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Expression, Purification, and Characterization of the Mast Cell Proteases Chymase and
Cathepsin G

A dissertation
presented to
the faculty of the Department of Biochemistry and Molecular Biology
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy in Biomedical Sciences

by
Brent Lockhart
May 2008

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Keywords: Cathepsin G, Chymase, Mast Cells, Serine Proteases

ABSTRACT

Expression, Purification, and Characterization of the Mast Cell Proteases Chymase and Cathepsin G

by
Brent Lockhart

Human mast cells have been associated with wound healing, allergies, inflammation, and defense against pathogens and have been detected in tissues close to blood vessels especially in the areas between the inside of the body and the external environment, such as the skin, lungs, digestive tract, mouth, and nose. Previous studies have shown that mast cells contain large granules filled with histamine, heparin, cytokines, eicosanoids, and the serine proteases, tryptase, Chymase, and cathepsin G (CatG). These proteases are stored and released from mast-cell granules upon activation by antigen binding to IgE immunoglobulins on the cell surface or by direct injury. In this study, chymase and CatG were expressed as active enzymes in the yeast *Pichia pastoris* by homologous recombination of the cDNA coding for the mature active proteases into the *Pichia* genome. Methanol induction resulted in the secretion of active enzyme into the *Pichia* growth media and increasing levels of enzyme were detected in the media for 5 days. Cells that secreted the highest levels of activity were selected by kinetic assay. Active chymase was purified from the culture media with a 22% yield of activity by a simple two-step procedure that involved hydrophobic-interaction chromatography followed by affinity chromatography on immobilized heparin. The major peak from the heparin column contained a single band of 30.6 kDa on SDS/PAGE. The purified recombinant human chymase was 96% active and the yield was 2.2 mg/l of

growth media. Active CatG was partially purified from culture media using an ultrafiltration. Mass Spectroscopy (Maldi-Tof) data confirmed that the major protein band was CatG, resulting in the first active human CatG to be produced recombinantly. Additionally, the partially purified enzyme was active against both chymotrypsin and trypsin substrates, and its reaction with inhibitors was consistent with CatG. Although the protein yields were low, these results confirm that CatG was recombinantly expressed.

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ABBREVIATIONS:

AS	ammonium sulfate
Asp	aspartic acid
BMGY	buffered glycerol-complex medium
BMMY	buffered methanol-complex medium
CatG	Cathepsin G
cDNA	complimentary deoxyribonucleic acid
DTT	dithiothreitol
EC	enzyme classification
Glu ₂₂₆	glutamic acid
HSPs	hemapoietic serine proteases
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic
HDL	high-density lipoprotein
HPQYNQR	histidine proline glutamine tyrosine asparagine glutamine arginine;
His	histidine
HNE	human neutrophil elastase
pH	hydrogen ion concentration
⁺ H	hydrogen ion
⁻ OH	hydroxide ion
IC50	Inhibitory concentration for 50% reduction of activity
ICatG	immature Cathepsin G
IIGGR	isoleucine isoleucine glycine glycine arginine
Leu	leucine
mCatG	mature Cathepsin G
MES	2-(4-morpholino)-ethane sulfonic acid
Met	methionine
nCatG	native cathepsin G purified from neutrophils
NK	natural killer cells
OG	octyl glucoside
PNGase F	peptide N-glycosidase F
PCR	polymerase chain reaction
PAR3	protease associated receptor 3
PDB	Protein Data Bank

PVDF	polyvinylidene difluoride
rhCatG	recombinant human cathepsin G
rhChymase	recombinant human chymase
iCatG	recombinant Immature cathepsin G
mCatG	recombinant mature cathepsin G
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SERPINS	serine protease inhibitors
Ser	serine
Suc-AAPF-Pna	succinyl-ala-ala-pro-phe p-nitroanilide
Suc-VPF-SBzl	succinyl-valine-proline-phenylalanine thiobenzyl ester
Suc-VPK-SBzl	succinyl-valine-proline-lysine thiobenzyl ester
TCA	trichloroacetic acid
3D	three dimensional
TPCK	tosyl phenylalanyl chloromethyl ketone
TLCK	tosyl-L-lysine-chloromethyl ketone
TRIS	tris(hydroxymethyl)aminomethane
Trp	tryptophan
IgE	type E immunoglobulin
Tyr	tyrosine
YPDS	yeast extract peptone dextrose medium

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CHAPTER 1
INTRODUCTION

HSP In Mast and Other Cells

The human hemopoietic serine proteases (HSPs) have been found in neutrophils, T-lymphocytes, basophils, natural killer cells (NK) and mast cells (Garwicz et al. 1998; Gullberg et al. 1999; MacIvor et al. 1999). Human mast cells have proven to play an important role in wound healing, allergy, and defense against pathogens. These cells have been found in tissues close to blood vessels primarily in the areas between the inside of the body and the external environment. Examples of these areas would be the skin, lungs, digestive tract, mouth, nose or in the skin. Mast cells have been found to contain large granules filled with histamine, heparin, cytokines, eicosanoids and serine proteases that are released upon antigen binding to IgE immunoglobins attached to the cell membrane or by direct injury (Table 1). In addition, mast cells have been implicated in rheumatoid arthritis and asthma (Faber et al. 1993; Guay et al. 2006). The HSPs in

Cell Type	Neutrophils	Mast Cells
Proteases	Cathepsin G	Cathepsin G
	Elastase	Chymase
	Proteinase 3	Tryptase
	Azurocidin	

Table 1 Cell Types and Protease They Produce. *Gullberg et al, 1997, Biosynthesis, processing and sorting of neutrophil proteins.*

neutrophils have been shown to consist of a combination of the cationic proteases cathepsin G, elastase, protease 3, and azurocidin that although similar in sequence was thought to

be non-functional (Garwicz et al. 1998; Gullberg et al. 1999). Neutrophil HSPs have been proven to be stored in the cytoplasmic azurophil granules that contain other proteins such as defensins and myeloperoxidase or they are transported out of the cell by “constitutive secretion” where they can attach to the cell surface. Neutrophil proteases have also been found to be normally transported to and function in the phagosome intracellularly to help in degradation and killing phagocytosed microbes or toxins (Gullberg et al. 1997). Researchers have also shown that the extracellular secretion occurs from the azurophil granules (Griffiths, 1996; Gullberg et al. 1997) probably due to cellular contact with bacteria or viruses resulting in an inflammatory response (Bangalore, 1994) through a process known as “regulated secretion”.

Discovery, Sequence, and Structure of CatG and Chymase

Cathepsin G, also abbreviated as CatG, was first described in 1975 (Dewald et al. 1975; Rindler-Ludwig, 1975), with Starkey and Barrett being the first to purify the protease from human spleen and thus naming it (Starkey and Barrett, 1976b). CatG was designated as a member of the HSP family and was found to possess a unique structure that has been linked to several interesting aspects in the medical field that warrant additional study. Chymase (chymotrypsin-like enzyme activity) was first reported in Human Mast cells in 1963 (Lagunoff et al, 1963). Caughey (1991) deduced the nucleotide sequence and protein structure of human chymase. Both the CatG and chymase (Caughey et al. 1991) genes were located on chromosome 14, while azuorcidin, proteinase 3, and Human Neutrophil elastase (HNE) genes were located in a single locus on chromosome 19 (Gullberg et al. 1997). The nucleotide sequences coding for the enzymes were obtained for CatG (GenBank M16117), that was discovered by Salvesen (Salvesen et al. 1987), while chymase’s sequence (GenBank M64269) was credited to Caughey (Caughey et al. 1991). Information on CatG and chymase’s structure and function was found in several databases with their 3-D structure having been previously determined by X-ray diffraction. (CatG, PDB ID# 1CGH, Hof et al. 1996; 1KYN, Greco et al. 2002; and 1AU8, Medrano et al. 1997; Chymase PDB ID# 1NN6, Reiling et al. 2003; 1KLT, McGrath et al. 1997; and 1PJP, Pereira et al. 1999).

CatG has been found to exist in either a mature (mCatG) or immature (iCatG) form based upon processing, although the exact reason of the two forms has not yet been determined. CatG has been found to be synthesized as a 235 amino acid protein in its full-length or immature form (iCatG) but was found to be processed at both the N and the C-terminals prior to reaching maturation (mCatG) of 224 residues. The amino acid sequence of iCatG was first deduced from its cDNA sequence (Salvesen et al. 1987); however, X-ray crystallography data showed that the mature CatG was missing a C-terminal peptide of 10-12 residues. In addition, the immature form of CatG (iCatG) was found to have a slightly lower pI of 11.37 than its mature form and a molecular weight of 26757.6 and an extinction coefficient of 0.891 $A_{280}/\text{mg}/\text{ml}$ (<http://www.expasy.org/cgi-bin/protparam>). mCatG was found to have a molecular weight of 25441 with a pI of 11.51 and an extinction coefficient of 0.937 $A_{280}/\text{mg}/\text{ml}$ (Gasteiger et al. 2005).

Chymase was found to contain 226 residues with a molecular weight of 25029.9, a pI of 9.60, and an extinction coefficient of 0.957 $A_{280}/\text{mg}/\text{ml}$ (Gasteiger et al. 2005).

It should be noted that these enzymes were found to have many similarities including size, isoelectric points, and the first 4 residues of their amino acid sequences. In addition, their 3D structures (See Figure 1) both contained beta sheets and alpha helices in approximately the same regions.

Serine Protease Mechanism of Action

HSPs have been described as possessing a “trypsin-like” activity, as trypsin or “chymotrypsin-like”, as chymase, or both types of activity as CatG has been shown to exhibit. These two enzymes have been designated as members of the chymotrypsin family (Barrett, 1981b) and were proven to have a catalytic triad consisting of the amino acid residues His 57, Asp102, and Ser 195 (based on the chymotrypsin numbering system) that catalyzes the cleavage of peptide bonds (See Figure 1). The aspartic acid residue was shown to act on the histidine to create a dipole that attracted the hydrogen from the serine residue forming an alkoxide ion, that was found to be a strong nucleophile. The peptide carboxyl oxygen becomes negatively charged creating an

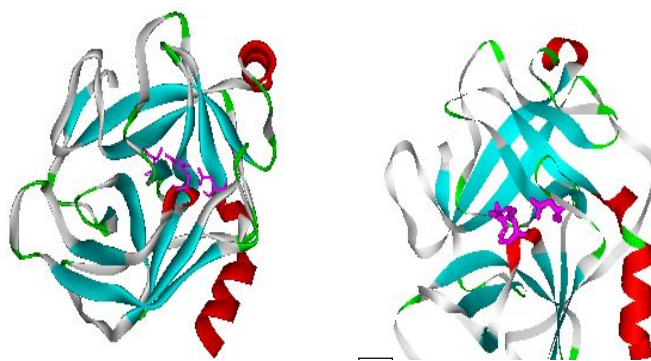


Figure 1 3D Structure of Proteases. A) CatG 1CGH B) Chymase 1PJP with the catalytic triad (His 57, Asp 102 and Ser 195) highlighted in purple. Beta Sheets are denoted in blue and alpha helices are denoted in red. Figure adapted from Protein Data Bank. H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne: The Protein Data Bank. Nucleic Acids Research, 28 pp. 235-242 (2000).

oxyanion hole, while the alkoxide ion of the serine provides a nucleophilic attack on the carbonyl carbon of the peptide bond. This, in turn, causes the double bond to be changed into a single bond, forming a tetrahedral intermediate. The peptide bond was broken, relieving the negative charge on the oxygen and resulted in an acyl-enzyme intermediate. The N-terminal section of the cleaved peptide was released with the donation of a hydrogen ion (^+H) to the histidine and a hydroxide ion (^-OH) to the esterified serine by a water molecule that created a second oxyanion hole. The release of the carboxyl component of the peptide caused the serine's hydrogen bond to be released from the histidine's nitrogen, thus returning the catalytic site to its original condition.

Binding and Substrate Specificity

Binding must occur between the protease and substrate in order for the cleavage interaction to take place. Schechter-Bergren nomenclature (Schechter and Berger, 1968) denoted the different locations of the residues of both the substrate and the enzyme in peptide bond cleavage interactions (Figure 2). The substrate was defined with "P" with the residues on the N-terminus side of the scissile bond labeled 1, 2, 3... and the residues on the C-terminus side of the scissile bond labeled 1', 2', 3', etc. The residues of the protease that formed the binding pocket and interacted with the substrate residues were denoted with an "S". The protease residues that interact with the P1, P2, P3 substrate residues were denoted as S1, S2, S3 and the residues that

interact with the P1', P2', P3' substrate residues were denoted as S1', S2', S3'. The scissile bond was designated as the bond between the P1 and P1' substrate residues that the protease cleaved.

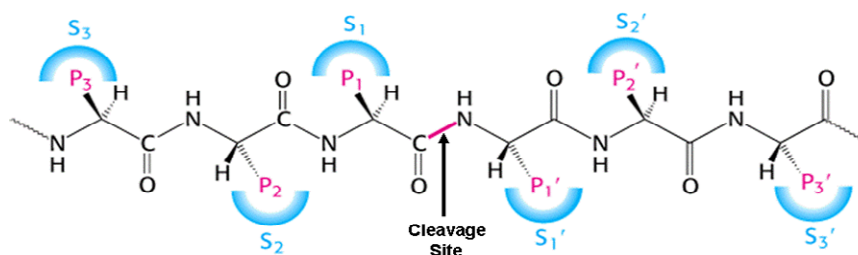


Figure 2 Schechter-Berger nomenclature for Proteases.
Schechter I. Berger A. (1968) Biochem Biophys Res commun. 32:898-902.

Activity of the two recombinant enzymes was determined by used either a Suc-Val-Pro-Phe or Suc-Val-Pro-Lys thio benzyl ester substrate in kinetic plate assays. When the enzyme cleaved the thio benzyl ester substrate, a benzyl-thiol group was released that, in turn, reacted with the DTNB, releasing the nitro benzoate thiol anion that produced an intense yellow color with an extinction of $13,600 \text{ M}^{-1} \text{ cm}^{-1}$ at 405 nm (See Figure 3).

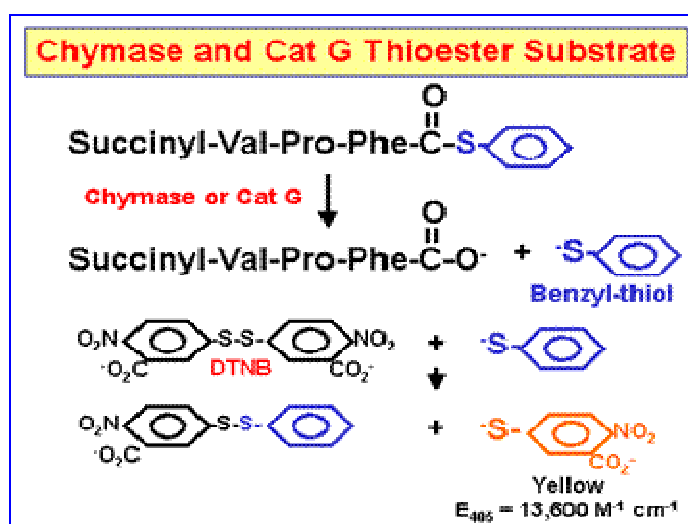


Figure 3 ThioBenzyl Ester Reaction for CatG and Chymase. Suc-VPF-Thio benzyl ester is cleaved by enzyme releasing the Benzyl-thiol group that forms with DTNB emitting a yellow color at a wavelength of 405 nm.

All serine proteases have been shown to function using the same catalytic triad (His, Asp, Ser), but their differences in specificity have been found to depend primarily on the structure of the S1 binding pocket of the protease. “Chymotrypsin-like” proteases have been proven to have a specificity for a substrate with a P1 hydrophobic residue, such as phenylalanine, tyrosine, tryptophan, leucine, or methionine, while “trypsin-like” proteases have been found to have a specificity for the positively charged residues arginines and/or lysines. As a general rule, “chymotrypsin-like” proteases do not cleave trypsin substrates and “trypsin-like” proteases do not cleave chymotrypsin substrates. CatG has been proven to be the only known exception to this rule and displayed “dual specificity” by being able to cleave both chymotrypsin and trypsin substrates (See Table 2).

		H⁵⁷	
Chymo	IVNGEDAVPGSWPWQVSLQDK---	TGFHFCGGSLISEDWVVTAAHCGVRTSDVVVAGEFD	57
Chymase	IIGGTECKPHSRPYMAYLEIVTSNPGPSKFCGGFLIRRFVLTAAHC-AGRSITVTLGAHN		59
CatG	IIGGRESRPHSRPYMAYLQIQSPAGQSR-CGGFLVREDFVLTAAHC-WGSHINVTLGAHN		58
Trypsin	IVGGYNCEENSVPYQVSLN-----	SGYHFCGGSLINEQWVVSAGHC-YKSRIQVRLGEHN	54
		D¹⁰²	
Chymo	QGSDEENIQVLKIAKVFKNPKFSILTVHNDITLLKLATPARFSQTVSAVCLPSADDDFPA		117
Chymase	IITEEDTWQKLEVIKQFRHPKYNTSTLHHDIMLLKLEKASLTLAVGTLPPFSQFNFPVP		119
CatG	IQRRENTQQHITARRAIRHPQYNQRTIQNDIMLLQLSRRVRRNRNVNPVALPRAQEGLRP		118
Trypsin	IEVLEGNEQFINAAKIIRHPQYDRKTLHNDIMLIKLSRAVINARVSTISLPTAPP--AT		112
		S¹	
Chymo	GTLCAATGWGKTKYNANKTPDKLQQAALPLLNAECKKSWGRRITDVMICAG--ASGVS		175
Chymase	GRMCRVAGWGRIG-VLKPQSDTLQEVKLRMLDPQACS-HFRDFDHNQLQCVGNPRKTKSA		177
CatG	GTLCTVAGWGRVS-MRR-GTDTLREVQLRVQRDRQCLRIFGSYDPRRQICVGDRRERKAA		176
Trypsin	GTKCLISGWNTASSGADYPDELQCLDAPVLSQAKCEASYPGKITSNMFVGVFLEGGKDS		172
	S¹⁹⁵	E²²⁶	
Chymo	CMGDSGGPLVCQKDGAWTLVGVISWGSDDCSTSSPGVYARVTKLIPWVQKILAN-----		230
Chymase	FKGDSGGPLLCAQ----VAQGIVSYGRSDA--KPPAVFTRISHYRPWINQLQAN-----		226
CatG	FKGDSGGPLLCAQ----VAHGIVSYGKSSG--VPPVFTTRVSSFLPWIRTTRSFKLLDQ		230
Trypsin	CQGDSGGPVVCNG----QLQGVVSWGDGCAQKNKPGVYTKVYNYVKWIKNTIAANS----		224
Chymo	-----	% Identity to Chymotrypsin	
Chymase	-----	34	
CatG	METPL 235	31	
Trypsin	-----	42	

Table 2 Clustal W Sequence Alignment for CatG, Chymo (Chymotrypsinogen) and Trypsin. Protein sequences of chymotrypsin (Chymo), chymase, cathepsin G (CatG), and trypsin were aligned and compared based on their single letter abbreviations. The active site histidine (57), aspartic acid (102) and serine (195) are bolded along with glutamic acid residue a potential secondary binding residue (Higgins D., 1994).

