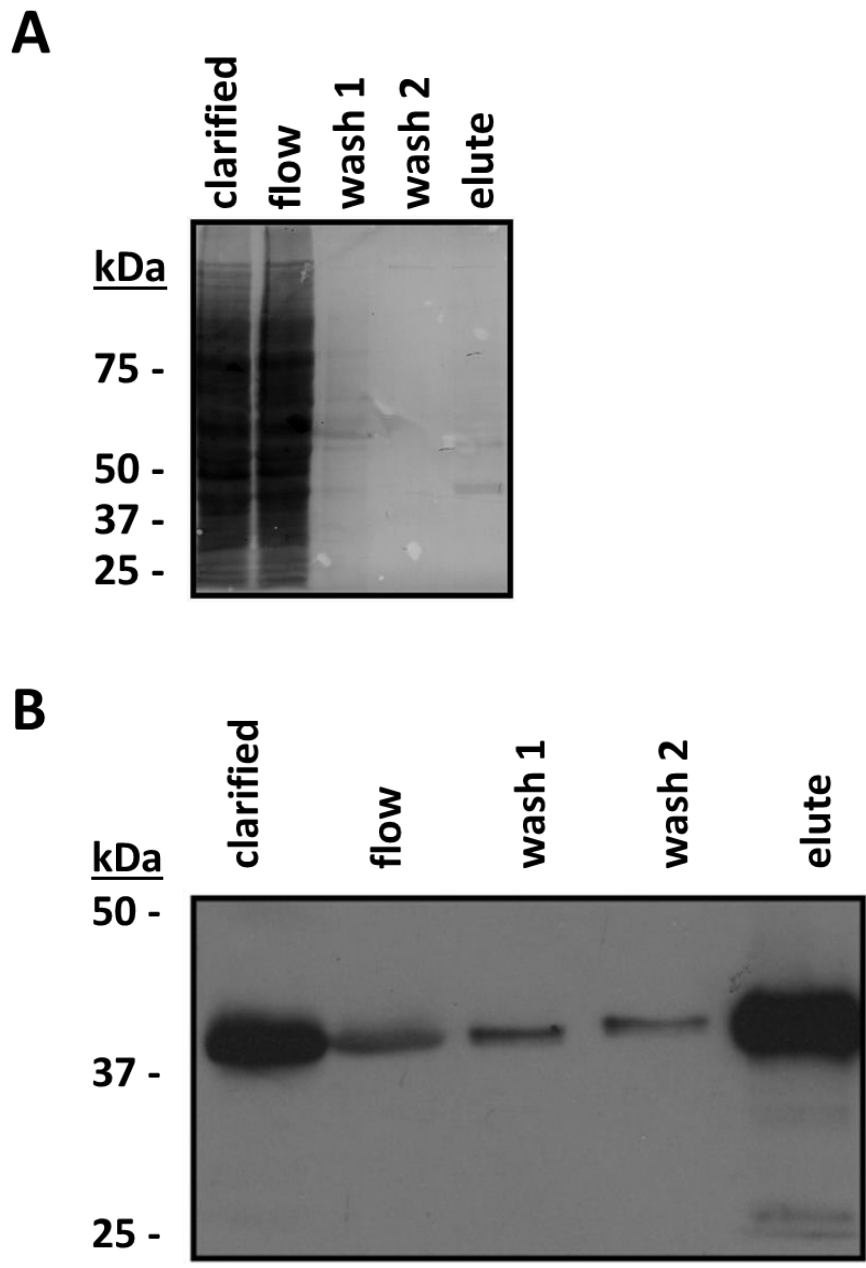
Figure 6. Histidine-tagged Ecm14 can be purified using Cobalt-metal affinity chromatography.

Proteins were extracted from Sf9 cells and clarified by centrifugation (clarified). Proteins that did not bind were discarded (flow). Two washes with native wash buffer (wash 1/2) were performed to further reduce the number of contaminants in the sample. Purified Ecm14 was eluted from the column with imidazole (elute). Following purification of Ecm14 by metal affinity chromatography, a 40 kDa protein predicted to be Ecm14 was detected by Ponceau S staining (A) and histidine-tagged Ecm14 was detected by western blotting (B).
