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UNIVERSITY OF CALIFORNIA, IRVINE

In Silico Exploration and Experimental Validation: A Story of Protein Discovery, Aggregation and Degradation

DISSERTATION

submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Chemistry

by

Megha Hemant Unhelkar

Dissertation Committee: Professor Rachel W. Martin, Chair Professor Carter T. Butts Professor Douglas J. Tobias

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DEDICATION

To my parents, Aarti and Hemant Unhelkar, for their constant encouragement, unwavering support and unconditional love.

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- "NMR structure determination and biophysical characterization of J2 crystallin- a unique eye lens protein from the box jellyfish" Unhelkar, M. H.; Khago, D.; Kelz, J; Bierma, J.; Sengupta, S.; Kingsley, C.; Valentic, T. R.; Dizon, P. A.; Martin, R. W. Manuscript in preparation, December 2020

INVITED TALKS

The fascinating story of Drosera capensisNovember 2019Annual Gilliam Biomedical Research Conference, Irvine, CaliforniaMarch 2019Protein Discovery and Characterization using Molecular Modeling, Experimental Chemistry and Bioinformatics
Columbia University in the City of New York, NYMarch 2019Structure Prediction and Network Analysis of Enzymes
from Carnivorous Plant Drosera capensis
Women in Statistics and Data Science Conference, La Jolla, CaliforniaOctober 2017

CONFERENCE PRESENTATIONS

Network-based modeling of amyloid fibril formationMAmerican Chemical Society National Meeting, Philadelphia, PennsylvaniaM	/Iarch 2020
Leveraging molecular modeling, experimental chem- istry and bioinformatics to discover and characterize novel proteinsFebrBiophysical Society Annual Meeting, San Diego, CaliforniaFebr	ruary 2020
Biophysical characterization and solution-state structureAnd the solution state structureture determination using NMR of J2 crystallin: NovelAnd the solution state structureeye lens protein from the box jellyfishAmerican Chemical Society National Meeting, San Diego, California	ugust 2019
NMR structure determination and biophysical charac- terization of J2 crystallin- a unique eye lens protein from the box jellyfish Experimental Nuclear Magnetic Resonance Conference, Asilomar, California	April 2019
Protein Discovery and Characterization using Molec- ular Modeling, Experimental Chemistry and Bioinfor- matics Biophysical Society Annual Meeting, Maryland, BaltimoreMaryland Maryland Maryland Maryland Maryland Maryland Maryland 	Aarch 2019
Design and Construction of a static ssNMR Probe for M the Investigation of Oriented Membrane Samples and Single Crystals Experimental Nuclear Magnetic Resonance Conference, Asilomar, California	Aarch 2017
Design and Construction of ssNMR Probes for the In- vestigation of Oriented Solids and Liquids Rocky Mountain Conference on Magnetic Resonance Breckenridge, Colorada	July 2016

ABSTRACT OF THE DISSERTATION

In Silico Exploration and Experimental Validation: A Story of Protein Discovery, Aggregation and Degradation

By

Megha Hemant Unhelkar Doctor of Philosophy in Chemistry University of California, Irvine, 2020 Professor Rachel W. Martin, Chair

Proteins are fundamental building blocks of life: understanding protein structure, function, aggregation, and degradation is, therefore, one of the central questions in biology. My work investigates protein aggregation and degradation through computational modeling, protein structure network analysis, and experimental verification.

One theme of my work is the discovery of new enzymes from the carnivorous plant, *Drosera* capensis (*D. capensis*). With the ever-expanding genomic data, it is imperative to swiftly move from raw genomic data to chemical results. Using the "target selection pipeline" that we invented, in silico protein structures can be predicted rapidly, to direct the subsequent experimental characterization of the promising candidates. Subsequent network analysis predicts interesting protein properties such as potential enzyme activity, enzyme specificity and the functional pH range, aiding the selection of functionally useful proteins for experimental characterization. This approach illustrates a generally applicable way to leverage the wealth of information provided by whole genome shotgun sequencing for proteomics. Computational techniques, despite their limitations, are now powerful enough to allow potentially useful proteins to be identified directly from the genome and filtered for strong indicators of biochemical function. So far, this work has resulted in three publications including proteases,

chitinases, and esterase/lipases.