Powers et al (Powers et al. 1989) discovered that CatG cleaved both trypsin and chymotrypsin peptide substrates referred to CatG as a “Janus faced” enzyme and proposed that Glu₂₂₆ provided a negative charge to attract a lysine in the P1 position, indicating that CatG did not possess a second active site for the “trypsin-like” substrates. When the crystal structure was determined, researchers (Hof et al. 1996) found the proximity of the Glu₂₂₆ to the active site peptide supported the hypothesis of a secondary S1 site for accepting lysine and arginine (P1) substrates. (Hof et al. 1996; Polanowska et al. 1998) (See Figure 4). However, Glu₂₂₆ has not been proven to be the secondary S1 binding site for trypsin substrates primarily because this enzyme previously has not been expressed recombinantly.

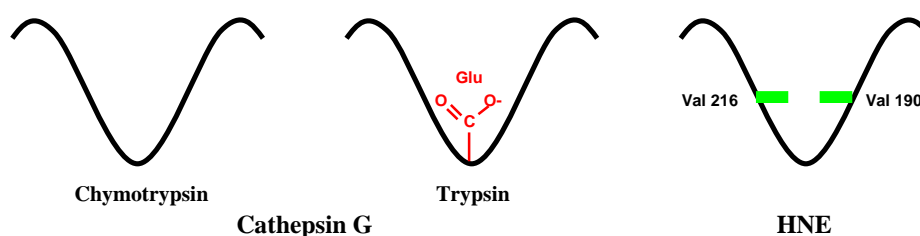


Figure 4 Cathepsin G and HNE Specific Binding Pockets.

The enzymes chymotrypsin, trypsin, and elastase binding pockets are shown with trypsin having a glutamic acid (glu) residue. Elastase has 2 valines that limit only linear residues entering the pocket.

CatG has been found to cleave many biological proteins after bulky aromatic residues such as phenylalanine, histidine, isoleucine, valine and tyrosine or after positively charged residues such as lysine and arginine (<http://www.hprd.org/protein/00289?selectedtab=PTMs>; Saravana, 2005). CatG interacted in vitro with Thrombospondin I (Hogg et al. 1993a); (Hogg et al. 1993b), Kininogen (Selim et al. 2001), Protease inhibitor 13 (Mitsudo et al. 2003), Coagulation Factor V (Camire et al. 1998), Syndecan 1 (Kainulainen et al. 1998), Integrin (Molino et al. 1993), and beta thromboglobulin (Cohen et al. 1992). CatG interacted in vivo with alpha 1 antichymotrypsin (Gibson, 1999) and PAR3 (Cumashi et al. 2001). Chymase has “chymotrypsin-like” activity allowing it to cleave after bulky aromatics (FLYW).

Medical Relevance

CatG and chymase have been found to play important roles in many biological processes and conditions. CatG has been associated with blood clotting and was proven to cleave coagulation Factor X and Factor V (Camire et al. 1998). The protein also was found to have the sequences IIGGR and HPQYNQR that have been considered to be antimicrobial agents toward *Neisseria gonorrhoeae* and *Staphylococcus aureus* (Odeberg et al. 1975) even though its active site was inhibited (Shafer et al. 1996; Shafer et al. 1991; Bangalore et al. 1990). The two antimicrobial sequences were determined by purifying neutrophil CatG and digestion of the enzyme into fragments. The fragments were then purified and the two antimicrobial sequences corresponding to residues 1-5 (N-terminus IIGGR) and 77 - 83 (HPQYNQR) were identified. Bangalore et al. (1990) also synthesized the corresponding antimicrobial sequences and showed that they were also bacteriicidal. CatG was also found to degrade the flagella of *Pseudomonas aeruginosa* (Lopez-Boado et al. 2004) and to cleave and inactivate the neutrophil chemoattractants tumor necrosis factor-alpha (TNF- α) (Scuderi et al. 1991), interleukin-1 (IL1) (Hazuda et al. 1990), and interleukin 8 (IL8) (Padrines et al. 1994). CatG was shown to produce apoptosis in cardiomyocytes by activation of caspase 3 (Sabri et al. 2003) and conversion of angiotensin I into angiotensin II (Reilly et al. 1982). DNA fragments of 30 bp have been shown to bind CatG with a Kd of 8.5nM; furthermore this complex decreases the inhibition of α -1-proteinase inhibitor by 3190 fold (Duranton et al. 2000b). As Duranton explains, "DNA thus renders CatG virtually resistant to inhibition by these irreversible serpins" and makes CatG a major player in inflammatory lung disease (Duranton et al. 2000a).

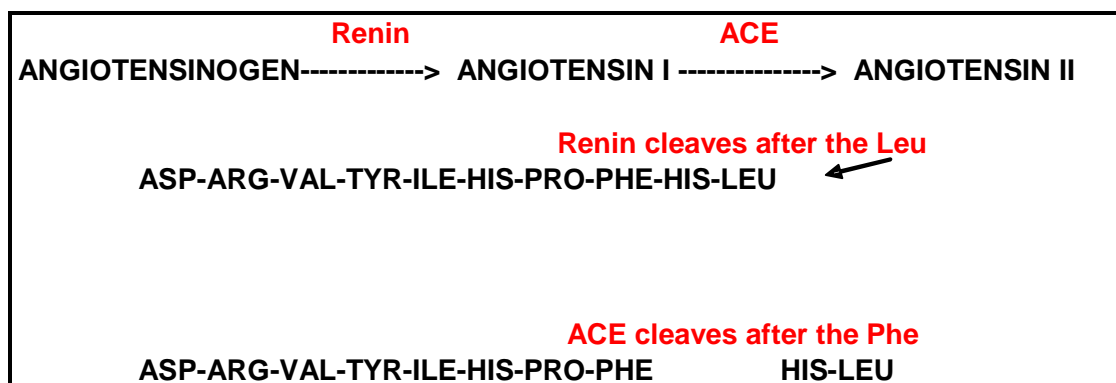


Figure 5 Conversion of Angiotensinogen to Angiotensin II. This Figure depicts the event that Angiotensinogen is converted into Angiotensin I by the enzyme renin and then into Angiotensin II by the enzyme ACE. Renin cleaves after the amino acid Leu, while ACE cleaves after Phe. This classic conversion mechanism has also been found to occur from reactions of CatG or chymase.

Chymase was found to have several medically related functions such as degradation of HDL (Lee et al. 2002), production of apoptosis in rat smooth muscle (Leskinen et al. 2006) and the conversion of angiotensin I into angiotensin II (Caughey et al. 2000). Angiotensinogen was converted by renin into angiotensin I by cleavage following a leucine residue and into angiotensin II with cleavage after a phenylalanine amino acid by ACE (See Figure 5). CatG and chymase can also produce the same conversion effects with their proteolytic abilities.

Experimental Purpose

The overall objective of this work was to clone and express the active enzymes chymase (EC 3.4.21.39) that has chymotrypsin-like activity and cathepsin G (EC 3.4.21.20) that possesses both trypsin-like and chymotrypsin-like activity using the *Pichia pastoris* expression system. Recombinant CatG was expressed in two different forms, an immature full length CatG protein (iCatG) and a mature protein (mCatG) that had 11 amino acids eliminated from the C terminus. A mature, native form of CatG (nCatG), previously isolated from neutrophils, was used as a positive control during characterization studies. It has been hypothesized that the purpose of the C-terminal extension was to inactivate the protease prior to its transport to the granule to allow cell survival (Garwicz et al. 1998). However, it was not known whether the C terminus of

iCatG may affect its catalytic activity because this protein has not been expressed in sufficient quantities to allow isolation and careful characterization study prior to this research. A hypothesis of this dissertation was that the presence of the 11 amino acid C-terminus of CatG does not prevent catalytic activity but existed for a different role, such as cell surface attachment or reduced SERPIN inhibition.

An active recombinant form of CatG, either in its immature or mature form, has not been previously expressed, presumably due to the protease activity against the intracellular machinery of the host cell. Garwicz was able to produce an inactive enzyme that had both its catalytic site mutated and its propeptide removed, with the protease still being transported to the granule and able to bind to aprotinin after further processing in the granule (Garwicz et al. 1998). However, when an active form of the same enzyme that had its propeptide removed was expressed, it resulted in unviable cells (Garwicz et al. 1998). Garwicz also proved that the protease was processed in the granule and not in golgi through disruption of the organelle with Brefeldin A that disassembles the apparatus (Garwicz et al. 1998). In previous studies Garwicz also mutated the N-glycosylation site to eliminate the mannose-6-phosphate tag as a means for transportation (Garwicz et al. 1995).

Incorporating the Invitrogen Easy Select Expression System allowed the enzymatically active protease to be produced without further processing as it was attached to the Kex2 cleavage site. In addition, the alpha-secreting factor allowed the protease to be secreted extracellularly, thus preventing damage to the host and allowing activity to be determined by kinetic assays. Used the homologous recombination of the yeast expression prevented the CatG sequence from being removed from the host cell genome as can be the case with a plasmid. This system was also beneficial in that Pichia has an optimal growth pH of 5, while CatG was found to be less active at the same pH thus reducing any potential catalysis of protein. The ingredients for media were also minimal for this expression system and the viral threat has been removed through recombinant expression as the enzyme was not isolated from tissue. The zeocin resistance gene also proved to be beneficial in that it allowed selection of positive transformants for both E. coli and Pichia.

The expression of active recombinant CatG in both its immature and mature forms by this expression system now may allow confirmation of other issues such as the secondary binding pocket, C terminal processing, and function.

CHAPTER 2

RECOMBINANT HUMAN MAST CELL CHYMASE: IMPROVED EXPRESSION IN PICHIA PASTORIS AND PURIFICATION OF HIGHLY ACTIVE ENZYME

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Running title: Active Recombinant Human Chymase

SYNOPSIS

Human mast cell chymase (EC: 3.4.21.39) has been shown to be a chymotrypsin-like serine protease stored in and released from mast cell granules. This enzyme has been expressed in *Pichia pastoris* via homologous recombination of the cDNA coding for the mature active chymase into the *Pichia* genome. Cells producing the highest levels of recombinant human chymase (rhChymase) were selected by activity screening and they were grown in a fermentor. Methanol induction resulted in the secretion of active chymase into the *Pichia* growth media and increasing levels of enzyme were detected in the media for 5 days. Active enzyme was purified from the culture media with a 22% yield of activity via a simple two-step procedure, using hydrophobic interaction chromatography followed by affinity chromatography on immobilized heparin. The major peak from the heparin-column contained a single band of 30.6 kDa on SDS-PAGE. The purified rhChymase was 96% active and the yield was 2.2 mg per liter of growth media.

Key words: Active, Chymase, Fermentation, Heparin, Human, Secretion

INTRODUCTION

Chymase (EC: 3.4.21.39) has been proven to be a chymotrypsin-like serine proteinase found in the cytoplasmic granules of some human mast cells (Irani and Schwartz, 1994), and stored and released as an active enzyme when mast cells degranulate (Sayama et al. 1987; Schechter et al. 1994; Welle, 1997). Chymase has been shown to have multiple functions, with its ability to convert angiotensin I to angiotensin II (Reilly et al. 1982) receiving considerable attention. Human skin tissue served as the initial source of mast cell chymase (Schechter et al. 1983). There have been several reports on the expression of recombinant human mast cell chymase (rhChymase). Mammalian cell lines COS1 (Urata et al. 1993) and CHO-K1 (Ferry et al. 2001) have been used to produce recombinant human chymase (rhChymase) in the pro-enzyme form. *Escherichia coli* expression systems yielded unglycosylated protein requiring refolding and activation with enterokinase (Takai et al. 2000; Wang et al. 1995). *Bacillus subtilis* was used to produce active rhChymase in the culture media that lacked glycosylation (McGrath et al. 1997). The baculovirus insect expression system yielded the highest amounts of rhChymase in a zymogen form requiring activation with enterokinase (Suzuki et al. 2002; Wang et al. 1998) or dipeptidyl peptidase I (McEuen et al. 1998). *Pichia pastoris* was found to secrete active enzyme (Nakakubo et al. 2000a) and to accumulate inactive rhChymase in the cells (Nakakubo et al. 2000b). In this report we have described an improved *Pichia pastoris* system for the stable expression of active rhChymase and purification of the enzyme from the growth media.

EXPERIMENTAL PROCEDURES

Materials

Succinyl-Ala-Ala-Pro-Phe-p-Nitroanilide (Suc-AAPF-pNA S-7388), goat Anti-mouse IgG (Fab specific) conjugated to alkaline phosphatase (A-2179), antifoam B, and octyl glucoside (O-8001) were obtained from Sigma Chemicals. Western Blue™, NotI, BstXI, and XhoI were Promega products. MES, glycerol, octyl glucoside, ammonium sulfate, sodium azide, and sodium chloride were purchased from Fisher Scientific. Tosopearl butyl 650-M and AF-Heparin resins were obtained from Tosohaas. NuPAGE SDS gels (12% acrylamide) pre-cast with Bis-Tris buffer, NuPAGE MOPS buffer (20X), True Blue pre-stained protein standards, Simply Blue coomassie stain, and *Pichia pastoris* EasySelect Pichia Expression Kit were purchased from Invitrogen. The GelCode Blue Stain was purchased from Pierce. DeepVent DNA polymerase and Peptide N-glycosidase F (PNGase F) were obtained from New England Biolabs. Competent JM109 *E. coli* cells were produced by the method of Inoue et al. (Inoue et al. 1990). Micro-titer 96-well half-diameter plates (Product Number 3695) were obtained from Corning. Recombinant Eglin C was graciously provided by H.P. Schnebli (Ciba-Geigy). The full-length cDNA in the pVL1392 vector (Invitrogen) was generously provided by Dr. George Caughey (UCSF). The chymase specific CC1 mouse monoclonal Ab was generously provided by Dr. Andrew Walls (Southampton).

A 10X Yeast Nitrogen Base (YNB) solution was prepared by dissolving 134 g of Yeast Nitrogen Base with ammonium sulfate but without amino acids in 1 L of deionized water and filter sterilized. A 500X Biotin solution was prepared by dissolving 20mg in 100ml of deionized water and filter sterilized. The Trace Metals Solution contained 6 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.08 g NaI, 3 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.5 g CoCl_2 , 20 g ZnCl_2 , 0.02 g H_3BO_3 , $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 4 ml of 6 N H_2SO_4 and 0.2 g Biotin per liter that was filter sterilized. The basic salts fermentation media contained 0.93 g $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 18.2 g K_2SO_4 , 14.9 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.13 g KOH, 4.3 g NH_4Cl , 26.7 ml H_3PO_4 , 40 ml glycerol, and after sterilization 4.6 ml of the Trace Metals solution were added. Agar Yeast Extract Peptone Dextrose Medium plates with Zeocin (YPDS) contained 1% yeast extract, 2% peptone, 2% dextrose (glucose), 1 M sorbitol, 2% agar and 100 $\mu\text{g/ml}$ Zeocin. Buffered Glycerol-complex Medium (BMGY) media was prepared by dissolving 10 g yeast extract and 20 g peptone in 700ml of water followed by sterilization to that was added 100 ml of 1 M K_2HPO_4 pH 6.0, 100 ml 10XYNB, 2 ml 500X Biotin and 100 ml

10% glycerol. Buffered Methanol-complex Medium (BMMY) media was the same as BMGY with 50 ml of methanol in place of the glycerol.

Construction of pPICz α -rhChymase

Recombinant human chymase (rhChymase) was engineered and cloned into the pPICz α vector for expression in *Pichia pastoris* strain X-33. The full-length human chymase cDNA was amplified and engineered via PCR using the primers shown in Figure 1. PCR removed the signal and pro-peptides and added a Kex2 protease cleavage site just prior to the region coding for the N-terminal of the active enzyme. These primers also added restriction sites for XhoI at the 5' end and for NotI at the 3' end, and a stop codon at the 3' end. The PCR product was analyzed on a 3% Nusieve agarose gel. After digestion with XhoI and NotI the PCR product was ligated into the similarly cut pPICz α vector that had been gel purified and isolated using a Qiagen kit. The ligation mixtures were used to transform competent JM109 *E. coli*. Positive colonies were selected on low-salt LB plates with 25 μ g/ml of Zeocin. Only 1 of the 10 positive colonies contained the chymase gene insert based on the analysis of plasmid #6 DNA on 1% agarose gels after digestion with XhoI. PCR of plasmids #6 and #10 using the chymase primers yielded a product of 800 bp with plasmid #6, whereas plasmid #10 was negative as expected. Sequencing of this rhChymase-pPICz α plasmid (#6) using 5' α factor and 3'AOX1 specific primers (Invitrogen), proved that the correctly engineered chymase gene was in-frame with the alpha factor initiation codon and that the chymase coding region ended with a stop codon. The rhChymase-pPICz α DNA (3 μ g) linearized with BstXI was used to transform the X-33 strain of *Pichia pastoris* that had been made chemically competent using the EZComp Reagents. After growing the transformed *Pichia-X-33-rhChymase* on YPDS (yeast extract peptone dextrose medium) 1% yeast extract, 2% peptone, 2% dextrose (glucose), 1 M sorbitol, 2% agar plates containing 25 μ g/ml Zeocin for 4 days, 20 positive colonies were picked and re-screened on YPDS-Zeocin plates. Of the 20 colonies 16 were again positive and these were screened for chymase expression by first growing 10 ml cultures in glycerol-containing BMGY media overnight at 30 °C with shaking. The cells were then switched to methanol-containing BMMY media and cultured overnight at 30 °C with shaking. After centrifugation at 3000 x g for 5 minutes the media was assayed using 50 μ M Suc-AAPF-pNA as the substrate. Three colonies (#2, #8, & #10) produced the most

chymase activity, but colony #10 had a little more activity and was used for expression in a fermentor.