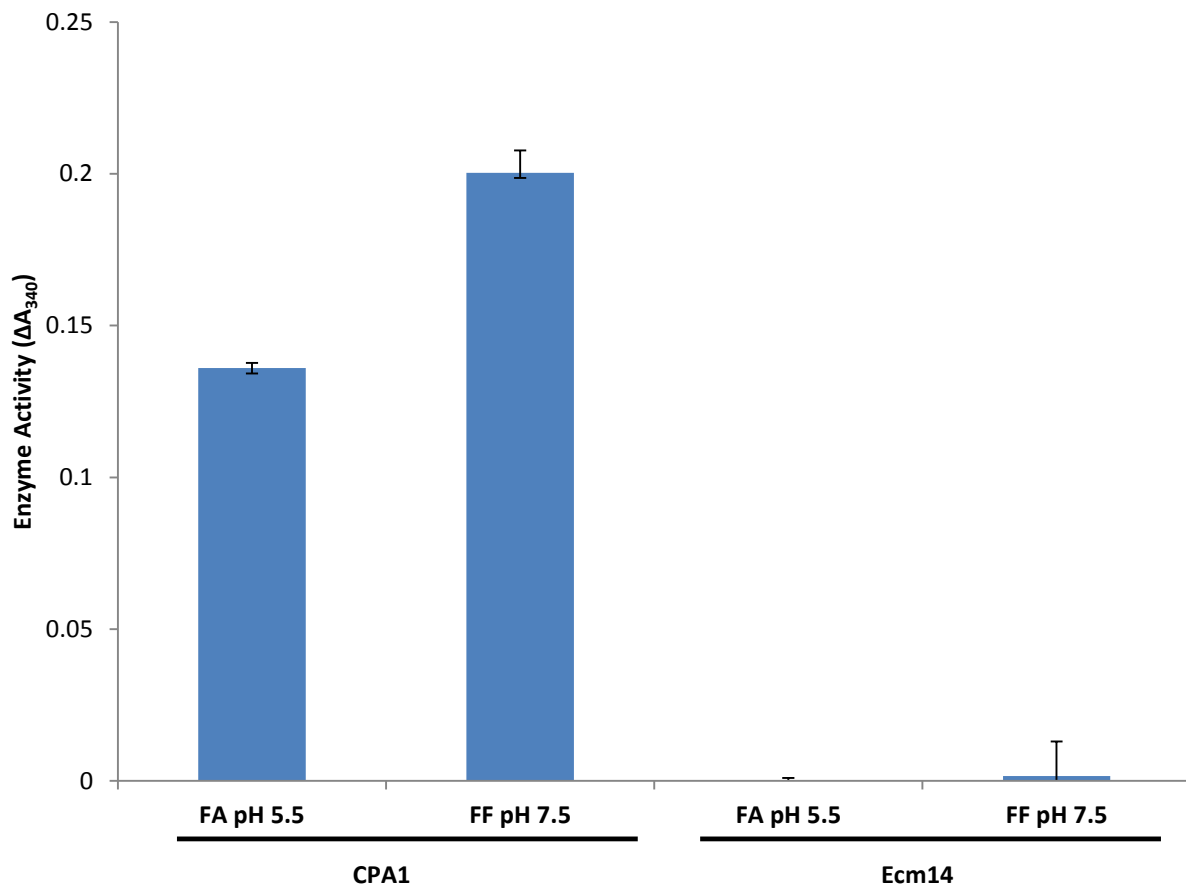
Figure 7. Purified ProEcm14 is inactive.

Ecm14 and CPA1 (10 μg) were incubated for 90 minutes at room temperature with carboxypeptidase substrates FA-PhePhe and FA-PheAla (0.5 mM). Cleavage of these substrates resulted in a decrease in absorbance at 340 nm. While the control CPA1 showed robust activity, no activity was observed for Ecm14. Error bars indicate standard error, $n=3$.