The protease resistance of amyloid fibrils and their central role in more than 40 human diseases, including Alzheimer's, makes them an attractive target to test the activity of new proteases from *D.capensis*. To advance and streamline scientific discovery related to amyloid fibrils, it was crucial to have a standardized nomenclature. With collaborators, I introduced a systematic approach to the nomenclature of fibril topology using graph theoretic concepts to abstract the structure. The scheme encompasses all amyloid fibrils currently in the Protein Data Bank (PDB), and can be easily extended to accommodate newer discoveries. The work also showed that the vast majority of known fibril structures fall into just three topological categories, something that was previously unnoticed. My work has improved the discussion of fibril structures by condensing the descriptions of complicated structural features using a set of universal structural motifs.

The other theme of my work includes solving the protein structure of J2 crystallin, an aggregation resistant protein. J2-crystallin is a novel eye lens protein, highly expressed in *Tripedalia cystophora* (box jellyfish) and is an interesting target because of its very high stability and water-solubility. Unlike most non-cephalopod invertebrates, box jellyfish have camera-type eyes; therefore, their crystallins present an interesting system from an evolutionary biology perspective, making them an intriguing model system for vertebrates. Interestingly, Basic Local Alignment Search Tool (BLAST) search of J2 in the Protein Data Bank (PDB) found no proteins above 32 % similarity. Therefore, the structure determination of J2 is not only important from the evolutionary standpoint but also because of the hypothesis that J2 possesses a novel protein fold, due to lack of known homology. Here, I present the biophysical characterization, and solution-state NMR assignments of J2 crystallin, a previously uncharacterized eye lens protein, addressing the interplay of sequence, structure and function in the eye lens crystallins.

Chapter 1

Significance of rapid, *in silico* discovery of novel proteins from the carnivorous plant *Drosera capensis*

Proteins are fundamental building blocks of life: understanding protein structure, function, aggregation, and degradation is, therefore, one of the central questions in biology. It is not only important to discover new proteins as a source to study novel systems but also to expand the toolkit for chemical biology, biotechnology and proteomics. My work investigates protein aggregation and degradation through discovering new protein from the carnivorous plant *Drosera capensis*, using bioinformatics and genomics tools for analysis and testing the activity interesting of the newly discovered enzyme candidates experimentally. One may ask why *D. capensis*? The digestive enzymes of carnivorous plants have been a topic of biological interest at least since Darwin's 1875 monograph on insectivorous plants; in fact, in his book "Insectivorous plants" [17, 18] Darwin says he cares more about *Drosera* than the origin of all the species in the world!

Darwin observed that the mucilage secretions of plants in the genus *Drosera* contained a "ferment" that he conjectured to be similar to mammalian pepsin (now known to be an aspartic protease) [17, 18]. Only recently scientists are beginning to characterize the carnivorous plant digestive enzymes. Before *Drosera capensis*, only two carnivorous plant genomes, *Genlisea aurea* and *Utricularia gibba*, both members of the asterid order Lamiales, were sequenced [19, 20]. Both these plants feed on small, often microscopic, prey and perform their digestive functions in closed traps in a relatively thermostable environment (underground or under water), therefore they are less subject to the environmental constraints faced by carnivorous plants that perform their prey capture in exposed environments [3]. On the other hand, carnivorous plants require stable and highly active digestive enzymes that would allows the plant to digest its prey to its component amino acid over relatively long time spans and usually under milder chemical conditions than those of their animal counterparts [3]. As the digestive process must occur without any mechanical disruption of the prey tissue while competing with bacterial and fungal growth, carnivorous plants are attractive targets for enzyme discovery.