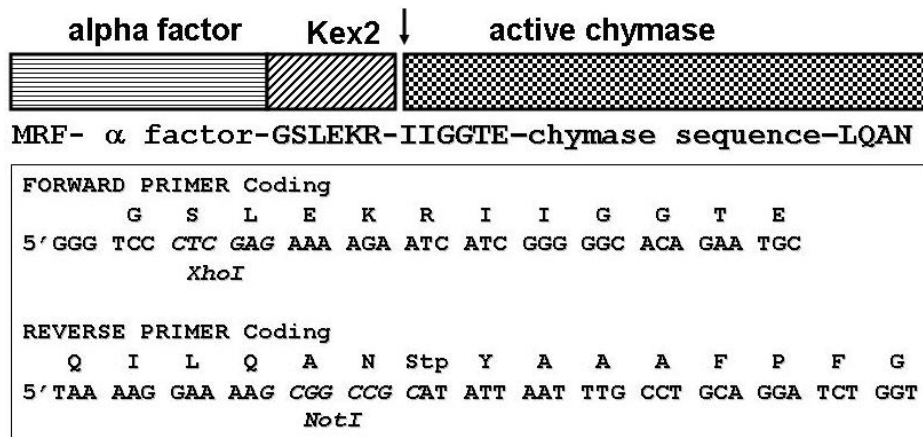


Figure 6 Schematic of rhChymase Genetic Engineering.

Diagram shows the expression product from the starting Met of alpha factor to the chymase N-terminus. The Kex2 processing site is marked with an arrow. Primer sequences, restriction sites (italics), and the coded amino acids are also shown.

Figure 6 has depicted a schematic diagram of the genetic engineering showing the expression product from the starting Met of alpha factor to the chymase N-terminus, with the Kex2 processing site marked by an arrow. Primer sequences, restriction sites (italics), and the coded amino acids have also been shown. The pePICz α vector contains sequences homologous to the alcohol oxidase 1 (AOX1) gene in *Pichia* and the transformation results in homologous recombination of the engineered sequences under the control of the AOX1 gene, producing a stable cell line. The *Pichia pastoris X-33-chymase* grew well on media containing glycerol as the carbon source, but when the carbon source was changed to methanol the AOX1 gene was turned on and growth was slowed. *Pichia pastoris X-33-chymase* secreted the alpha factor- Kex2-chymase protein because the alpha factor contained secretion recognition sequences. Because the *Pichia pastoris* contained the Kex2 protease, this enzyme cleaved at the engineered Kex2 cleavage site, freeing it from alpha factor and releasing active chymase into the media.

Fermentation

To obtain enough rhChymase for purification, *Pichia pastoris*- X-33-chymase was grown using a New Brunswick BioFlo 110 fermentor with a 7-liter vessel. The colony producing the highest level of rhChymase activity, designated X33-rhChymase, was grown 24 hours in a 0.5 L baffled shaker flask containing 200 ml of BMGY media. When this culture reached an A_{600} of 6.6, it was used to inoculate 2500 ml of basal salts media with glycerol media in the fermentor. Air was pumped through the stirred culture to maintain the dissolved oxygen level at 40% by varying the stirring rate from 200-1,000 RPM and temperature was maintained at 30°C. The pH was maintained at 5 ± 0.2 by pH probe controlled addition of 7.5% NH_4OH or 8% H_2SO_4 . The culture was fed 1 ml/min of 50% glycerol containing 12 ml/L Trace Metals Solution. After 46.5 hours the A_{600} was 220 and the cells were separated from the media by centrifugation at 3000 x g for 30 minutes and re-suspended in BMMY and returned to the fermentor. After 30 minutes, 100% methanol containing 12 ml/L of Trace Metals Solution was added at a rate of 1ml/hour using a peristaltic pump. Samples were removed daily and assayed for chymase activity. The media was harvested on day 5, after 120 hours of induction and separated from the cells by centrifugation at 10,000 x g for 30 minutes. The media (3280 ml) was stored at -20 °C until it was used for purification

In Vitro Enzymatic and Protein Assays

Chymase activity was measured using 0.5 mM succinyl-Ala-Ala-Pro-Phe-p-Nitroanilide (Suc-AAPF-pNA) in 0.1 M HEPES, 1 M NaCl, 10% glycerol, 0.1 mg/mL Heparin, 0.01% Triton X-100, 0.02% Sodium Azide, pH 7.5. Assays were performed in Corning 96-well half diameter plates with 25 μL samples. Reactions were started by adding 25 μL of 1 mM substrate in the assay buffer. Kinetic rate data were obtained by following product development at 405 nm using a BIO-TEK Instruments EL 312e with KC Junior software to measure initial velocities. Rate data were collected every 10 seconds for a total of 10 minutes at 22 – 25 °C. Linear regression fits to the initial portion of the rate curves had r^2 values of 0.99 or higher. A unit of enzyme activity was defined as an increase of 1 absorbance unit at 405 nm per minute at room temperature. Protein was measured by A_{280} and the conversion to mg/ml was based on the A_{280} value 0.952 for a 1% solution calculated from the amino acid composition of the active enzyme (Pace et al. 1995). For the determination of K_m and k_{cat} kinetic constants with

the Suc-AAPF-pNA substrate the buffer used was 0.45 M Tris-HCl, 1.8 M NaCl, 9% DMSO, pH 8.0 (Schechter et al. 1997) and regular microplates with full-sized wells were employed to assure adequate mixing. The substrate range was 0.1 – 7 mM and initial rate data were analyzed by non-linear regression of a plot of rate versus substrate concentration with fitting to a hyperbolic curve using Sigma Plot.

Electrophoretic Analyses

Protein samples of equal A_{280} were concentrated and de-salted via TCA precipitation in the presence of 0.02% deoxycholate (Brown et al. 1989) for SDS-PAGE. After washing the precipitated protein with 10% TCA followed by 1% TCA, 3 acetone washes were used to remove the TCA. Air dried samples were dissolved in NuPage LDS Sample Buffer (Invitrogen) with 50 mM dithiothreitol as the reducing agent. Samples were analyzed on NuPAGE 12% Bis-Tris pre-cast gels (1.0 mm X 10 well) using NuPAGE MOPS buffer. Following electrophoresis at 200 volts for 45 minutes protein bands were stained with GelCode Blue (Pierce) coomassie stain. Imaging was accomplished with an Alpha Innotech Alpha Imager and software, except for the Western blot that was imaged on a flat bed scanner. For the analysis of expression in media samples 5 ml from each time point were first bound to 1 ml of AF-heparin resin followed by batch elution with 0.5 ml of 1 M NaCl. Samples were concentrated and desalted via TCA precipitation.

For Western blot analyses, samples were subjected to SDS-PAGE under reducing conditions and electro-blotted onto nitrocellulose, blocked with 1% BSA in 10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 8.0 (TBST), and probed with anti-human chymase mAb, clone CC1 1:5000 in TBST. Then the blots were incubated with a 1:10,000 dilution of goat Anti-mouse IgG (Fab specific) conjugated to alkaline phosphatase in TBST and developed with Western Blue™ alkaline phosphatase detection reagent.

Deglycosylation

Both rhChymase peaks A and B off the heparin column were subjected to PNGase F digestions according to the manufacturer's protocol. Briefly, the chymase peaks (0.012 A_{280} units of each) were denatured and desalted via TCA precipitation. Protein pellets were dissolved in the deglycosylation denaturing buffer containing 0.5% SDS and 1%

beta-mercaptoethanol and heated at 100 °C for 10 minutes. Samples were then made 50 mM in sodium phosphate pH 7.5 with 1% NP-40 and incubated with 1000 units of PNGase F at 37 °C for 24 hours. The reaction was stopped by adding SDS-PAGE sample buffer and heating to 100 °C for 5 minutes prior to analysis on 12% a NuPAGE gel. Protein bands were detected by staining with Simply Blue coomassie stain (Invitrogen).

Eglin C Active Site Titration

Recombinant Eglin C, a leech derived inhibitor of chymase with a molecular weight of 8222, was used to measure the activity of the purified rhChymase. A known and constant amount of rhChymase (5.5 pmol) was incubated with increasing amounts of Eglin C in microplate wells followed by enzyme assays of the residual chymase activity. A stock solution containing a known amount of Eglin C was prepared based on its A_{280} extinction coefficient of 0.934 for a 0.1% solution. Because each mole of Eglin C inhibits 1 mole of chymase the resulting titration plot provides a measure of the absolute activity of the rhChymase.

RESULTS and DISCUSSION

Expression Kinetics

Cell-free supernatants were collected at 0, 24, 48, 96 and 120 hours post-induction and analyzed by SDS-PAGE (Figure 7), Western blotting (Figure 8), and assayed for chymase activity (Figure 9). SDS-PAGE analysis of the heparin-binding proteins in the media showed an increasingly darker band at approximately 33 kDa. Western blotting using a monoclonal to chymase showed no reaction prior to methanol induction and an increasingly positive band corresponding to the expected molecular weight of approximately 32 kDa (Figure 7) after induction. As expected, no chymase activity was detected in the *Pichia pastoris* X33-rhChymase media prior to induction, whereas following induction, chymase enzymatic activity increased up to 120 hours post induction (Figure 8). Taken together these data show that *Pichia pastoris* X33-rhChymase expressed active rhChymase following induction.

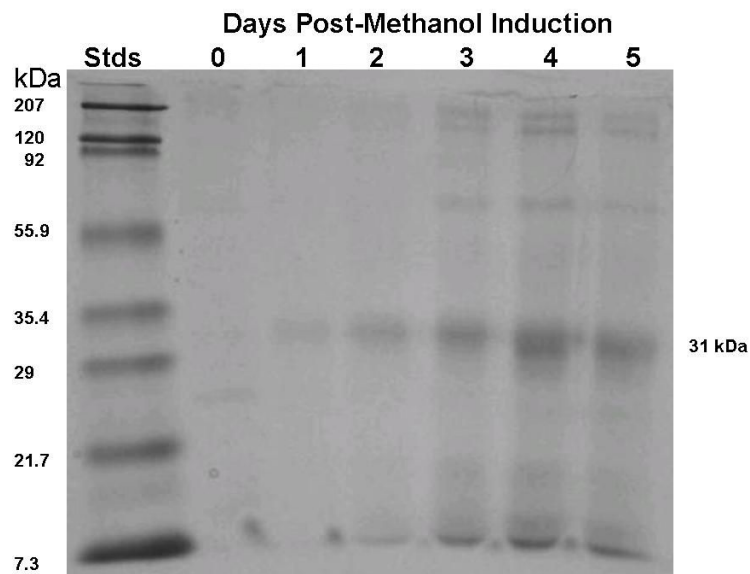


Figure 7 SDS-PAGE of Fermentation Media. Media (5 ml) taken at different times was analyzed by SDS-PAGE with reduction. Samples had been bound to AF-heparin column material and prepared as described in Methods.

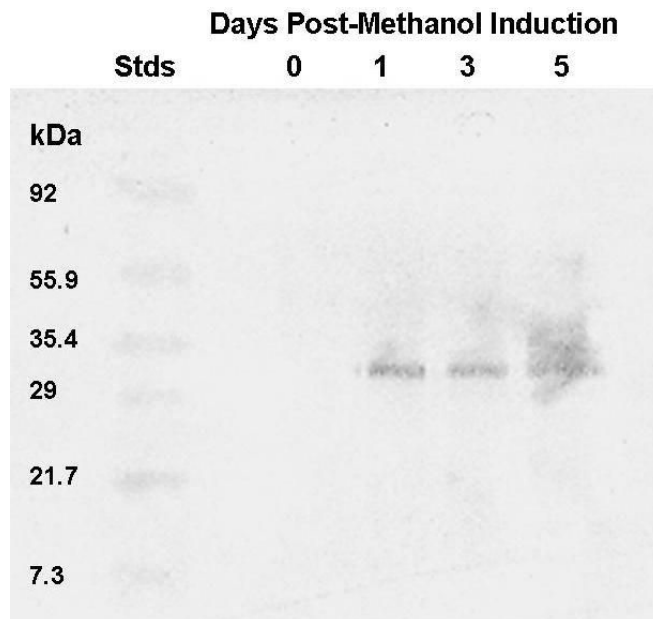


Figure 8 Induction of Immuno-reactive rhChymase. Western blot of fermentation

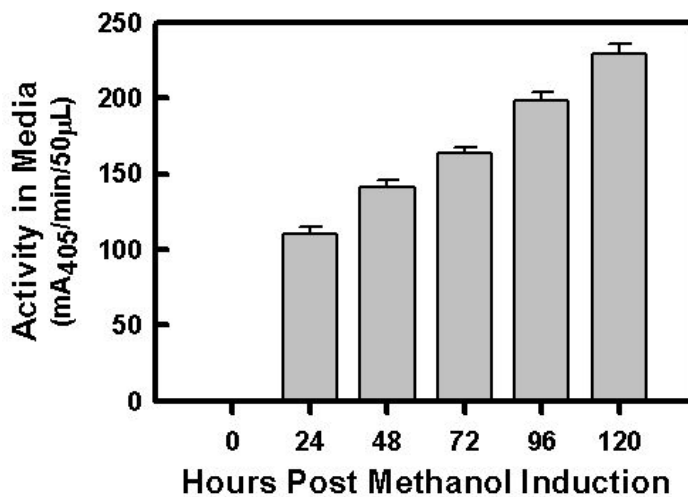


Figure 9 Induction of rhChymase Activity. Secreted chymase activity was measured each day in the growth media starting with Day 0, just prior to the addition of methanol. Chymase activity was assayed using 50 µL of media in a standard plate with a total volume of 100 µL.

Purification

Cell-free media was obtained by centrifugation at 10,000 x g for 30 minutes and stored at -20 °C for 3 months. Upon thawing the media was made 2 M in ammonium

sulfate by slowly adding the solid while stirring. After the ammonium sulfate was dissolved, the media was centrifuged at 21,000 x g for 30 minutes, resulting in only a minor pellet and a clear supernatant fraction. A 150 ml sample of this media was then applied to a TosoHaas 650M butyl hydrophobic interaction column (2.5 x 8.5 cm; 41.7 ml) equilibrated in 10 mM MES, 10% glycerol, 0.01% octyl glucoside (OG), 2 M ammonium sulfate (AS), 0.02% sodium azide, pH 6.0. After washing extensively with the equilibration buffer, rhChymase was eluted with 0.5 M ammonium sulfate, 10 mM MES, 10% glycerol, 0.01% OG, pH 6.0 at a flow rate of 2 ml per minute. Fractions were collected at 1.5 min intervals, yielding ~3 mL/fraction (Figure 10). Pooled fractions (12-24) were made 1 M in ammonium sulfate and concentrated in an Amicon stirred cell to

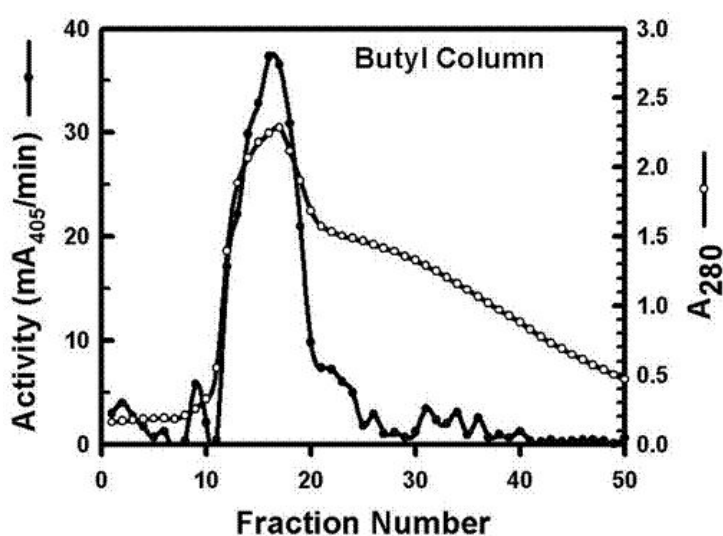


Figure 10 Butyl Column Chromatography. Media (150 ml) that had been made 2 M in AS was applied to a TosoHaas 650 M butyl hydrophobic interaction column (2.5x8.5 cm; 41.7 ml) equilibrated in 10 mM MES, 10% glycerol, 0.01% OG, 2 M AS, 0.02% NaN₃, pH 6.0. After washing extensively with the equilibration buffer, rhChymase was eluted with 0.5 M AS, 10 mM MES, 10% glycerol, 0.01% OG, pH 6.0 at a flow rate of 2 ml per minute. Fractions (3 ml) were collected at 1.5 min intervals and 2 μ L of each were assayed for activity. Fractions 12-24 were pooled for chromatography on the heparin column.