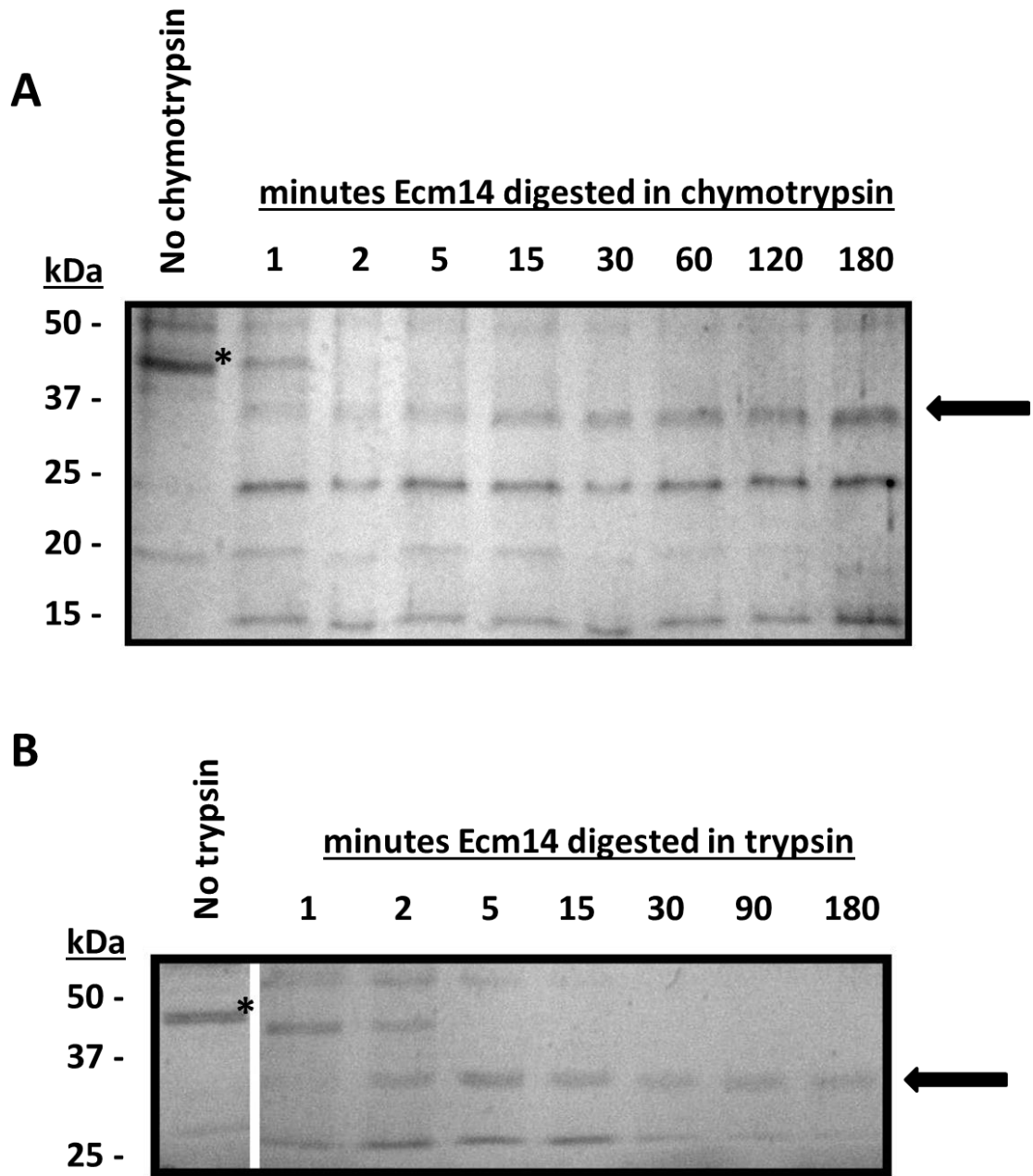
Figure 8. The prodomain of Ecm14 can be cleaved with the addition of trypsin or chymotrypsin.

Ecm14 was incubated with chymotrypsin (A) or trypsin (B), resolved by SDS-PAGE, and detected by Coomassie stain. Both enzymes resulted in the appearance of a 35 kDa band likely to be mature Ecm14 (arrows). Ecm14 not incubated with trypsin or chymotrypsin resolves as a 40 kDa protein (*). Bands observed at about 25 kDa are the trypsin and chymotrypsin used to cleave Ecm14.

Figure 9. Chymotrypsinized Ecm14 exhibits weak enzymatic activity.

Ecm14 cleaved with chymotrypsin (10 µg) shows activity after 2 hours of incubation with carboxypeptidase substrate FA-PhePhe (0.5 mM) at pH 7.5. No activity is observed for either purified Ecm14 or trypsinized Ecm14. Chymotrypsin and trypsin were incubated without Ecm14 as a control. Error bars indicate standard error, $n=3$. ** $p < 0.01$.