Carnivorous plant digestive enzymes are stable, are substrate specific, possess unique cleavage patterns, and have the ability to function over different pH ranges, presenting a rich resource for chemical biology and biotechnology laboratory applications [3, 21, 2]. We selected the Cape sundew (*Drosera capensis*), native to the Cape region of South Africa and belonging to the order Caryophyllales [3, 21, 2]. *D. capensis* is an excellent model organism for the study of carnivory in plants as it can easily be cultivated, is capable of self-pollination, matures quickly, requires no period of dormancy, and is large and robust, facilitating tissue collection for multiple experiments from the same specimen [3, 21, 2].

The sequencing and assembly of a high-quality draft genome for D. capensis by the Martin lab and the Butts lab can be found in the paper [3] from which a plethora of enzymes were discovered. However, the journey of a protein sequence to an experimentally studied protein takes many years, as observed in Uniprot which has more than 5 million protein sequences without a structure [22]. To study genomics effectively, it is imperative to quickly move from raw data to chemical results. Using the target selection pipeline, *in silico* protein structures can be predicted rapidly, to direct the subsequent experimental characterization of the promising candidates [3, 21, 2]. Subsequent network analysis predicts interesting protein properties such as potential enzyme activity, enzyme specificity and the functional pH range, aiding the selection of functionally useful proteins for experimental characterization. This approach illustrates a generally applicable way to leverage the wealth of information provided by whole genome shotgun sequencing for proteomics. Computational techniques, despite their limitations, are now powerful enough to allow potentially useful proteins to be identified directly from the genome and filtered for strong indicators of biochemical function [3, 21, 2]. So far, this work has resulted in three publications including proteases, chitinases, and esterase/lipases [18, 21, 2]. As the target selection pipeline forms a basis to my PhD, I will be discussing the target selection pipeline (Figure 1.1) in detail in this chapter.

1.0.1 Genomic DNA assembly and gene discovery

Genomic DNA was isolated using a protocol developed for recalcitrant plants by Prof. Rachel Martin [18] and details can be found in the paper.

1.0.2 Feature annotation

Sequence alignments are performed using ClustalOmega [24], with settings for gap open penalty = 10.0 and gap extension penalty = 0.05, hydrophilic residues = GPSNDQERK, and the BLOSUM weight matrix [23]. The presence and position of a signal sequence flagging the protein for secretion was predicted using the program SignalP 4.1 [24], while other localization sequences were identified using TargetP [25]. The alignment figures are annotated

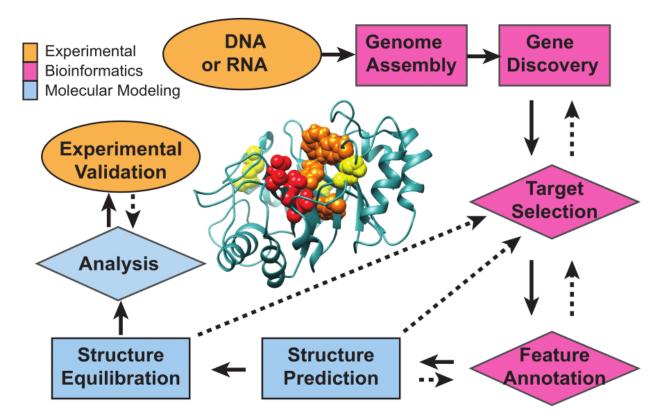


Figure 1.1: Flow chart illustrating the overall strategy for identifying enzymatic targets from genomic DNA. The workflow is indicated with solid arrows, while dotted arrows represent steps where information from a later stage of the pipeline enables refinement of earlier stages in an iterative manner. After genome sequencing, assembly, and gene discovery, target proteins are identified based on putative enzymatic activity. Functional sequence features are identified by analogy to annotation reference sequences found in the UniProt database. Structures are predicted using the Rosetta software, and equilibrated in explicit solvent after removal of sequence regions not present in the mature enzyme. Structures are compared using network analytic methods, enabling strategic selection of enzymes for experimental characterization in a future study. [1, 2, 3]