23 ml. This sample was diluted 10 fold with deionized water and loaded onto a TosoHaas-Heparin-AF column (1.5x13 cm; 23 ml) equilibrated with 10 mM MES, 0.2 M NaCl, 10% glycerol, 0.01% OG, 0.02% NaN₃, pH 6 and washed with this buffer until the

A_{280} was essentially zero. The bound rhChymase was eluted with a 150 ml linear gradient from 0.2 – 0.75 M AS in the same buffer at a flow rate of 2 ml per minute, collecting 3ml fractions. As shown in Figure 11, two peaks of chymase activity were obtained with the second peak containing most of the activity and being separated from

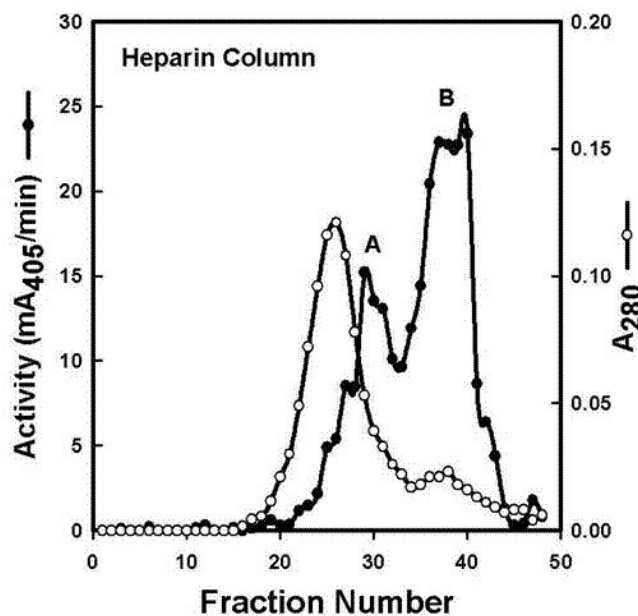


Figure 11 Heparin Column Chromatography. The rhChymase pool off the Butyl column was applied to a TosoHaas-Heparin-AF column (1.5x13 cm; 23 ml) equilibrated with 10mM MES, 0.2 M NaCl, 10% glycerol, 0.01% OG, 0.02% NaN₃, pH 6. The bound rhChymase was eluted with a 150 ml linear gradient from 0.2 - 0.75 M AS in the same buffer at a flow rate of 2 ml per minute. Fractions of 3 ml were collected and assays were performed on 2 μ L of each fraction. Activity peaks A (25-33) and B (34-43) were pooled separately and analyzed.

the major A_{280} peak. Fractions 35-44 were pooled and concentrated to 2.5 mL using an Amicon stirred cell with a 10 kDa Pall Omega membrane. The A_{280} of this solution was 0.13 that was calculated to be equivalent 0.137 mg/mL for a total protein yield of 342 micrograms from 150 mL of media. The data from 1 purification procedure have been shown in Table 1. The yield of pure rhChymase in peak B off the heparin column from 3 purifications averaged 2.23 ± 0.2 mg per liter of *Pichia* media. SDS-PAGE analysis of

both peaks showed that Peak A had a much fainter band at 33 kDa than Peak B (Figure 12). While

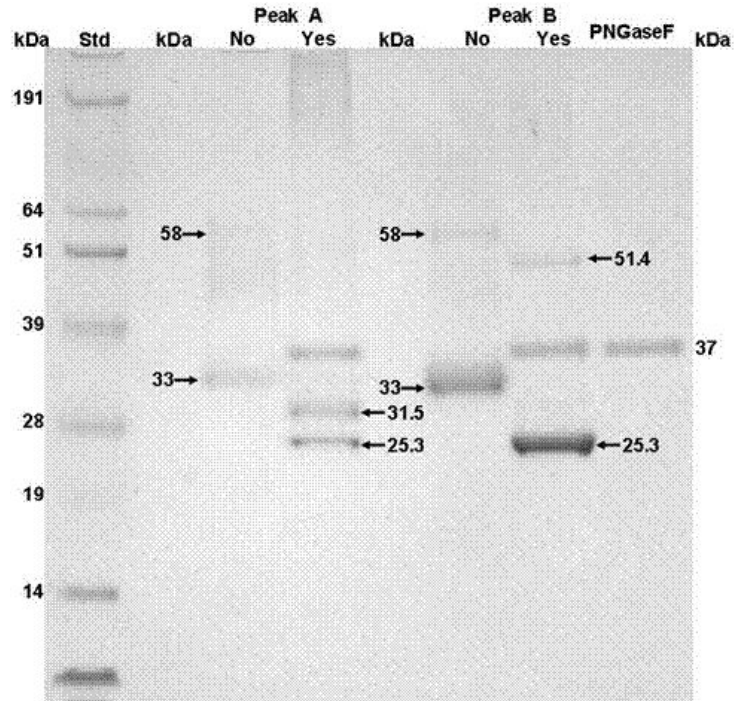


Figure 12 SDS-PAGE Analysis of Purified and Deglycosylated rhChymase. SDS-PAGE with reduction and deglycosylation of Heparin Peaks A and B. Lanes labeled **NO** are untreated Peaks A and B from the Heparin column. Lanes labeled **YES** were deglycosylated with PNGaseF. The lane labeled PNGaseF contained only the PNGaseF enzyme.

both peaks contained a faint band at 58 kDa, Peak A also contained a broad diffuse band from 40 to 58 kDa. The yield of activity in Peak B was 22% with this material having been purified more than 1000 fold (Table 3).

Step	<u>Purification Data</u>			
	Total Protein (A ₂₈₀)	Total Activity (units)	Specific Activity (units/A ₂₈₀)	Yield %
Media	1548	891.6	0.576	100
Butyl Column	87	527.1	6.06	59
Heparin Peak B	0.325	199.7	614.5	22

Table 3 Purification Data. A table is displayed detailing each of the 3 purification steps. The A₂₈₀ of each step is seen in Column 2. Column 3 shows total activity with the specific activity (units/A₂₈₀) in Column 4; and the % Yield from purification in Column 5.

Deglycosylation

Both rhChymase peaks A and B were treated with PNGase F to remove Asn-linked carbohydrate and analyzed on SDS-PAGE (Figure 12). Comparison of the bands in lanes treated with PNGase F (YES) with the untreated control samples (NO) show that the products were glycosylated. Peak A bands decreased to two bands of 31.5 and 25.3 kDa that stained more intensely. The Peak B band at 58 kDa was reduced to 51.4 kDa and the major band at 33 kDa was reduced to 25.3 kDa. The bands at 25.3 kDa in both samples probably represent the deglycosylated rhChymase, that has a calculated molecular weight of 25.03 kDa. The identity of the 31.5 kDa band in Peak A after deglycosylation was unknown, but it may be a contaminant or possibly rhChymase with Ser or Thr-linked carbohydrate, resistant to PNGase F. The Peak B band at 51.4 kDa may represent a small amount of dimeric rhChymase.

Activity Measurement

To determine the percentage of rhChymase in Peak B that was active, samples were incubated with increasing molar ratios of Eglin C, a tight binding inhibitor. As shown in Figure 13, chymase activity decreased with increasing Eglin C molar ratios and linear regression of the data intersected at 0.96 mole of Eglin C per mole of rhChymase, indicating that the rhChymase was 96% active.

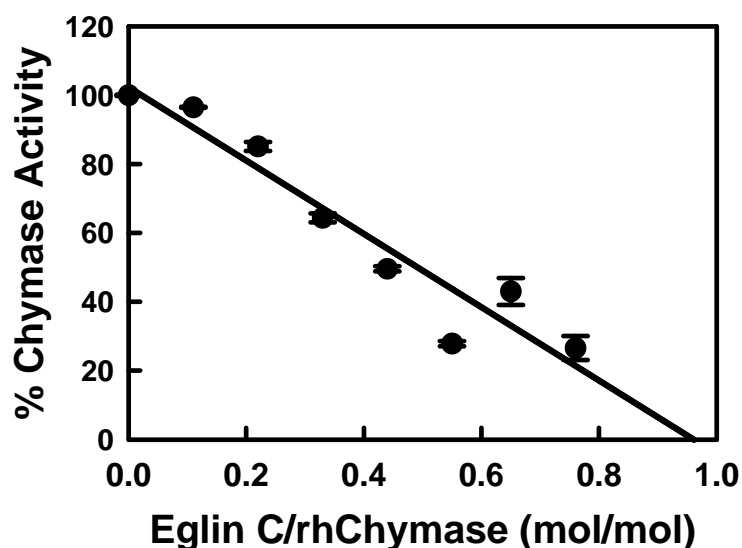


Figure 13 Active Site Titration with Eglin C. rhChymase was incubated with increasing molar ratios of Eglin C followed by enzyme assays of the remaining rhChymase activity as described. Linear regression of the data ($r^2 = 0.92$) intersected at 0.96 mole Eglin C per mole of rhChymase. Error bars represent the standard deviation of the mean of triplicate assays.

Kinetic analysis of the purified rhChymase with the Succinyl-Ala-Ala-Pro-Phe-pNA substrate yielded a K_m of 2.96 mM and a k_{cat} of 17.5 sec^{-1} resulting in a calculated k_{cat}/K_m catalytic efficiency constant of $5,912 \text{ M}^{-1}\text{s}^{-1}$. This K_m value was lower than the value of 5.93 mM previously reported for *Pichia* expressed rhChymase (Schechter et al. 1997), but higher than the K_m of 0.95 mM reported for natural human skin chymase. The k_{cat} we observed was lower than the 50 sec^{-1} reported for natural human skin chymase for this substrate. These kinetic differences were not considered to be unreasonable and could be due to differences in glycosylation.

Concluding Remarks

Presented data clearly demonstrate the stable expression of rhChymase by transformed *Pichia pastoris* with secretion into the media. Normally, proteases are expressed as zymogens that require activation. The addition of a Kex2 site prior to the amino terminus of the chymase used the ability of the *Pichia* to activate the translational product. The activation of the expressed enzyme via an additional step was avoided when this approach was used. A simple two-step purification protocol was developed

that yielded highly purified and highly active rhChymase. An amount of 2.2 mg of rhChymase was obtained from a liter of media, that represented a considerable improvement in the yield of rhChymase compared to other methods. While a number of different systems have been used to produce rhChymase (Table 4), only expression in *Baculovirus* systems produced greater amounts of enzyme. However, the *Pichia* system has the considerable advantage of producing active enzyme, rather than a zymogen form that requires activation. Additionally, the rhChymase produced in *Pichia* was found to be structurally similar to natural chymase,

Comparison of Various rhChymase Expression Systems

System	Product	mg/L	Reference
COS	Pre-pro-	ND	Urata et al., 1993
CHO-K1	Pro-	0.800	Ferry et al., 2001
<i>E. coli</i>	Ub-Ek-	0.125	Wang et al., 1995
<i>E. coli</i>	His-tag-Ek-	ND	Takai et al., 2000
<i>B. subtilis</i>	active	0.500	McGrath et al., 1997
Baculovirus	Pro-	0.960	McEuen et al., 1998
Baculovirus	Ub-Ek-	3	Wang et al., 1998
Baculovirus	hemolymph-Ek-	32	Suzuki et al., 2002
<i>P. pastoris</i>	active/media	0.190	Nakakubo et al., 2000
<i>P. pastoris</i>	inactive/cellular	ND	Nakakubo et al., 2000
<i>P. pastoris</i>	active/media	2.2	Present report

Table 4 Comparison of Various rhChymase Expression Systems. A table is displayed containing the different expression systems used in Column 1. Column 2 details their generated product; with the final purified quantity displayed in Column 3; and the Researcher in Column 4.

in that both the natural and recombinant proteins were glycosylated. The stable expression of active rhChymase in *Pichia pastoris* at increased levels will facilitate further study of this interesting serine protease. Isolation of rhChymase from *Pichia* eliminates the hazards of working with human tissues and greatly increases the amount of available enzyme. In particular, rhChymase can be used as a primary standard in immunological assays and for comparison with other natural and recombinant proteases.

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CHAPTER 3

RECOMBINANT HUMAN MAST CELL CATHEPSIN G: NOVEL EXPRESSION IN PICHIA PASTORIS AND PURIFICATION AND CHARACTERIZATION OF A DUAL SPECIFICITY ENZYME

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Running title: Active Recombinant Human Cathepsin G

SYNOPSIS

Human mast cell cathepsin G (EC 3.4.21.20), that had not previously been expressed as a recombinant protein, was found to be a “Janus faced” serine protease possessing both a chymotrypsin-like and a trypsin-like activity stored in and released from mast cell granules and in neutrophils. This enzyme was expressed in *Pichia pastoris* via homologous recombination of the cDNA coding for the active immature (iCatG) and mature (mCatG) forms into the *Pichia* genome. Cells producing the highest levels of recombinant human CatG (rhCatG) were selected through kinetic activity screening during a pilot expression test. *Pichia* colonies of both forms of CatG, that displayed superior enzyme production, were induced for 4 days and stabilized with dextran sulfate in the media. The proteases were partially purified using ultrafiltration. A major peak was detected on SDS-PAGE gel at 26 kDa with the sequence being confirmed as iCatG through MALDI-TOF Mass fingerprinting. Kinetic activity assays were performed on both iCatG and mCatG using two different substrates Suc-APF-SBzl and Suc-APK-SBzl that presented similar V_{max} . Inhibition studies using TPCK, TLCK, Bowman-Birk, and Eglin C eliminated the dual activity of both forms of rhCatG confirming the dual activity.

Key words: Active, Cathepsin G, Dextran Sulfate, Human, Secretion, Protease, Mast Cell

Abbreviations: Recombinant human cathepsin G, rhCatG; Recombinant Immature cathepsin G, iCatG; Recombinant mature cathepsin G, mCatG; native cathepsin G purified from neutrophils, nCatG; Serine Protease Inhibitors, SERPINS; Suc-VPF-SBzl, succinyl- Valine-Proline-Phenylalanine thiobenzyl ester; Suc-VPK-SBzl, succinyl- Valine-Proline-Lysine thiobenzyl ester; polymerase chain reaction (PCR); SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; Tosyl phenylalanyl chloromethyl ketone (TPCK); Tosyl-L-lysine-chloromethyl ketone (TLCK); DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic; IC₅₀, Inhibitory concentration for 50% reduction of activity.

INTRODUCTION

Human mast cell cathepsin G (CatG; EC 3.4.21.20), that had not previously been expressed in recombinant form, has been named a “Janus faced” serine protease possessing both chymotrypsin-like and trypsin-like activities. CatG had been found in both neutrophils (Barrett, 1981a) and in mast cell granules (Schechter et al. 1990). Although the CatG mRNA (NM_001911) had been found to code for an active enzyme of 235 amino acids, only a mature form (mCatG) of approximately 224 residues has been isolated from neutrophils and studied. X-ray crystal structures show that mCatG had lost 10 to 12 residues from the C-terminal based on Protein Data Bank IDs 1KYN and 1CGH. Although they were shown to possess similar molecular weights and isoelectric points, the exact reason for C-terminal processing has not been discovered. Neutrophil elastase also undergoes C-terminal processing to remove 20 residues and elimination of this processing has been linked to cyclic neutropenia (Horwitz et al. 2004). Although CatG has been generally regarded as a chymotrypsin-like protease cleaving at hydrophobic residues, it has also been shown to cleave synthetic substrates after lysines (Powers et al. 1989), that has been confirmed in this laboratory (unpublished data). Additionally, the crystal structure of CatG showed a Glu at position 226 (chymotrypsinogen numbering) situated at the bottom of the S1 specificity pocket (Hof et al. 1996) that could form an ionic bond to lysine (Hof et al. 1996). Hydrophobic residues, such as phenylalanine, bind at the entrance of the S1 pocket, but they do not fully occupy the S1 pocket as research has concluded for normal chymotrypsin-like proteases. Hof et al. (1996) termed CatG a “Janus-faced” enzyme because of the dual specificity for trypsin-like and for chymotrypsin-like substrates. The inability to fully bind chymotrypsin-substrates may explain the slow reaction kinetics of CatG with chymotrypsin substrates (Powers et al. 1989). This dual specificity was confirmed using p-nitroanilide substrates (Polanowska et al. 1998). CatG’s dual specificity allows cleavage of many biological proteins either after bulky aromatics (F, H, I, V, Y) or positively charged (L and R) residues of different proteins. Although first purified from human tissue and characterized in 1976 (Starkey and Barrett, 1976a), CatG had not been produced in a recombinant form prior to this research. There have been several factors that have made CatG impossible to express as a recombinant protein, including its bactericidal activity (Reeves et al. 2003; Shafer et al. 1991) and its high isoelectric point that had recently been shown incorrectly to be a negative factor for protein

expression in *Pichia pastoris* (Boettner et al. 2007). Garwicz's attempt at expression of active CatG, with its propeptide deleted, resulted in non-viable cells (Garwicz et al. 1998) upon transformation of RBL-1 cells; however, they expressed an inactive mutant. Salvesen found that CatG (and neutrophil elastase) are "therefore, transiently present as zymogens, presumably to protect the biosynthetic machinery of the cell from adventitious proteolysis (Salvesen and Enghild, 1990)", until packaged into the acidic azurophilic granules of neutrophils that protect the cytoplasmic proteins from these destructive proteases. *Pichia pastoris* has been used in this laboratory as a useful host for the expression of the recombinant mast cell serine proteases tryptase (Niles et al. 1998) and chymase (Lockhart et al. 2005). Consequently, the *Pichia pastoris* system was chosen for the stable expression of active rhCatG, that has previously been described.

EXPERIMENTAL PROCEDURES

Materials

Substrates Succinyl-Val-Pro-Phe-thiobenzyl ester (Suc-VPF-SBzl) was purchased from Bachem and Suc-Val-Pro-Lys-SBzl ester (Suc-VPK-SBzl) was synthesized for us by GenScript. Stock solutions of the substrates of 20mM were prepared in dry isopropyl alcohol. Octyl glucoside (O-8001) and Bowman-Birk (C7268) were obtained from Sigma Chemicals. TRIS, glycerol, ammonium sulfate, sodium azide, methanol and sodium chloride were purchased from Fisher Scientific. PAGEgel Cassette Gel-12 well SDS gels (12% acrylamide) pre-cast with Bis-Tris buffer were purchased from PAGEgel, Inc. NuPAGE MOPS buffer (20X), MultiMark® Multi-Colored Standard, Simply Blue™ SafeStain, Magic Marker Western Standards pre-stained protein standards, and *Pichia pastoris* EasySelect Pichia Expression Kit were purchased from Invitrogen. SuperSignal™ Chemiluminescent Substrate horseradish peroxidase enhancer and ImmunoPure® Antibody Anti-goat, rabbit Horseradish Peroxidase, GelCode Blue Stain were purchased from Pierce. PfuUltra™ Hotstart High-Fidelity DNA Polymerase was obtained from Stratagene. Rabbit Anti-CatG human IgG (Fab specific) was purchased from Calbiochem. Yeast Nitrogen Base with ammonium sulfate but without amino acids was purchased from Research Products International Corporation. DeepVent DNA polymerase was obtained from New England Biolabs. Western Blue™, XbaI, BstXI, and XhoI were purchased from Promega. Competent JM109 *E. coli* cells were produced

by a modification of the method of Inoue et al (1990) (Detailed protocol in Appendix). Micro-titer 96-well half-diameter plates (Product Number 3695) were obtained from Corning. Recombinant Eglin C was kindly provided by Dr H. P. Schnebi (formerly of Ciba-Geigy, Basel, Switzerland). The CatG-cDNA in the pUC-9 vector was provided by Dr. Guy Salvesen (Burnham Institute, San Diego, CA). Native human CatG was isolated from neutrophils in the laboratory following the method of Martodam et al. (1979) and the Trasyolol affinity chromatography step was repeated.