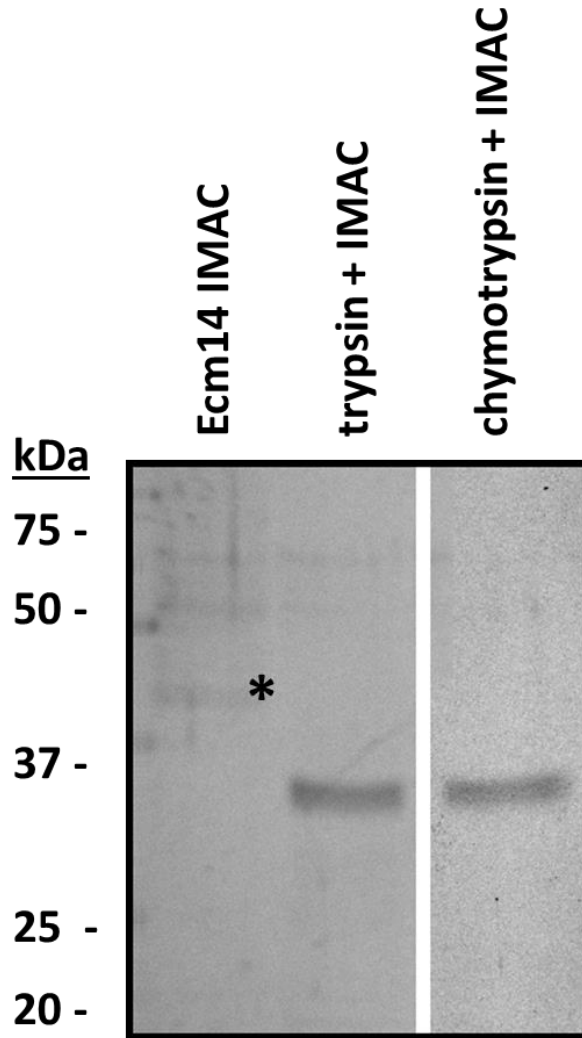
Figure 10. Cleaving the prodomain from Ecm14 with trypsin or chymotrypsin prior to IMAC purification increases purity of eluted Ecm14.
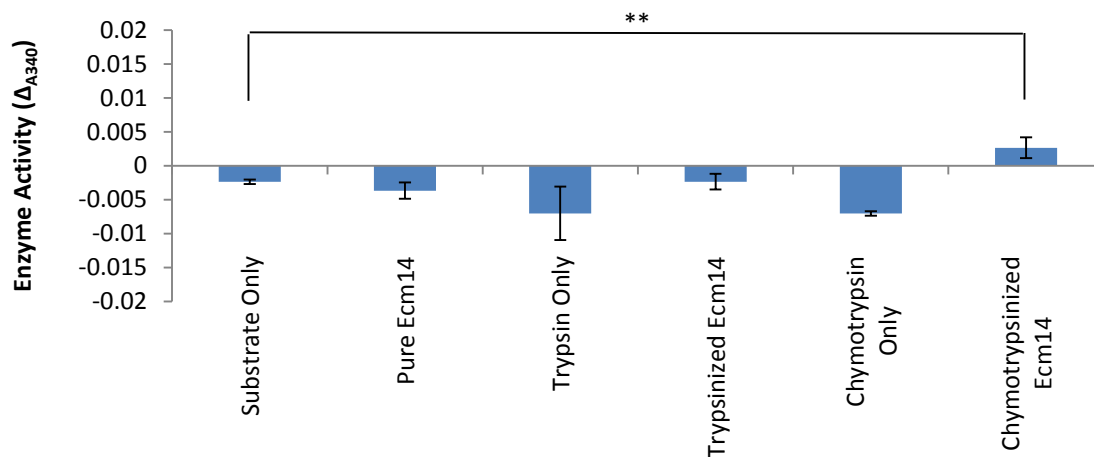
Ecm14 purified by cobalt-metal affinity chromatography is seen as a 40 kDa protein (*) by Coomassie staining. This Ecm14 was previously quantified as being roughly 50% pure. In order to cleave the prodomain prior to purification, 10 mL of crude Ecm14 was incubated with 500 μg of trypsin or chymotrypsin for 10 minutes at room temperature. This incubation was followed by purification as performed before. A roughly 35 kDa protein is clearly visible in both trypsinized and chymotrypsinized Ecm14. Cleavage of the prodomain prior to purification increased the purity of Ecm14 within the sample for both trypsin (80% pure) and chymotrypsin (> 95% pure) as determined by ImageJ.