to highlight chemical properties of the amino acid residues as well as important sequence features. The amino acid attributes are color-coded as follows: cysteines are yellow, positively charged residues are blue, negatively charged residues are red, hydrophobic residues are green, and all others are black [1, 2, 3]. Highly conserved residues are indicated with a dot above the sequence position. The catalytic triad residues are marked with colored arrows. SignalP 4.1 [26] is used to predict the signal peptide cleavage site, which is specified by underlining the residues on either end of the cleavage point. The signal peptide itself is highlighted in light orange. Strikethrough text indicates sequence regions that are absent in the active enzyme, in this case the N-terminal signal peptide that is expressed but removed during maturation. Annotations were performed by homology to the annotations reference sequences from found in the UniProt database and identified by their UniProt IDs. An example of chitinase Family 18 is seen in Figure 2.2 [21]. More details on chitinases can be found in the following chapter.

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DCAP_2209				PVLNLAGHCD PSSGGCTSLT DDINTCO	
C7F821_NEPMI C7F817 9CARY				F PVLNLAGHCD PSSNGCTGLS TDITSCO F PVLNLAGHCD PSSNGCTGLS TDITSCO	
I7HCY7 NEPAL				PVLNLAGHCD PSSNGCIGLS IDIISCO	
C7F818 9CARY				PVLNLAGHCD PSSNGCIGLS IDIISC	
Q06SN0 9CARY				PVLNLAGHCD PSSNGCTGLS TDITSC	
C7F824 9CARY				PVLNLAGHCD PSSNGCTGLS TDITSCO	
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DCAP_2879				/ PQLNLAGHCD PQSGGCVSLA SEIEHCQ	
DCAP_4799				PVLNLAGHCD PSSGGCVALA SEIEYC	
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DCAP 2209				A LNAYGS-VVLSAAP QCPYPDA	
C7F821 NEPMI				LKGYSQSVLVSAAP QCPYPDA	
C7F817 9CARY				A LKGYSQSVLVSAAP QCPYPDA	
17HCY7 NEPAL				A LKGYSQSVLVSAAP QCPYPDA	
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Q06SN0_9CARY	IKVLLSLGGA SGSYSLVSTD DADQVA	YLW NNYLGGQ-SD SRPLGSA	/LD GIDFDIESGS DNYWGDLAT.	A LKNYSQSVLVSAAP QCPYPDA	AHLD
C7F824_9CARY				A LKNYSQSVLVSAAP QCPYPDA	
C7F822_9CARY				A LKGYSQSVLVSAAP QCPYPDA	
C7F819_9CARY				A LKGYSQSVLVSAAP QCPYPDA	
C7F823_NEPGR				A LKGYSQSVLVSAAP QCPYPDA	
CHIT3_VITVI				A LSRIEFQQER GRKVYLTAAP QCPFPDP	
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Figure 1.2: Sequence alignment for Family 18 chitinases, annotated by homology to the reference sequence CHIT3_VITVI. The "DXDXE" motif, in which the acidic residues are marked with red arrows, is imperative for the enzyme activity. Orange arrows indicate residues implicated in substrate binding.

1.0.3 Structure prediction, equilibration and Protein Structure Network (PSN) Analysis

In collaboration with Prof. Carter Butts, we developed the *in silico* maturation method. Preliminary models of the newly discovered enzymes are produced using the online Robetta implementation [27] of Rosetta [28]. The Rosetta structures contain the full sequences, simulated in vacuum, and without any post-transnational modifications. *In silico* maturation matures the preliminary structures obtained from Rosetta, adds the post-translational modifications and chemical changes needed to make initial model match its native chemical environment, which includes, but not limited to pro-sequence removal, protonation state correction, and solvation, and adding disulfide bonds, introducing backbone cuts, oligomerization and metal coordination. During *in silico* maturation, the signal sequence is removed and the structure is equilibrated for 500 ps in explicit TIP3P solvent using NAMD[29], using the CHARMM22 forcefield with the CMAP correction and sodium or chlorine ions were added as necessary to neutralize the charge of the resulting structure [30]. Examples of chitinases, esterase/lipases, phospholipases and proteases are represented in the following chapters.