Methods

A 10X Yeast Nitrogen Base (YNB) solution was prepared by dissolving 67 g of Yeast Nitrogen Base with ammonium sulfate (but without amino acids) in 1 L of deionized water followed by filter sterilization. A 500X Biotin solution was prepared by dissolving 20 mg in 100 ml of deionized water followed by filter sterilization. The Trace Metals Solution contained 6 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.08 g NaI, 3 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.5 g CoCl_2 , 20 g ZnCl_2 , 0.02 g H_3BO_3 , $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 4 ml of 6 N H_2SO_4 , and 0.2 g Biotin per liter that was filter sterilized. A vitamin mix was prepared by dissolving 20 g casamino acids, 1 g sodium glutamate, 1 g/L tryptophan, 100 mg dextran sulfate, 400 mg histidine, 40 mg EDTA in 100 ml of water followed by filter sterilization. *Pichia pastoris* strain SMD1163 that was considered to be deficient in yeast proteases A and B and a histidine requiring strain was generously provided by Invitrogen. Basic growth media was prepared by combining 100 mL of 20% glucose, 100 mL of 100% glycerol, 776 mL of 1X YNB with ammonium sulfate, 12 mL/L Trace Metals Solution, 12 mL/L Vitamin Solution, 40 μg of histidine, 1 $\mu\text{g}/\text{mL}$ of dextran sulfate, 25 $\mu\text{g}/\text{mL}$ of ampicillin, and 100 $\mu\text{g}/\text{mL}$ zeocin in a final volume of 1 L. Expression Induction Media contained 0.05% methanol in place of glucose and glycerol. Agar Yeast Extract Peptone Dextrose Medium plates with Zeocin (YPDS) contained 90 mL deionized water, 2 g peptone, 1 g yeast extract, 10 mL of 20% dextrose, 2 g agar and 100 $\mu\text{g}/\text{mL}$ Zeocin YPDS (yeast extract peptone dextrose medium with sorbitol) plates contained 1% yeast extract, 2% peptone, 2% dextrose (glucose), 1 M sorbitol, 2% agar plates, and 100 $\mu\text{g}/\text{mL}$ Zeocin.

Construction of pPIC α -rhCatG

The full-length cathepsin G cDNA in pUC9 vector was propagated by transformation into *E. coli* JM-109 cells and purified using a Qiagen MIDI-Prep. The CatG cDNA was

then amplified by PCR to add a XhoI site and a KEX2 protease cleavage site at the N-terminus using the primer 5' TCT CTC GAG AAA AGA ATC ATC GGA GGC CGG GAG AGC AGG 3' as shown in Figure 14.

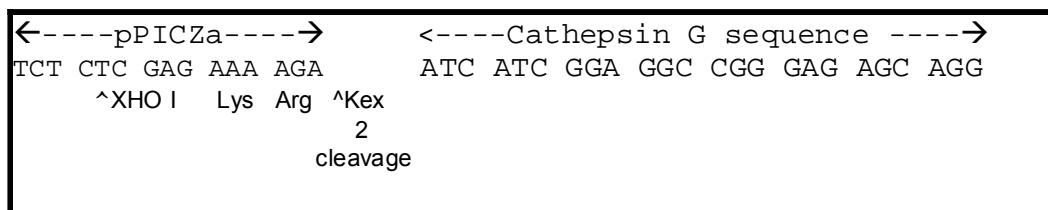


Figure 14 N terminus primer for CatG immature and Mature Forms. CatG was ligated into the pPICZa vector. Row 1 contains the regions of the vector and CatG's sequences. Row 2 has the DNA sequences of pPICZa and CatG. Row 3 shows the XHOI restriction site, the amino acid sequences, and the KEX2 cleavage site designating the area of the protein to be cleaved upon secretion from the cell.

The reverse primer used to generate immature (iCatG; full-length) with a full-length C-terminus had the sequence 5' CAA ATT TCT AGA TTA CAG TGG AGT TTC CAT CTG ATC CAG 3' (Figure 15). Mature (mCatG) missing the C-terminal 11 amino acids, but having a C-terminal His Tag sequence to assist in purification, was bioengineered as shown in Figure 3 with the primer sequence 5' CTA CTA TCT AGA TTA CTA ATG ATG ATG ATG ATG GCT TCT CAT TGT TGT CCT TAT CCA 3' (Figure 16).

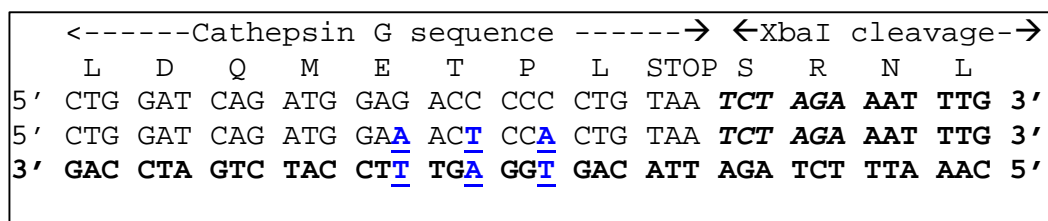


Figure 15 C terminus primer for CatG immature form. CatG and XbaI cleavage site sections are listed in Row 1. Row 2 has the amino acid residues of CatG and XbaI. The Corresponding 5' to 3' DNA sequences are grouped into codons in Row 3. Row 4 contains modified DNA sequences for primer optimization. Row 5 contains the complimentary strand. iCatG sequence is thought to end in METPL but has a 13 amino acid extension than the mature form. Blue letters denote bioengineering sites to improve PCR.

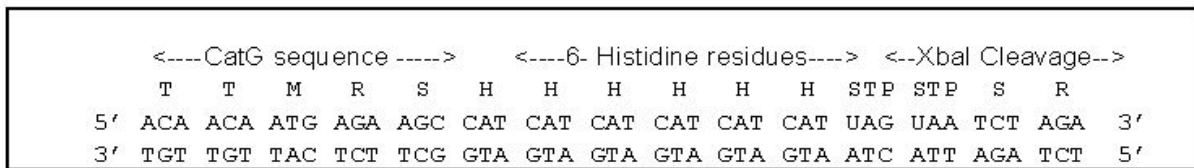


Figure 16 C terminus primer for CatG mature form. Sequence ends in TTMRS and is 13 amino acids shorter than the immature form. This sequence includes 6 histidine residues, 2 stop codons, and an Xbal site for cleavage. CatG, 6X Histidine, and Xbal cleavage site sections are listed in Row 1. Row 2 has the amino acid residues of CatG, Histidine, and Xbal. The Corresponding 5' to 3' DNA sequences are grouped into codons in Row 3. Row 4 contains the complimentary strand. mCatG sequence is thought to end in TTMRS.

PCR of the ligated pPICz α -CatG sequence was completed with denaturing at 94° C for 15 seconds, extension at 68° C for 60 seconds and 55° C for 30 seconds. Both C-terminal primers included Xbal restriction sites for cloning into pPICz α , with Vector NTI (Invitrogen) used to design the primers. This design placed a Kex2 protease cleavage site that was cleaved by the yeast Kex2 protease to remove the yeast alpha mating factor sequence and generate the N-terminal of active CatG. The PCR product was analyzed on a 3% Nusieve agarose gel followed by digestion with XhoI and XbaI, gel purification, and ligation into a previously purified and similarly digested pPICz α vector. Vector NTI was also used to confirm that the XhoI and XbaI restriction digestion sites did not exist in the center of the rhCatG DNA sequences.

Transformation

The ligated CatG- pPICz α vector was transformed into competent JM109 cells with selection being achieved on low-salt LB plates with Zeocin (25 μ g/ml). DNA plasmid mini-preps for zeocin resistant colonies of both iCatG and mCatG were digested with XhoI and XbaI and then analyzed on a 1% agarose gels to confirm they contained the 750 base CatG insert. In addition, positive samples were sequenced with the 5' alpha factor and 3' AOX1 specific primers (Invitrogen) to confirm the sequence was in-frame with the alpha factor initiation codon. Both iCatG- pPICz α and mCatG- pPICz α vectors were linearized with BstXI for homologous recombination into the protease deficient SMD1163 strain of *Pichia pastoris* that had been made electro competent (Lin-

Cereghino et al. 2005). The 50 μg of cells were transformed with 3 μg of DNA in an Electro Cell Manipulator 600 (BTX Electroporation System, San Diego, California, 92121) at 21.5 kV (2 mm gap) for 9 milliseconds in a total volume of 50 μL . A volume of 1 mL of cold 1 M sorbitol was added to the cuvette, followed by transfer of the cells to a sterile 15 mL tube. The tube was incubated at 30°C for 2 hours with shaking and 50-100 μL of cells were spread on YPDS plates with Zeocin (100 $\mu\text{g}/\text{mL}$) and incubated at 30°C. Seven iCatG (designated 1-7) and two mCatG positive colonies (designated A and B) were selected and confirmed through PCR using the 5' and 3' AOX1 primers, based on the criterion that PCR products from non-transformed cells yield a single band (2200 bp) whereas transformed samples yield an additional band at 1200 base pair (588 bp plus the expected size the CatG insert- 700 bp). The presence of CatG DNA was confirmed to be incorporated into the *Pichia* genome through sequencing at the Molecular Biology Core Facility (ETSU).

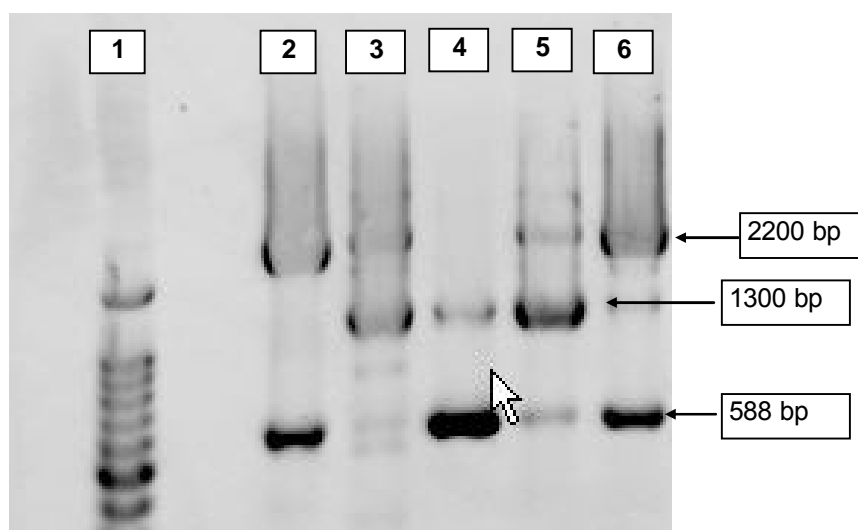


Figure 17 Analysis of *Pichia* Genomic DNA by PCR.

PCR was used to amplify the CatG sequences in the *Pichia* genomic DNA resulting from homologous recombination. Lane 1, 100bp DNA Standard; Lane 2, SMD1163 control that had not been transformed with pPICZ α (negative control); Lane 3, *Pichia* strain X-33 transformed with pPICZ α chymase (positive control); Lane 4, pPICZ α CatG in X33 insert; Lane 5, *Pichia* strain SMD1163 transformed with pPICZ α CatG ; Lane 6, *Pichia* strain X-33 pPICZ α .

Genomic DNA PCR and Sequencing

Confirmation of homologous recombination of CatG DNA sequences into *Pichia pastoris* was performed by isolating genomic DNA samples from iCatG and mCatG colonies followed by PCR with 3' and 5'AOX1 primers (Invitrogen). Polymerase Chain Reaction had a denaturing of 94° C for 15 seconds, an extension of 68° C for 1 minute per kb, and an annealing of 60 ° C for 30 seconds, with samples analyzed on a 1% agarose gel ran for 1 hour at 150 volts followed by ethidium bromide staining (See Figure 17). Duplicate sample combined with orange loading dye were loaded onto a 3% (Nuseive) gel that was run for 70 minutes at 150 mvolts. The gel was stained for 10 minutes in dilute (1:20,000) cybergreen and excised from the gel and sent to the ETSU Core Facility who confirmed of the integration of the sequences.

Expression and Growth

Enzyme expression was examined by growth of positive colonies and a non-transformed negative control in 250 mL baffled flasks containing 50 mL of Basic Growth Media overnight at 30 °C with agitation of 250 RPM. After 24 hours, samples were switched to Expression Induction Media by the daily addition of 0.5% of methanol and 10 µL /mL histidine (2 gm /100 mL). Enzymatic activity of the protease in the media was analyzed by removing 2 ml aliquots daily followed by centrifugation at 3000 x g for 5 minutes to yield cell-free media that was then assayed using 50 µM Suc-VPF-SBzl as substrate.

In Vitro Enzymatic and Protein Assays

CatG activity was measured using either Suc-VPF-SBzl or Suc-VPK-SBzl in assay buffer comprised of 0.1 M HEPES, 1 M NaCl, 10% glycerol, 0.1 mg/mL heparin, 0.02% sodium azide, pH 7.5 at a final substrate concentration of 0.5 mM and 4mM dithiodinitrobenzoic acid (DTNB). The DTNB reacts with the thiobenzene product to form thiodinitrobenzoic acid that has a 405nm extinction coefficient of 13,000 M⁻¹ (Johnson, 2006). Kinetic assays were performed with 25 µL of sample and reactions were started by adding 25 µL of 1 mM substrate in assay buffer for a total volume of 50 µL in Corning 96-well microtiter plates, that were monitored at 405nm at room temperature on a BIO-TEK Instruments EL 312e with KC Junior software. Kinetic data

were collected by taking readings every 10 seconds over 10 minutes and linear regression rate curves had a r^2 of not less than 0.99. A unit of enzyme activity was defined as an increase of 1 absorbance unit at 405 nm per minute at room temperature. Enzyme kinetic analysis were performed on the full length immature CatG to determine the V_{max} (maximum reaction velocity), and K_m used the substrate Suc-VPF-SBzl under the previously mentioned reagents and conditions. The substrate concentration ranged from 0.07 – 0.99 mM in assay buffer with 0.05 μ L of iCatG added to each well for a total of 150 μ L per well. Velocity/Reaction rate (V) data were plotted against the substrate concentrations (S) and analyzed by non-linear regression with fitting to a hyperbolic curve using Hyper32 by J.S. Easterby (University of Liverpool), resulting in an r^2 of 0.99 and Lineweaver-Burk and Eadie-Hofstee Plots

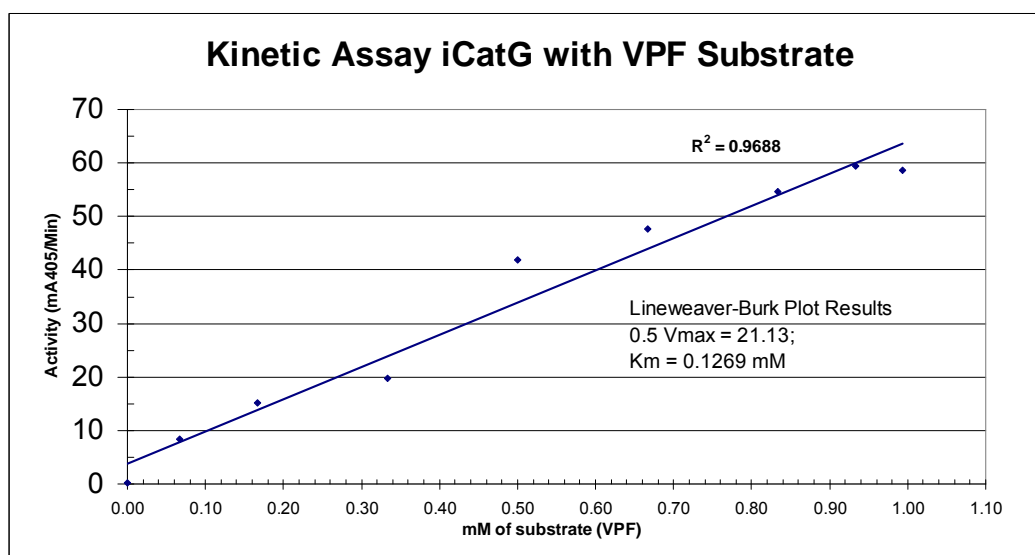


Figure 18 Km Determination of iCatG with the Suc-VPF-SBzl substrate. The activity (mA405/minute) was plotted against the concentration (mM) of Substrate. Concentration of substrate was increased, providing a linear response ($R^2 = 0.9688$) to determine V_{max} and K_m using the chymotrypsin-like substrate.

were used to calculate V_{max} and K_m (See Figure 18). Protein molecular weights were calculated from their amino acid sequences using the ProtParam software tools on the ExPASy.org web site (Gill and von Hippel, 1989; Pace et al. 1995). Literature showed that nCatG has a K_m of 0.019 mM and a K_{cat} of 22, while these experiments produced a K_m of 0.1296. A K_{cat} value could not be calculated due to incomplete purification.

Purification

Following a 4-day methanol induction period, the 50 mL of media containing the mCatG Colonies (A and B) and iCatG Colonies (2 and 4) was centrifuged at 21,000 x g for 30 minutes. The clear supernatant samples were made 0.25 M NaCl, pH 8.8 followed by storage at 4° C. Samples were concentrated on a Pall Omega 10,000 MW cut-off membrane in an Amicon stirred cell to a volume of approximately 15 mL, after 3 15 mL washings with 0.25 M MgCl₂, that has been reported to stabilize nCatG activity (Salvesen, 2004). Samples were further concentrated to 5 mL using a 10,000 MW cut-off Macrosep (Pall Corporation) filter by centrifugation at 3000 x g, followed by concentration on a Nanosep 10K Omega column (Pall Corporation) to approximately 500 µL. The samples were analyzed on an SDS-Page gel, as seen in Figure 19.

Electrophoretic and Mass Spectral Analysis

Protein samples of approximately equal concentrations based on activity, were de-salted and dissolved in NuPage LDS Sample Buffer (Invitrogen) with 50 mM

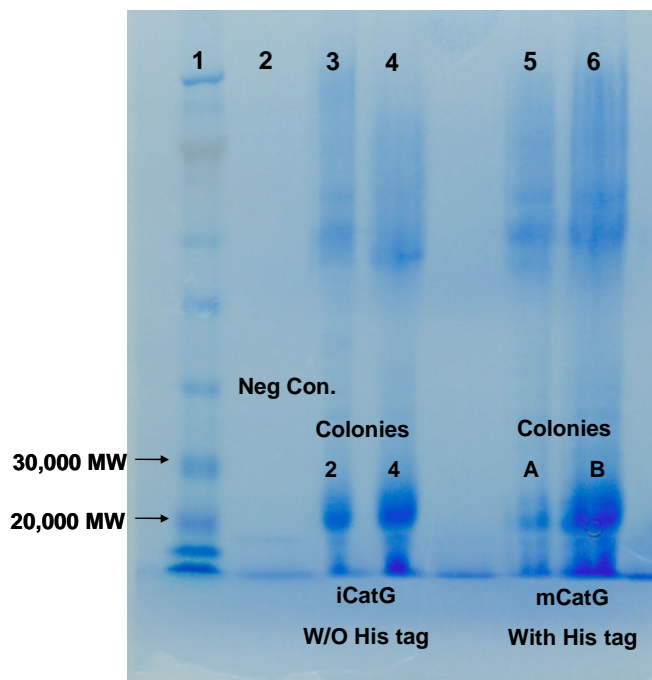


Figure 19 Qualification of iCatG and mCatG by size and purity by SDS Page Gel. Samples were found to be approximately 25,000 daltons. Lane 1, SeeBlue Plus2 SDS-PAGE standard; Lane 2, Negative Control; Lane 3, iCatG Colony 2; Lane 4, iCatG Colony 4; Lane 5, mCatG Colony A; Lane 6, mCatG Colony B.

dithiothreitol. Samples were analyzed on NuPAGE 12% Bis-Tris pre-cast gels (1.0 mm X 10 well) using NuPAGE MOPS buffer, with electrophoresis at 200 mvolts for 45 minutes, followed by staining with GelCode Blue (Pierce) Coomassie stain. Protein imaging and densitometry were accomplished with an Alpha Innotech Imager and software (See Figure 19). The major band of approximately 26kDa in lane 4 (Colony 4, iCatG) was excised from the gel and submitted to Columbia University for sequence confirmation by MALDI-TOF Peptide Mass Fingerprinting (See Figure 20). Theoretical peaks and actual peak results were determined with the results being placed in Table 7.