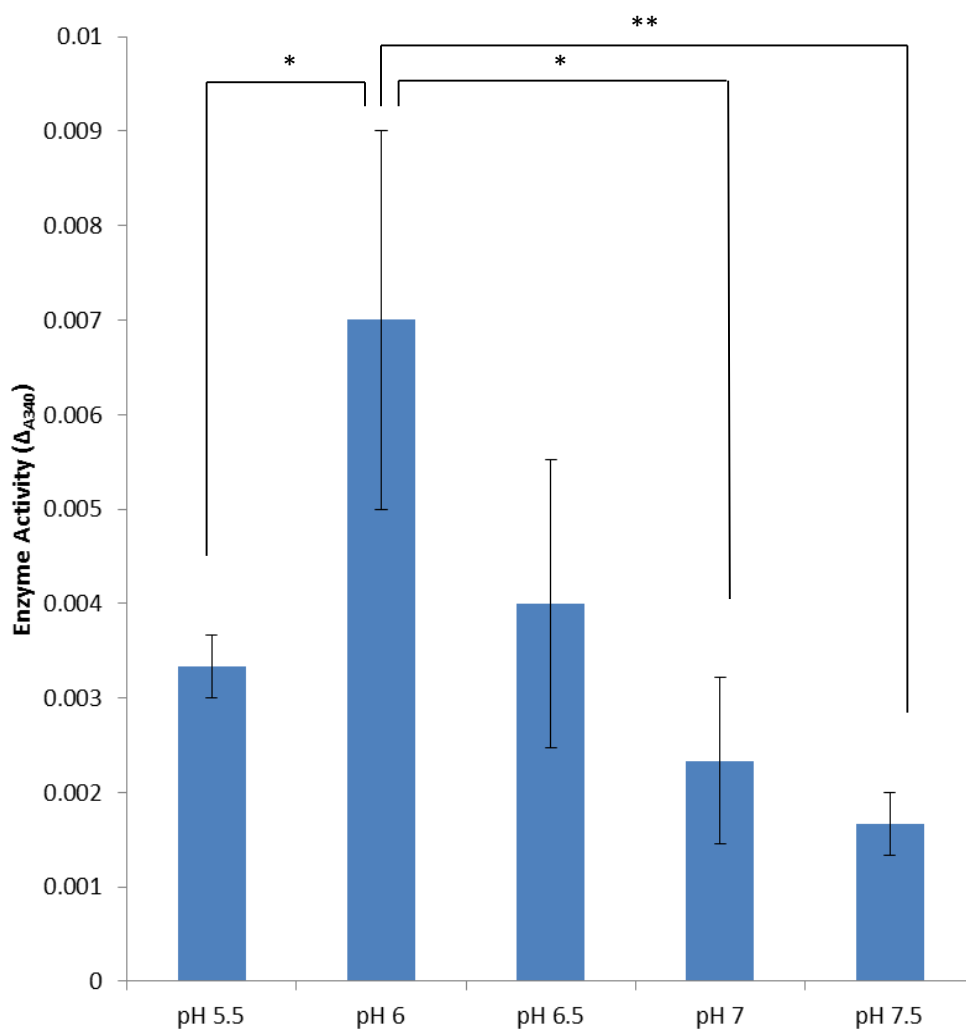
Figure 11. Ecm14 cleaved by chymotrypsin shows greatest enzymatic activity for carboxypeptidase substrate FA-PhePhe at pH 6.0.

Ecm14 cleaved with chymotrypsin prior to purification (10 µg) shows activity after 5 minutes of incubation with carboxypeptidase substrate FA-PhePhe (10 µM) at a range of pH values. The greatest activity is seen with a substrate pH of 6.0. Error bars indicate standard error, $n=3$. * $p < 0.05$; ** $p < 0.01$.