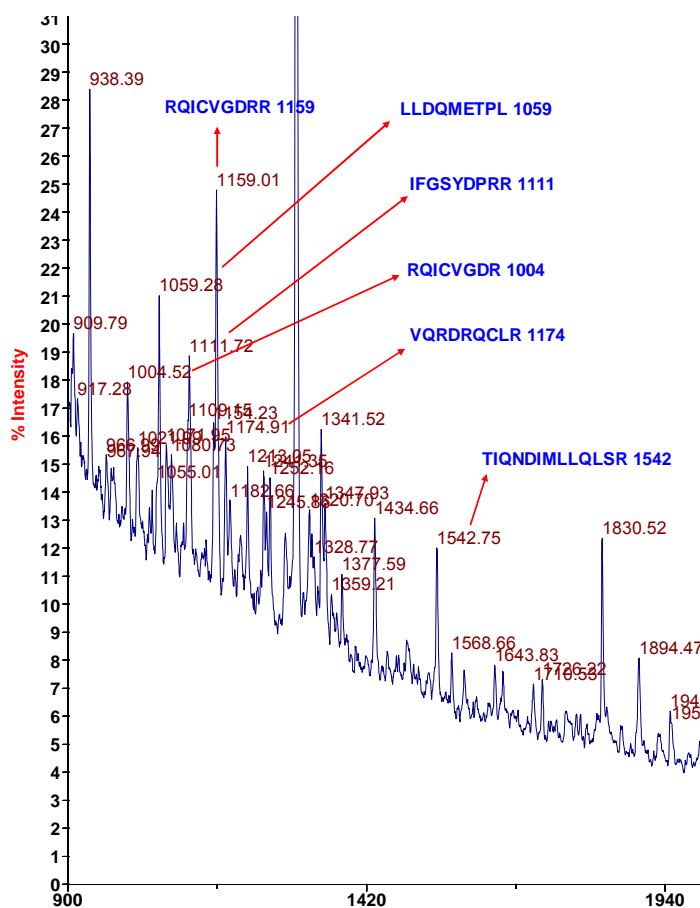


Figure 20 MALDI-TOF Peptide Mass Fingerprinting of iCatG. CatG fragments are analyzed and depicted on chromatograph after trypsin digestion. Actual mass of the fragment is depicted in black, while theoretical sizes are shown in blue.

It should be noted that the peptide with a mass of 1059 corresponds with the sequence LLDQMETPL (See Figure 20), that was consistent to the C terminus of iCatG. Other identified peptide masses were 1159 (RQICVGDRR), 1111 (IFGSYDPRR), 1004 (RQICVGDR), 1174 (VQRDRQCLR), AND 1542 (TIQNDIMLLQLSR) (See Figure 20 and Table 5).

Peak No.	Peak Intensity	Residue Numbers	Segment of Sequence	Experimental Peak Mass (Da)	Theoretical Peak Mass (Da)
1	31663.47	163-170 / 164-171	RQICVGDR / QICVGDRR	1004.52	1003.51
2	29859.97	227-235	LLDQMETPL	1059.28	1059.54
3	31001.19	155-163	IFGSYDPRR	1111.72	1110.57
4	74274.82	163-171	RQICVGDRR	1159.01	1159.61
5	22961.19	146-154	VQRDRQCLR	1174.91	1173.63
6	21041.41	84-96	TIQNDIMLLQLSR *Potential deamidation on asparagine	1542.75	1544.85
7	7953.84	149-162	DRQCLRIFGSYDPR	1726.22	1725.85
8	11766.33	179-199	GDSGGPLLCCNNVAHGIVSYG K	2059.36	2058.01
9	11808.8	36-61	EDFVLTAAHCWGSNINVTLG AHNIQR	3302.54	3301.68

Table 5 MALDI-TOF Peptide Mass Fingerprinting of iCatG.

Column 1, depicts each of the 10 peaks, with peak 1 having 2 possible segments. Column 2, provides peak intensity of fragments. Column 3, is the corresponding residues. Column 4, depicts the residues of the segment of the sequence. Columns 5 and 6, shows the experimental and theoretical sizes of the segments.

Inhibition Studies

Inhibition studies were conducted on iCatG and mCatG preparations for comparison with nCatG using 0.5 mM Suc-VPF-SBzl and Suc-VPK-SBzl substrates using the inhibitors Eglin C (0.00195 nmol/ μ L for the chymotrypsin-like substrate; 0.00002 nmol/ μ L for the trypsin-like substrate), TPCK (7.50 mmol/ μ L), TLCK (2.50 mmol/ μ L), and Bowman-Birk, a soybean trypsin and chymotrypsin inhibitor (Birk, 1985) (0.13 nmol/ μ L for the chymotrypsin-like substrate; 1.27 nmol/ μ L for the trypsin-like substrate)

were used. For comparison, the activities of iCatG and mCatG preparations were diluted with assay buffer to match the activity of 0.22 pmol of nCatG so that 10 μ L of the diluted enzymes could be used in each inhibition assay. The iCatG preparation was diluted 50 μ L into 500 μ L and mCatG was diluted 75 μ L into 525 μ L. Each micro plate well received 10 μ L of each enzyme, followed by 0-50 μ L of inhibitor in assay buffer was added. The total volume of the well was adjusted to 60 μ L with assay buffer then mixed. Incubation of inhibitor for 20 minutes was allowed for binding with the enzyme, followed by an assay of activity through addition of 50 μ L of 1mM substrate in assay buffer. Plates were shaken at high speed for 10 seconds to mix the reagents prior to the first read and were then read every 10 seconds for a total of 10 minutes. The initial velocities of the reactions were determined by linear regression resulting in correlation coefficients of 0.9 or greater. Each of the 3 forms of CatG were assayed for activity with the 4 previously mentioned inhibitors, using both the chymotrypsin and trypsin substrates for a total of 24 inhibition assays and 12 control assays without inhibitor (See Figures 21, 22, 23, 24).

The IC₅₀ data were calculated by graphing initial velocity of each assay (mA405/min) on the y-axis versus inhibitor concentration on the X-axis using Microsoft Excel. These

Inhibition of 50% Activity of CatG Enzymes (nCatG, iCatG and mCatG) by TPCK (MW 351), a chymotrypsin inhibitor using Suc-V-P-F-SBzl Substrates						
CatG type	C terminal	Avg Units of Activity Lost / Quantity of Inhibitor	Quantity of Inhibitor for total inhibition	R2 value of data points	CatG Concentration	IC50 (mMol)
nCatG	none	Max slope 0.26 lost/mmol	375 mmoles	0.8800	20 μ L > 200 μ L; 10 μ L > 1000 μ L	225
Colony B	with 6 His Tag	Max slope 0.24 lost/mmol	330 mmoles	0.9651	50 μ L > 500 μ L;	146
Colony 2	with 13 aa tail	Max slope 0.55 lost/mmol	225 mmoles	0.9693	75 μ L > 525 μ L	111

Table 6 Inhibition of 50% Activity of CatG Enzymes (nCatG, iCatG and mCatG) by TPCK (MW 351), a chymotrypsin inhibitor using Suc-V-P-F-SBzl Substrate. This table summarizing the results from the inhibition analysis with Column 1 depicting the type of CatG used, column 2 showing the type of C terminus associated with each enzyme, column 3 shows the activity lost per inhibitor. Column 4 shows the quantity needed for total inhibition, column 5 shows the R² value, column 6 shows the concentration of the enzyme, and column 7 depicts the IC₅₀ values for each reaction.

plots had correlation coefficients as follows: nCatG (0.88-0.98), iCatG (0.93-0.99), and mCatG (0.77-0.98). The “Quantity of Inhibitor for 50% inhibition” (IC₅₀) was extrapolated from these graphs and plotted as bar graphs (Figures 25, 26, 27, & 28) and recorded in table form along with the “Average Units of Activity Lost per Quantity of Inhibitor” and r² values (See Tables 6, 7, 8, 9).

Inhibition of 50% Activity of CatG Enzymes (nCatG, iCatG and mCatG) by TPCK (MW 351), a chymotrypsin inhibitor using Suc-V-P-K-SBzl Substrates						
CatG type	C terminal	Avg Units of Activity Lost / Quantity of Inhibitor	Quantity of Inhibitor for total inhibition	R2 value of data points	CatG Concentration	IC50 (mMol)
nCatG	none	Max slope 0.30 lost/mmol	300 mmoles	0.9241	20 uL > 200 uL; 10 uL > 1000 uL	125
Colony B	with 6 His Tag	Max slope 0.14 lost/mmol	270 mmoles	0.9487	50 uL > 500 uL;	114
Colony 2	with 13 aa tail	Max slope 0.25 lost/mmol	205 mmoles	0.9188	75 uL > 525 uL	90

Table 7 Inhibition of 50% Activity of CatG Enzymes (nCatG, iCatG and mCatG) by TPCK (MW 351), a chymotrypsin inhibitor using Suc-VPK-SBzl Substrate. This table summarizing the results from the inhibition analysis with Column 1 depicting the type of CatG used, column 2 showing the type of C terminus associated with each enzyme, column 3 shows the activity lost per inhibitor. Column 4 shows the quantity needed for total inhibition, column 5 shows the R² value, Column 6 shows the concentration of the Enzyme, and column 7 depicts the IC₅₀ values for each reaction.

Inhibition of 50% Activity of CatG Enzymes (nCatG, iCatG and mCatG) by Eglin C (MW 5185), a chymotrypsin inhibitor using Suc-V-P-F-SBzl Substrates						
CatG type	C terminal	Avg Units of Activity Lost / Quantity of Inhibitor	Quantity of Inhibitor for total inhibition	R2 value of data points	CatG Concentration	IC50 (pMol)
nCatG	none	Max slope 116.9 lost/picomol	0.713 Picomols	0.9472	20 uL > 200 uL; 10 uL > 1000 uL	0.3
Colony B	with 6 His Tag	Max slope 0.823 lost/picomol	84 Picomols	0.9644	50 uL > 500 uL;	42
Colony 2	with 13 aa tail	Max slope 0.762 lost/picomol	164 Picomols	0.9423	75 uL > 525 uL	72

Table 8 Inhibition of 50% Activity of CatG Enzymes (nCatG, iCatG and mCatG) by Eglin C (MW 5158), a chymotrypsin inhibitor using Suc-V-PF-SBzl Substrate. This table summarizing the results from the inhibition analysis with Column 1 depicting the type of CatG used, column 2 showing the type of C terminus associated with each enzyme, column 3 shows the activity lost per inhibitor. Column 4 shows the quantity needed for total inhibition, column 5 shows the R² value, column 6 shows the concentration of the enzyme, and column 7 depicts the IC50 values for each reaction.

Inhibition of 50% Activity of CatG Enzymes (nCatG, iCatG and mCatG) by Eglin C (MW 5185), a chymotrypsin inhibitor using Suc-V-P-K-SBzl Substrates						
CatG type	C terminal	Avg Units of Activity Lost / Quantity of Inhibitor	Quantity of Inhibitor for total inhibition	R2 value of data points	CatG Concentration	IC50 (pMol)
nCatG	none	Max slope 246.2 lost/picomol	0.325 Picomols	0.9560	20 uL > 200 uL; 10 uL > 1000 uL	0.153
Colony B	with 6 His Tag	Max slope 0.442 lost/picomol	73 Picomols	0.9513	50 uL > 500 uL;	35
Colony 2	with 13 aa tail	Max slope 0.399 lost/picomol	77 Picomols	0.9523	75 uL > 525 uL	36.3

Table 9 Inhibition of 50% Activity of CatG Enzymes (nCatG, iCatG and mCatG) by Eglin C (MW 5158), a chymotrypsin inhibitor using Suc-VPK-SBzl Substrate. This table summarizing the results from the inhibition analysis with Column 1 depicting the type of CatG used, column 2 showing the type of C terminus associated with each enzyme, column 3 shows the activity lost per inhibitor. Column 4 shows the quantity needed for total inhibition, column 5 shows the R² value, column 6 shows the concentration of the enzyme, and column 7 depicts the IC50 values for each reaction.

Inhibition of 50% Activity of CatG Enzymes (nCatG, iCatG and mCatG) by Bowman-Birk (MW 7873), a dual inhibitor using Suc-V-P-F-SBzl Substrates						
CatG type	C terminal	Avg Units of Activity Lost / Quantity of Inhibitor	Quantity of Inhibitor for total inhibition	R2 value of data points	CatG Concentration	IC50 (nMol)
nCatG	none	Max slope 10.34 lost/nanomol	10 nM	0.9786	20 uL > 200 uL; 10 uL > 1000 uL	4.7
Colony B	with 6 His Tag	Max slope 4.692 lost/nanomol	16 nM	0.957	50 uL > 500 uL;	7.6
Colony 2	with 13 aa tail	Max slope 7.991 lost/nanomol	11 nM	0.982	75 uL > 525 uL	5.25

Table 10 Inhibition of 50% Activity of CatG Enzymes (nCatG, iCatG and mCatG) by Bowman-Birk (MW 7873), a dual inhibitor using Suc-V-P-F-SBzl Substrate. This table summarizing the results from the inhibition analysis with Column 1 depicting the type of CatG used, column 2 showing the type of C terminus associated with each enzyme, column 3 shows the activity lost per inhibitor. Column 4 shows the quantity needed for total inhibition, column 5 shows the R² value, column 6 shows the concentration of the enzyme, and column 7 depicts the IC50 values for each reaction.

Inhibition of 50% Activity of CatG Enzymes (nCatG, iCatG and mCatG) by Bowman-Birk (MW 7873), a dual inhibitor using Suc-V-P-K-SBzl Substrates						
CatG type	C terminal	Avg Units of Activity Lost / Quantity of Inhibitor	Quantity of Inhibitor for total inhibition	R2 value of data points	CatG Concentration	IC50 (pMol)
nCatG	none	Max slope 78.93 lost/picomol	1.45 pM	0.9233	20 uL > 200 uL; 10 uL > 1000 uL	0.57
Colony B	with 6 His Tag	Max slope 0.64 lost/nanomol	41 nM	0.9302	50 uL > 500 uL;	21,000
Colony 2	with 13 aa tail	Max slope 1.485 lost/nanomol	37 nM	0.7659	75 uL > 525 uL	13,800

Table 11 Inhibition of 50% Activity of CatG Enzymes (nCatG, iCatG and mCatG) by Bowman-Birk (MW 7873), a dual inhibitor using Suc-V-P-K-SBzl Substrate. This table summarizing the results from the inhibition analysis with Column 1 depicting the type of CatG used, column 2 showing the type of C terminus associated with each enzyme, column 3 shows the activity lost per inhibitor. Column 4 shows the quantity needed for total inhibition, column 5 shows the R² value, column 6 shows the concentration of the enzyme, and column 7 depicts the IC50 values for each reaction.

Inhibition of 50% Activity of CatG Enzymes (nCatG, iCatG and mCatG) by TLCK (MW 369), a trypsin inhibitor using Suc-V-P-F-SBzl Substrate.						
CatG type	C terminal	Avg Units of Activity Lost / Quantity of Inhibitor	Quantity of Inhibitor for total inhibition	R2 value of data points	CatG Concentration	IC50 (mMol)
nCatG	none	Max slope 0.31 lost/mmol	275 mM	0.9374	20 uL > 200 uL; 10 uL > 1000 uL	145
Colony B	with 6 His Tag	Max slope 0.48 lost/mmol	212 mM	0.9315	50 uL > 500 uL;	100
Colony 2	with 13 aa tail	Max slope 0.30 lost/mmol	240 mM	0.9284	75 uL > 525 uL	111

Table 12 Inhibition of 50% Activity of CatG Enzymes (nCatG, iCatG and mCatG) by TLCK (MW 369), a trypsin inhibitor using Suc-V-P-F-SBzl Substrate. This table summarizing the results from the inhibition analysis with Column 1 depicting the type of CatG used, column 2 showing the type of C terminus associated with each enzyme, column 3 shows the activity lost per inhibitor. Column 4 shows the quantity needed for total inhibition, column 5 shows the R² value, column 6 shows the concentration of the enzyme, and column 7 depicts the IC50 values for each reaction.

Inhibition of 50% Activity of CatG Enzymes (nCatG, iCatG and mCatG) by TLCK (MW 369), a trypsin inhibitor using Suc-V-P-K-SBzl Substrate.						
CatG type	C terminal	Avg Units of Activity Lost / Quantity of Inhibitor	Quantity of Inhibitor for total inhibition	R2 value of data points	CatG Concentration	IC50 (mMol)
nCatG	none	Max slope 0.36 lost/mmol	750 mM	0.9666	20 uL > 200 uL; 10 uL > 1000 uL	121
Colony B	with 6 His Tag	Max slope 0.28 lost/mmol	170 mM	0.9933	50 uL > 500 uL;	86
Colony 2	with 13 aa tail	Max slope 0.29 lost/mmol	115 mM	0.8947	75 uL > 525 uL	54

Table 13 Inhibition of 50% Activity of CatG Enzymes (nCatG, iCatG and mCatG) by TLCK (MW 369), a trypsin inhibitor using Suc-V-P-K-SBzl Substrate. This table summarizing the results from the inhibition analysis with Column 1 depicting the type of CatG used, column 2 showing the type of C terminus associated with each enzyme, column 3 shows the activity lost per inhibitor. Column 4 shows the quantity needed for total inhibition, column 5 shows the R² value, column 6 shows the concentration of the enzyme, and column 7 depicts the IC50 values for each reaction.

RESULTS and DISCUSSION

Expression Kinetics

Aliquots of 0.5 mL *Pichia pastoris*-mCatG media supernatant was obtained following centrifugation at 21,000xg for 5 minutes were prior to induction and at 24 hours intervals post-induction. A total of 10 μ L of the media containing enzyme was placed in each well along with 40 μ L of assay buffer for a total volume of 50 μ L. Colonies A and B (mCatG) contained activities of 17.9 mA405/minute/10 μ L and 18.9 mA405/minute/10 μ L, respectively. Colonies 1-4 (iCatG) contained activities of 18.0, 16.4, 14.9, and 16.4 mA405/minute/10 μ L, respectively). The activity from Colonies B and 2 were used to calculate the total units (mA045) for the 50 mL samples, with Colony B having a total activity of 94,500 mA405 and Colony 2 having a total activity of 82,000 mA405 (See Table 10).

Activity of Media Prior to Purification

Sample	Colony B	Colony 2
Volume of enzyme	10	10
Total volume in well	50	50
Dilution	5	5
activity / 10 uL	18.9	16.4
activity / 50 uL	94.5	82
activity / 1 uL	1.89	1.64
Total Volume (uL)	50,000	50,000
Total Units (mA405)	94,500	82,000

Table 14 Activity of Colony B and Colony 2 in the Media After 24 Hours. A table summarizing the Activity of each of the 2 Samples, the volume of enzyme, (Row 1) and their total volume in each well (Row 2); their dilution (Row 3); the activity per Well (Row 4) and their activity per 50 μ L (Row 5) and their Activity per 1 μ L (Row 6); the total volume of enzyme Media (Row 7); and Total Activity Units for the Entire Volume (Row 8).

The negative control of untransformed cells was found to contain only one-third of the activity (5.8 mA405/minute/10 μ L) when compared to the transformed samples. Although all iCatG (1-4) and mCatG (A, B) colonies cleaved the substrate at rates greater than the negative control, Colonies #2, #4 and A, B were selected for optimization of protein expression and were grown and induced in 200 mL baffled flasks

containing 50 mL of Yeast Growth Media under the same conditions as previously stated and were harvested after 4 days for purification. After purification, the 4 active samples (Colonies 2, 4, A, and B) were analyzed on SDS-PAGE producing a protein band at approximately 26 kDa corresponding to the theoretical values of both iCatG (26,758 MW) and mCatG (25,441 MW) (See Figure 19). The results from these two analyses were consistent with CatG enzymatic activity and expected size.

Purification and Confirmation

A 3-step purification procedure was employed, consisting of a combination of stirred cells and centrifugation columns, to concentrate the total volume from 50 mL to 0.5 mL. (See Table 11) Although 10,000 K cutoff was used, a larger pore diameter (20,000 MW) would probably yield a purer product and reduce the overall purification time.

Purification Step Employed on Cathepsin G

Purification Step	Beginning Quantity (mL)	Ending Quantity (mL)
Amicon stirred cell (10K Cutoff)	50	15
10,000 MW cut-off Macrosep column	15	5
10K Nanosep Omega column	5	0.5

Table 15 Purification Steps of CatG Enzymes (nCatG, iCatG and mCatG). A table summarizing the purification steps (column 1) and their Beginning and Ending Quantity of Enzyme (columns 2 and 3). An approximate reduction of over 100 fold was completed through the 3-stage process with a final volume of 500 µL.

The purified samples were analyzed on an SDS-Page gel, as seen in (See Figure 19) with each of the mCatg samples (Colonies A and B) and iCatG (Colonies 2 and 4) being approximately 26 KDa. Colony B band appearing to be more concentrated as compared with Colony A and Colonies 2 and 4 being of approximate concentrations.

Although the samples were not 100% pure, major bands were displayed in the expected size range of 25-30 kDa and a minor diffuse band in the 50 to 60 kDa range, that was approximately twice the size of the major bands. Diffuse bands of higher molecular size were observed with the expression of tryptase (Niles et al. 1998) and with chymase (Lockhart et al. 2005) when expressed by *Pichia pastoris*. These larger, diffuse bands may have resulted from hyper glycosylation (Macauley-Patrick et al. 2005).

A duplicate sample of Colony 4 protein was analyzed on SDS-Page gel and sequenced by MALDI-TOF Mass Fingerprinting after trypsin fragmentation. The resulting peptide masses were compared with the masses predicted by PEPTIDE MASS (ExPASy.org; Wilkins et al. 1997), resulting in 10 fragments, ranging from 8 to 22 residues, and all fragments being within 1 dalton of insilico predicted results. The C-terminal fragment sequence LLDQMETPL, that would be the last nine amino acids cleaved from the iCatG sequence, matched the predicted mass to within 0.26 daltons. The molecular weight of the fragments detected ranged from 1004.52 daltons to 3302.5 daltons and the intensity having a low of 7954 with 6 of the 10 fragments having intensities between 21,000 and 75,000 (See Figure 20 and Table 7).

A percent recovery of enzyme for Colonies 2, 4, A, and B was calculated based on the A_{280} of the purified samples and the extinction coefficient obtained from the

Quantification of Enzyme After Purification

Colony (Form)	A280	Extinction Coefficient	A280/ Ex. Coefficient (mg/mL)	Final Purified Volume (mL)	Total Protein (mg)	% of Total Protein of gel	Total CatG (mg)	Initial Volume of Media (mL)	mg of Protein /L of Media
A (Mature)	0.825	0.952	0.87	0.5	0.43	55.3	0.240	50	4.79
B (Mature)	1.332	0.952	1.40	0.5	0.70	68.0	0.476	50	9.51
2 (Immature)	1.561	0.891	1.75	0.5	0.88	59.1	0.518	50	10.35
4 (Immature)	1.704	0.891	1.91	0.5	0.96	60.8	0.581	50	11.63

Table 16 Quantification of Enzyme After Purification. A table summarizing each enzyme (mature/immature) and Colony along with its A_{280} and extinction coefficient (columns 2 and 3) that are used to calculate its concentration (column 4), the final purified volume of enzyme (column 5), and quantity of total protein (column 6). The percentage of enzyme of each gel band (column 7), the quantity of CatG (column 8), the initial volume of media (column 8) and the quantity of CatG produced per liter of media (column 9) are also displayed.

Expasy website. The concentration of total protein was calculated and the percent purity was determined from the SDS page gel (See Figure 19) using Adobe Photoshop. The total quantity of CatG was then calculated and ranged between 0.24 mg (Colony A) and 0.58 mg (Colony 4). The final volume of purified enzyme was 0.5 mL, reduced from the initial volume of 50 mL of media. After purification, a quantity of enzyme per liter was calculated and found to range of between 4.79 mg/ L (Colony A) and 11.63 mg/ L (Colony 4) (See Table 12).

Kinetic Comparison of iCatG with nCatG

The K_m of iCatG with the substrate Suc-VPF-SBzl was determined for comparison with the published K_m of nCatG with this substrate. The iCatG preparation was diluted 1:2 with water and 1 μ L of enzyme was used in each micro plate well. The substrate concentration was varied from 0 to 0.99 mM in a total assay volume of 150 μ L. Initial rates varied from 0.149 to 58.7 mA405/minute. Analysis of the rate data (J.S. Easterby University of Liverpool, Lineweaver-Burk and Eadie-Hofstee Plots) yielded a K_m of 0.13 mM (Figure 18), that was approximately 7 times higher than the published K_m of 0.019 mM with nCatG (Powers et al. 1989). This experiment, along with the other activity kinetics that were performed, indicated that the extended C-terminus of iCatG increased the substrate binding. Unfortunately, because the iCatG preparation used was not pure the kinetics could be influenced by a contaminating enzyme.

Inhibition Analysis

In order to compare iCatG and mCatG with nCatG, and with each other, inhibition assays were performed using two chloromethyl ketone inhibitors that function as irreversible inhibitors via reaction with the active site histidine of serine (Schoellmann and Shaw, 1963) and cysteine (Walker et al. 1994) proteases. TLCK and TPCK preferentially inhibit trypsin-like and chymotrypsin-like proteases, respectively (Shaw, 1975). In addition to the chloromethylketone inhibitors, two small naturally occurring proteins that inhibit nCatG were examined for inhibition of iCatG and mCatG. The first of these was Eglin C a 70 amino acid protein derived from the leech *Hirudo medicinalis* (Schnebli et al. 1985) that inhibits neutrophil elastase and CatG (Braun et al. 1987).

The second was the Bowman-Birk inhibitor, a small protein with a molecular weight of 7872 that was derived from the soybean (Birk, 1985). Although not shown, all 4 inhibitors totally inhibited all 3 enzymes using both substrates (See Figures 21, 22, 23, 24).

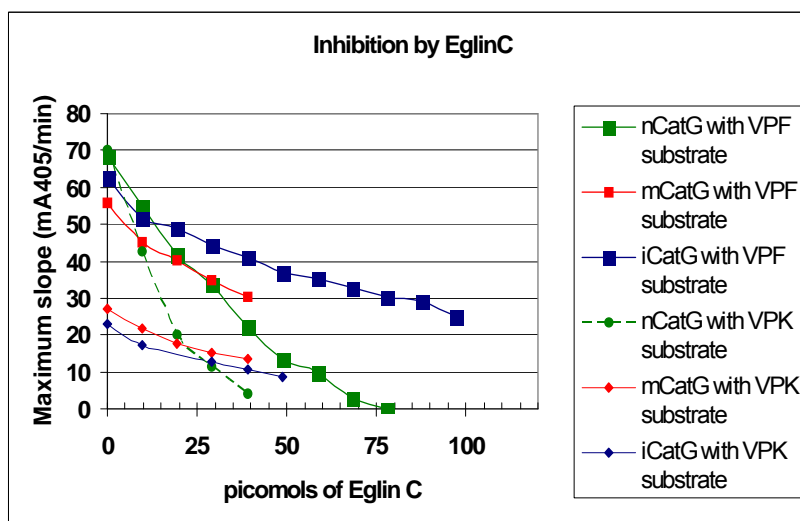


Figure 21 Inhibition of CatG (nCatG, iCatG and mCatG) with EglinC, a “chymotrypsin-like” inhibitor, using 2 different substrates (Suc-VPF-SBzl and Suc-VPK-SBzl) to Determine (IC₅₀). The activity (mA405/minute) was plotted against the increasing concentration (pM) of Inhibitor. The concentration of inhibitor producing a 50% reduction of activity was determined from these data.

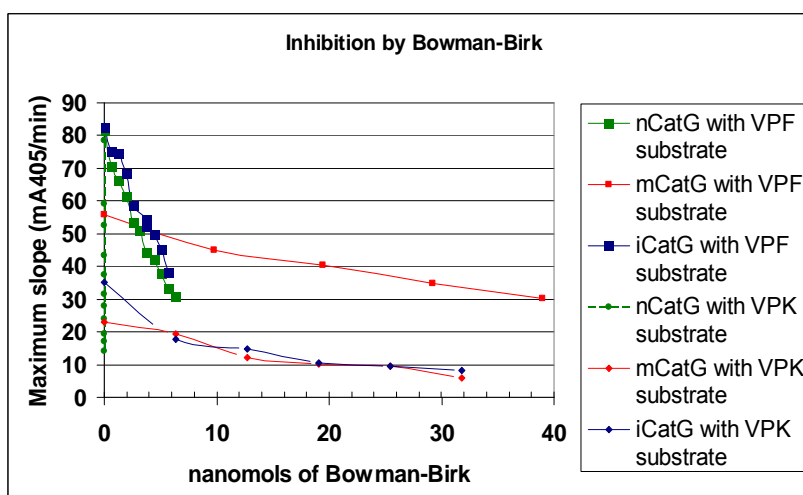


Figure 22 Inhibition of CatG (nCatG, iCatG and mCatG) with Bowman-Birk, a both a trypsin and chymotrypsin enzyme inhibitor, using 2 different substrates (Suc-VPF-SBzl and Suc-VPK-SBzl) to Determine (IC50). The activity (mA405/minute) was plotted against the concentration (nM) of Inhibitor. Concentration of inhibitor was increased, providing a decrease in activity. The concentration of inhibitor producing a 50% reduction of activity was determined from these data.

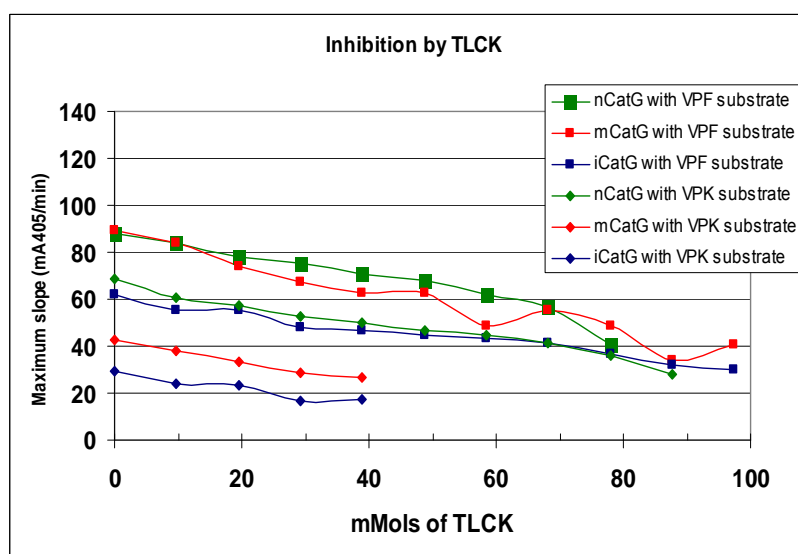


Figure 23 Inhibition of CatG (nCatG, iCatG and mCatG) with TLCK, a “trypsin-like” inhibitor, using 2 different substrates (Suc-VPF-SBzl and Suc-VPK-SBzl) to Determine (IC50). The activity (mA405/minute) was plotted against the concentration (mM) of Inhibitor. Concentration of inhibitor was increased, providing a decrease in activity. The concentration of inhibitor producing a 50% reduction of activity was determined from these data.

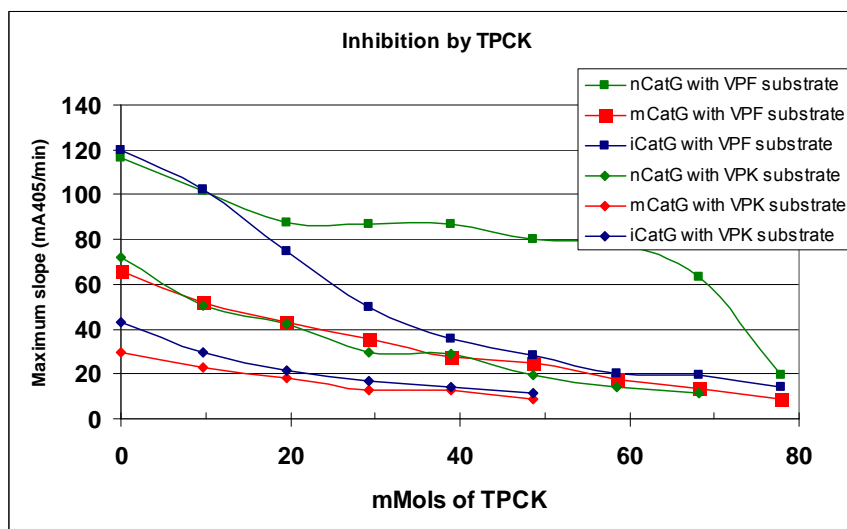


Figure 24 Inhibition of CatG (nCatG, iCatG and mCatG) with TPCK, a “chymotrypsin-like” inhibitor, using 2 different substrates (Suc-VPF-SBzl and Suc-VPK-SBzl) to Determine (IC₅₀). The activity (mA405/minute) was plotted against the concentration (mM) of Inhibitor. Concentration of inhibitor was increased, providing a decrease in activity. The concentration of inhibitor producing a 50% reduction of activity was determined from these data.

Eglin C concentrations needed for 50% inhibition (IC₅₀) of equal amounts of activity were more than 100 fold less for nCatG (0.34 pMols) as compared to mCatG (72 pMols) and iCatG (42 pMols). When inhibition was assayed using the Suc-VPF-SBzl substrate the IC₅₀s for Eglin C with nCatG (0.15 pMols) were more than 200 fold less than those of mCatG (36.3 pMols) and iCatG (35 pMols) (See Figure 25).

TPCK, that inhibits “chymotrypsin-like” enzymes, was similarly effective with each CatG form, resulting in IC₅₀s with nCatG (225 mMols), mCatG (111 mMols), and iCatG (146 mMols), using the Suc-VPF-SBzl substrate. Interestingly, by this measure TPCK was more effective against mCatG and iCatG than against nCatG. However, when using the Suc-VPK-SBzl substrate the IC₅₀s were virtually equal; (nCatG, 125 mMols; mCatG, 90 mMols; iCatG, 114 mMols) (See Figure 26). These data show that a chymotrypsin inhibitor also

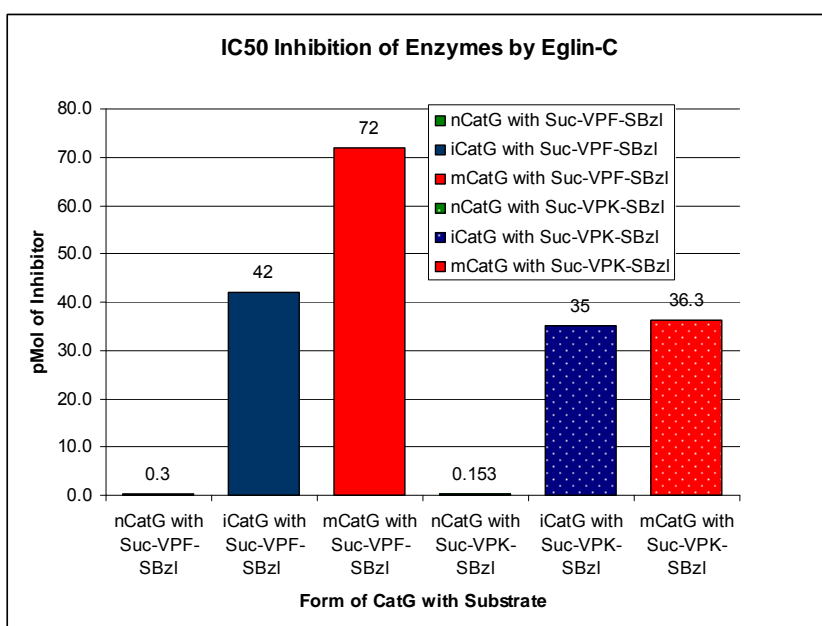


Figure 25 Comparison of IC₅₀ of CatG (nCatG, iCatG and mCatG) with EglinC, a “chymotrypsin-like” enzyme inhibitor. The concentration (pM) of Inhibitor resulting in 50% inhibition of activity, using 2 different substrates (Suc-VPF-SBzl and *Suc-VPK-SBzl*) was compared between enzymes. nCatG is depicted in green histograms; iCatG is depicted in blue histograms; and mCatG is depicted in red histograms.

inhibits the trypsin-like activity of all the CatG enzymes, that indicated that the recombinant enzyme activities were due to CatG rather than a contaminant. The “trypsin-like” inhibitor, TLCK had an IC₅₀ only slightly higher for nCatG (145 mMols) as compared to mCatG (111 mMols) and iCatG (100 mMols), using the Suc-VPF-SBzl substrate. These data showed that a trypsin inhibitor also inhibited the chymotrypsin-like activity of all the CatG enzymes, a further indication that the recombinant enzyme activities were due to CatG rather than a contaminant. Using the Suc-VPK-SBzl substrate (nCatG, 121 mMols; mCatG, 54 mMols; iCatG, 86 mMols) (See Figure 27) the IC₅₀ were again similar.