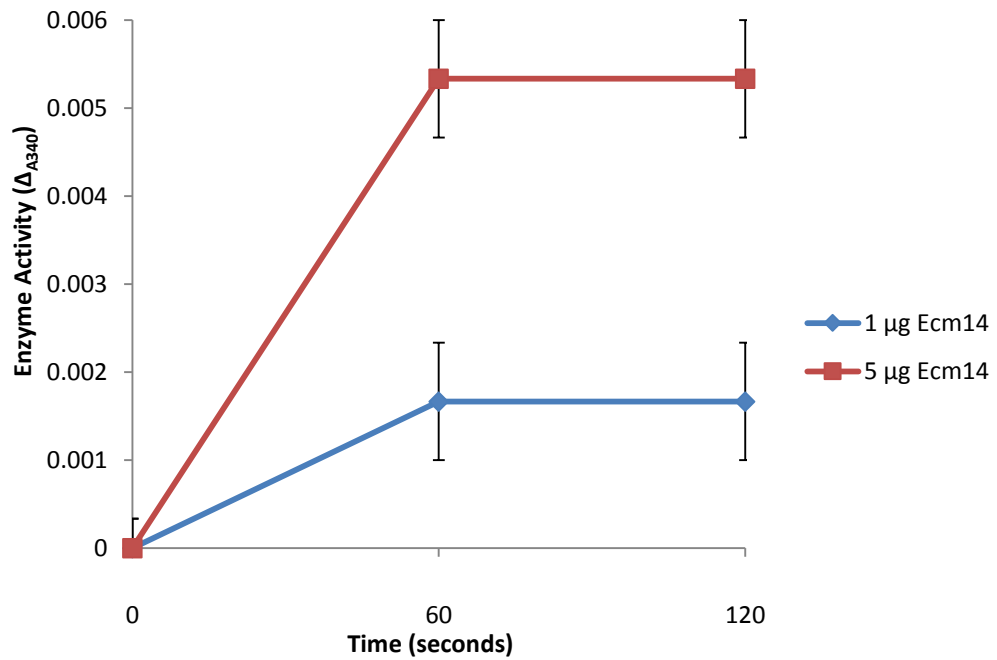
Figure 12. Chymotrypsinized Ecm14 acts in a non-catalytic manner.

Chymotrypsinized Ecm14 (1 µg and 5 µg) was incubated with FA-PhePhe substrate (10 µM) at pH 7.5 for 2 minutes at room temperature. Increasing the concentration of Ecm14 five-fold resulted in three to five times more activity. No activity was recorded for either sample of Ecm14 after 1 minute of incubation. Error bars indicate standard error, $n=3$. $p < 0.01$.
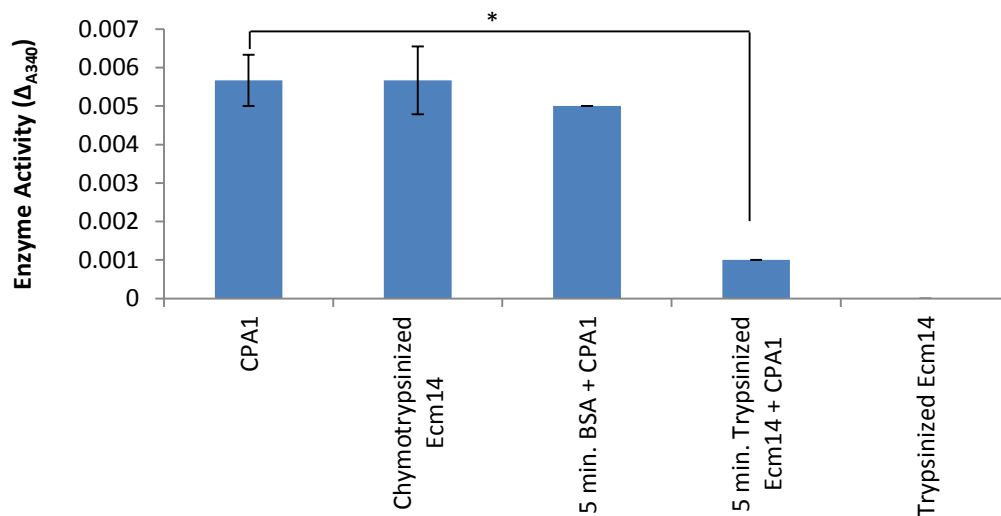
Figure 13. Trypsinized Ecm14 can bind to substrate and limit the activity of CPA1.

Ecm14 cleaved with chymotrypsin and CPA1 (10 μg) show similar activity after 1 minute of incubation with carboxypeptidase substrate FA-PhePhe (1 μM) at pH 7.5. Ecm14 cleaved with trypsin (10 μg) shows no activity as seen previously. Incubating substrate with trypsinized Ecm14 for 5 minutes prior to the addition of CPA1 greatly reduces the observed activity of CPA1. Error bars indicate standard error, n=3. * $p < 0.005$.

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