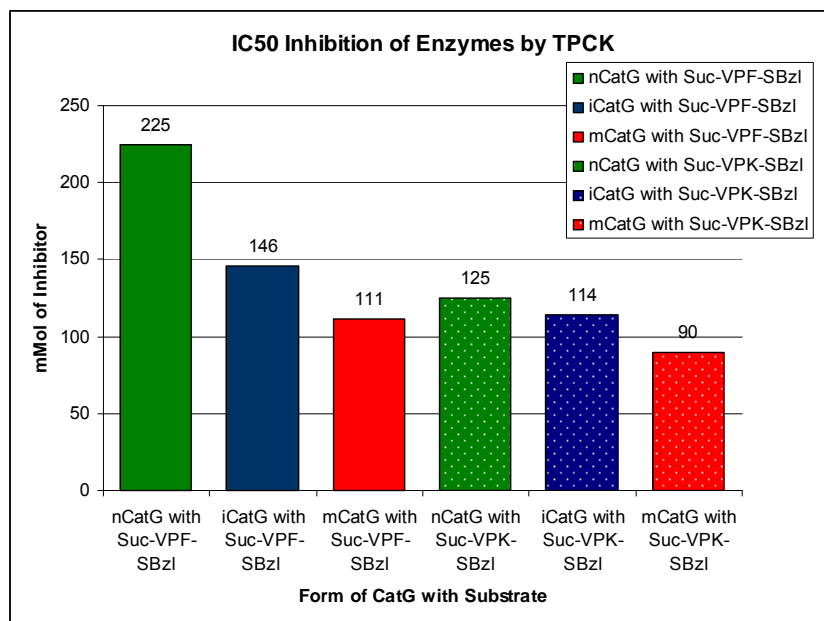


Figure 26 Comparison of IC₅₀ of CatG (nCatG, iCatG and mCatG) with TPCK, a “chymotrypsin-like” inhibitor. The concentration (mM) of Inhibitor resulting in 50% inhibition of activity, using 2 different substrates (Suc-VPF-SBzl and Suc-VPK-SBzl) was compared between enzymes. nCatG is depicted in green histograms; iCatG is depicted blue histograms; and mCatG is depicted in red histograms.

Bowman-Birk, a small compound from *Streptomyces hygroscopicus* with a molecular weight of 604, resulted in the greatest variation in IC₅₀s between recombinant enzymes (nCatG 0.57 pMols; mCatG 13,800 pMols; iCatG 21,000 pMols) using the Suc-VPF-SBzl substrate. However, the variances in IC₅₀ values were much less using the Suc-VPK-SBzl substrate (nCatG, 0.3 pMols; mCatG, 72 pMols; iCatG, 42 pMols) (See Figure 28). Comparisons between the immature and mature forms of the recombinant enzymes found iCatG to have an equal or greater IC₅₀ in 6 out of the 8 inhibition analyses, although more investigation needs to be conducted to determine if there may be a correlation between the 13 amino acid tail and enhanced inhibition. It should also be noted that the recombinant enzymes may have larger IC₅₀s than the native forms due to yeast’s tendency to hyperglycosylate. A second possibility may exist that dextran sulfate, that was added to help stabilize the recombinant enzyme, was still associated with the protein that may occlude specificity of the inhibitor. However, this

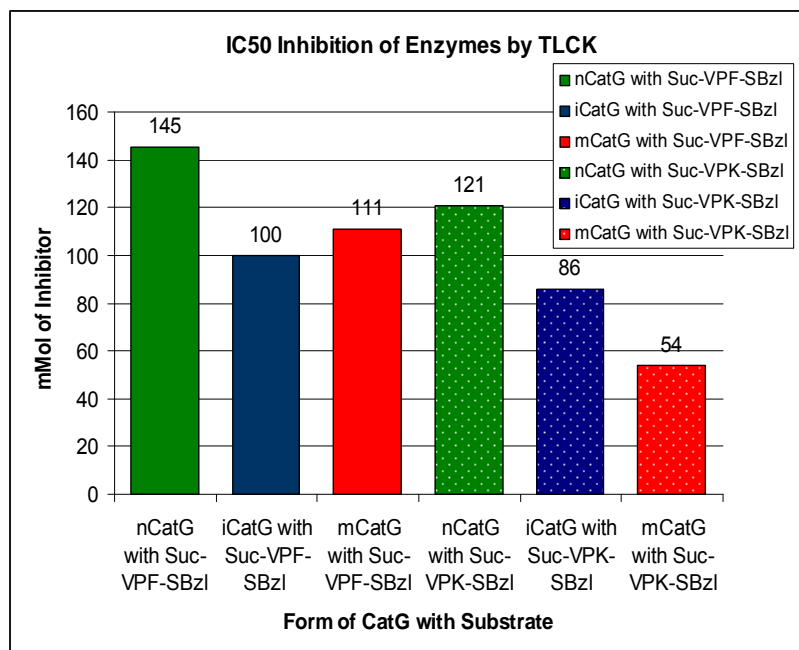


Figure 27 Comparison of IC₅₀ of CatG (nCatG, iCatG and mCatG) with TLCK, a “chymotrypsin-like” inhibitor. The concentration (mM) of Inhibitor resulting in 50% inhibition of activity, using 2 different substrates (Suc-VPF-SBzl and *Suc-VPK-SBzl*) was compared between enzymes. nCatG is depicted in green histograms; iCatG is depicted blue histograms; and mCatG is depicted in red histograms.

analysis also helps to verify that the first active recombinant CatG was produced because the chymotrypsin inhibitor, Bowman-Birk, was able to totally inhibit the activity on both trypsin-like and chymotrypsin-like substrates. The ability of TLCK (trypsin inhibitor) to inhibit the “chymotrypsin trypsin-like” activity using the Suc-VPF-SBzl substrate and both TPCK and Eglin C’s (chymotrypsin inhibitor) ability to inhibit the “trypsin-like” activity against Suc-VPK-SBzl substrate also substantiated the expression of an enzyme with dual specificity.

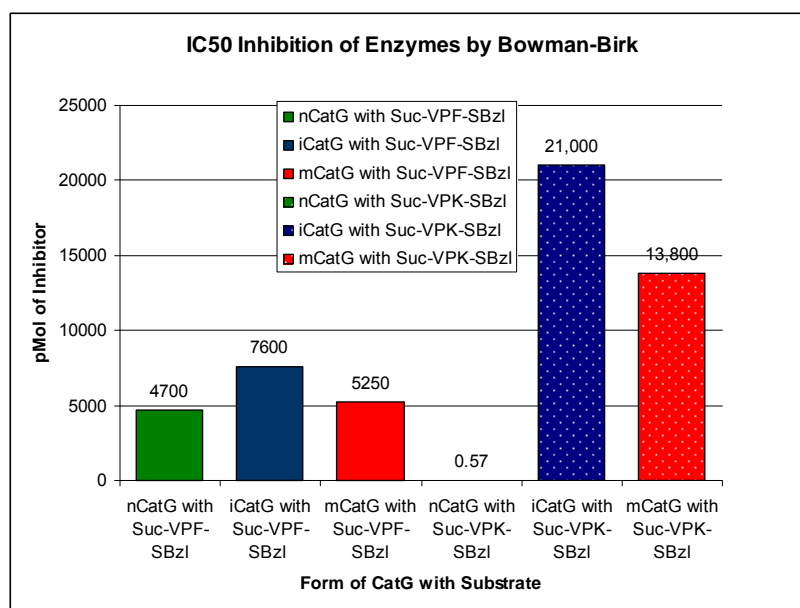


Figure 28 Comparison of IC50 of CatG (nCatG, iCatG and mCatG) with Bowman-Birk, a dual trypsin and chymotrypsin enzyme inhibitor. The concentration (pM) of Inhibitor resulting in 50% inhibition of activity, using 2 different substrates (*Suc-VPF-SBzI* and *Suc-VPK-SBzI*) was compared between enzymes. nCatG is depicted in green histograms; iCatG is depicted in blue histograms; and mCatG is depicted in red histograms.

Concluding Remarks

The findings presented were consistent with the *Pichia*-expressed enzyme activities being due to recombinant CatG in both its immature and mature forms. This highly toxic enzyme, normally expressed as a zymogen and then activated upon entering the azuorphilic granules in neutrophils and cytoplasmic granules in mast cells, has been expressed by transforming *Pichia pastoris*. Although insufficient amounts of enzyme was produced to quantitate final yields, the partial purification protocol consisting of only centrifugation and size exclusion columns could be modified to further enhance enzyme yield. Expression of recombinant CatG eliminated the hazards of working with blood products while providing forms for further investigation may advance understanding of this important enzyme.

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CHAPTER 4

SUMMARY OF EXPRESSION AND PURIFICATION OF RECOMBINANT HUMAN MAST CELL CHYMASE AND CATHEPSIN G

Discussion

This dissertation reports the expression and characterization of recombinant human mast cell chymase and recombinant human cathepsin G, that is found in mast cells and in neutrophils. The expression host for both enzymes was the yeast *Pichia pastoris*.

The work on recombinant chymase (Chapter 2) has been published in the journal *Biotechnology and Applied Biochemistry* (Lockhart et al. 2005). Additionally, this work was presented at the 2004 ETSU Appalachian Research Forum, winning the first place Student Choice Award in Division III, and at the 2004 June meeting of the American Society for Biochemistry and Molecular Biology held in Boston, MA. Chapter 3 has been written with the expectation that it will be submitted for publication.

Both enzymes were proven to be serine proteases with chymotrypsin-like activities; however, cathepsin G has been proven to be unique in that it also cleaved trypsin-like substrates. There were two major advantages in production of these enzymes in the recombinant form. The first advantage was that the availability of recombinant enzymes avoided having to purify these enzymes from blood and tissues, that has been shown to be laborious and potentially a hazardous process. Secondly, site-directed mutagenesis has been found to be possible with recombinant enzymes, that can now allow possible experiments to test the importance of single amino acids in CatG's structure and function. *Pichia pastoris* was chosen as the expression host because it worked well for the expression of human mast cell tryptase (Niles et al. 1998). Additionally, both chymase and cathepsin G are classified as glycoproteins and *Pichia*, a yeast, being eukaryotic, produced post-translational modifications similar though not identical to mammalian cells. Another advantage of expression of these enzymes in *Pichia* was the DNA coding for the recombinant protein was integrated into the host genome, rather than being in a plasmid vector. Whereas bacterial hosts can lose their plasmids, transformed *Pichia* has been shown to be very stable hosts as the genetic information was actually inserted into the genomic DNA by homologous recombination. The media

for growth of *Pichia* has proven to be inexpensive and induction of expression with methanol did not require any special chemicals or conditions. Finally, the pPICz α vector (Invitrogen) allowed for selection of transformed *E. coli* and *Pichia* based on zeocin resistance.

Chymase

Qualification of the experiments validated recombinant expression of chymase at larger quantities than previously documented. The expression of rChymase in *Pichia pastoris* resulted in secretion of the active enzyme at higher levels than previously reported and avoided an activation step required by other expression methods. The Kex2 site of *Pichia pastoris* expression system was located before the N-terminus of the chymase enzyme and activated the rChymase by removing the alpha mating factor fusion used to direct the rChymase for secretion, thus allowing the rChymase to be excreted from the cell without any additional folding or activation.

Purification of rChymase from the culture media was accomplished by hydrophobic interaction chromatography on a Tosopearl butyl column followed by affinity chromatography on Toyopearl AF-heparin. This method yielded at least a 10-fold increase of rChymase than previously reported. The purified rChymase was 96% active based on its reaction with recombinant Eglin C. Changes in media and fermentation conditions may also be explored to improve expression yields. Chymase expression levels achieved the highest documented quantities for an active enzyme. Although other systems had higher expression levels, they were inactive and required an additional activation step. In addition, with the secreted enzyme, the producing cells could potentially be used for a longer period of time because purification of enzyme does not require cell destruction.

The yeast expression system also tended to glycosylate proteins similarly to mammalian systems, thus producing an enzyme more comparable to the human protease. However, *Pichia* also tended to hyperglycosylate proteins, that could be potentially detrimental depending upon application. Future studies may include mutating glycosylation sites or using *Pichia* host strains that have been modified to glycosylate more like mammalian cells.

Cathepsin G

Fully active recombinant human chymase was expressed in larger quantity than had previously been reported in eight publications by other authors. Uniquely, our bioengineering approach coupled with the *Pichia pastoris* host resulted in the secretion of active enzyme, thus avoiding the two laborious tasks of cell lysis and enzyme activation. Through the previously described efforts, the first active recombinant human cathepsin G was expressed in both its immature and mature forms. Previous efforts have been unsuccessful in recombinantly expressing active CatG enzyme that was probably due to antimicrobial activity of CatG (Bangalore et al. 1990; Miyasaki, 1991; Shafer et al. 1991) toward the expression host *E. coli*. An active unregulated CatG protease released intracellularly could potentially destroy structural proteins and even the DNA of cells. Bioengineering of the active protease, along with its attachment to the Kex2 site, was devised to prevent the enzyme from destroying or killing its host. The alpha secretion factor allowed the active enzyme to be regulated internally then secreted externally, thus shielding the cellular machinery from the effects of the protease. However, even the active secreted protein has the ability to externally destroy some cell lines, but *Pichia* was not considered to be one apparently due to the fermentation ability of yeasts. In addition, *Pichia* was able to grow at an approximate pH of 5, that was not considered optimal for CatG activity.

Bioengineering of both an immature and mature version of CatG will allow further research to address the purpose of the C terminal extension and its deletion upon maturation in the granule. The N terminal deletion of CatG has been resolved, but the C terminal deletion has not been determined although several theories have been proposed including attachment, transport, and activation. This research has eliminated the theory that the C terminal was responsible for activity because both forms of CatG were active at comparable levels, that leads one to pursue the concepts of transport or attachment as the most viable options for the extensions. Additional research upon the immature form of CatG can help to resolve this issue.

Through 3D structural and sequence evaluation, it was also determined that CatG possesses “arginine-pairing” especially apparent on its outer residues. This novel design potentially allows ionic interactions to occur with other negatively charged surfaces or residues. The positively charged outer surface was attracted to negatively charged cellular glycoproteins and even the phosphate backbone of DNA. However,

this unique “hyper cationic charge” was also probably detrimental to purification methods as it could bind to silicates in glass and carboxyl groups of filters. Further work needs to be conducted on evaluating an uncharged or positively charged surface of purification products in order to improve expression quantities and yields.

Recombinant production of the serine proteases CatG and chymase reduced the potential dangers associated with handling of blood products as some methods stipulate for protein production. In addition, each of these enzymes was secreted in its active form and does not require any further processing for activity. The potential for the yeasts to hyperglycosylate the proteins could exist as compared to other expression systems; although this does not appear to have happened at least to the extent that activity has been affected.

During expression of both CatG and chymase with the Invitrogen Easy Select Expression System, we were able to determine that the untransformed cell lines (X33, SMD1163) provided us excreted small quantities of a protease with chymotrypsin activity when methanol was added during the induction phase. The enzyme responsible for this activity was probably associated with the methanol breakdown because the activity appears during the induction phase. Surprisingly, even the non-transformed “protease deficient” variant SMD1163 produced activity using the chymotrypsin-like substrate. However, we were able to use a control to eliminate the “background” activity with chymase and were able to exploit CatG’s dual specificity by using a trypsin substrate to overcome this problem. A transformable cell line with the deleted ability to produce an enzyme with chymotrypsin-like activity would be valuable to protease expression research.

Media compositions were also evaluated to identify the optimal growth ingredients while attempting to minimize impurities for enhanced purification. *Pichia* can be grown in several different media types but can be very selective as it may not express the recombinant proteins. Although Invitrogen recommends BMGY/BMMY media that has been shown to be excellent for cell growth, this media contains quantities of unwanted impurities that can hinder purification. CatG expression used YNB media, supplemented with additives (vitamins, trace metals, etc.), appeared to both optimize growth, with A_{600S} of over 200, and expression while reducing the quantity of adulterated materials. The YNB media provided all of the necessary nutrients while simplifying purification.

Dextran sulfate was added to stabilize the enzyme, although we did not confirm its effect on expression or purification. An alternative to dextran sulfate such as albumin, heparin, or even a smaller molecular weight of dextran sulfate could be a substitute if a stabilizer was determined necessary. Another possible explanation for not obtaining a positive Western Blot was that the *Pichia* hyperglycosylated the protein as yeasts tend to do that blocked the antigen recognition site. The activity of both forms of the protease was not affected by the size of dextran sulfate or hyperglycosylation of the protein. A stabilizer was not used for chymase expression and a successful Western Blot was achieved.

The media was introduced onto different gravity fed resins including hydrophobic, anionic, and Traysolol® in an effort to identify a simple but effective method of purification. The experimentation with each of these resins provided for minimal, if any, recovery of the enzyme (Data not shown). The lack of recovery could have been due to hyperglycosylation or, more probably, to the highly charged positive residues attaching themselves to columns or glass containers. Further research should be undertaken to identify uncharged purification materials to determine if resins could be employed. Although the percentage of CatG retained upon purification was not calculated due to low yields, further research in this area could yield results comparable with chymase's 2.2 mg/ L. It was considered that improvement in the simple purification steps employed, such as changing any vessels, flasks, filters, etc to an uncharged or positively charged design could potentially increase the yields of CatG because the levels of activity of this enzyme dissipated after contact on these surfaces.

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