In addition to the presence or absence of specific features, identifying broader patterns of structural differentiation can be helpful when selecting putative proteins for expression and characterization: proteins within different structural subgroups may differ with respect to other biophysically important properties such as thermal stability, substrate affinity pattern, overall activity, or aggregation propensity, and choosing a structurally diverse sample thus has the potential to maximize the chance of identifying proteins with functionally significant variation [30]. PSNs are a useful tool for such exploration, as they directly represent patterns of potential interaction among chemical groups rather than e.g. side chain dihedral angles or other properties that may vary substantially without inducing significant changes in protein function [30]. PSNs are created for each protein structure by Prof. Carter Butts, using

custom scripts using VMD and statuet tools [18] and more details can be found in the paper [30].

1.0.4 Experimental validation

Once the target enzymes were selected using the target selection pipeline, the enzymes can be expressed and studied experimentally. Projects in the Martin lab include expression of chitinases [21] and proteases [30]. As seen in subsequent chapters, I wanted to test the activity of proteases and I talk in-depth in the following chapters.

Chapter 2

In silico structure prediction and network analysis of chitinases from Drosera capensis

2.1 Background

Chitin, a β -(1,4)-N acetylglucosamine (GlcNAc) biopolymer, is the second-most abundant biopolymer [31]. Chitinases (EC 3.2.1.14) are ubiquitous even among organisms that do not produce chitin, with the latter employing them for purposes of digestion and/or defense. These enzymes cleave chitin at the α -1,4 linkage of N-acetyl glucosamine units, although substantial variation in activity and substrate specificity exists. Some chitinases can also cleave peptidoglycans at β -1,4 linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine, and chitodextrins between N-acetyl-D-glucosamine units. Plant chitinases sometimes have multiple functionalities; some display lysozyme activity [32], while others have a calcium storage function [33]. In humans, chitinases are produced in response to fungal infections, a feature of the innate immune system that is suppressed in immunocompromised individuals, including AIDS patients, transplant recipients, and burn victims [34]. These enzymes and related chitin-binding proteins are expressed in human lung tissue, where they are dysregulated in cystic fibrosis and asthma [35].

In plants, these enzymes are expressed in response to environmental stress and pathogen or pest infestation [36], driving efforts to overexpress particularly effective examples in transgenic crop plants [37]. Carnivorous plants use chitinases as part of the prey capture response: active chitinases have been found in the pitcher fluid of Nepenthes [38, 39], and in the digestive fluids of the Venus flytrap [6]. However, the extent to which chitin is used as a nitrogen source remains controversial. Drosera capensis plants fed on chitin incorporate its nitrogen into their leaf tissue; however nutrient uptake is less efficient than for plants fed on protein [40]. Examination of insect carcasses after digestion reveals that 40-60% of the total nitrogen is unused [41, 42], consistent with the observation that the remains of insect exoskeletons appear mostly intact [43]. However, chitinase expression is upregulated in the presence of prey in the related species Nepenthes alata. In Drosera rotundifolia, an increase in both expression of chitinase mRNA and chitinase activity was induced by addition of crustacean chitin with mechanical stimulation of the traps [44]. The prey-induced induction of chitinase activity, despite the low efficiency of chitin use, may indicate that chitinases primarily function to inhibit fungal growth in the traps, just as cytotoxic peptides discourage microbial growth in the fluid of *Nepenthes* pitchers [45, 46].

In this work, I compare novel chitinases recently discovered from the genome of the Cape sundew (*D. capensis*) [47], to those from other carnivorous plants in order Caryophylalles. The conservation of the overall protein folds and active site architectures suggests that many of the *D. capensis* chitinase sequences form functional enzymes. Using the 'Target Selection Pipeline' described in Chapter 2 which involves sequence analysis, comparative modeling with all-atom refinement followed by *in silico* maturation [48], and investigation of protein

structure networks, structurally distinct subgroups of proteins for subsequent expression and biochemical characterization could be identified. Author contributions can be found in the paper [21]. It is important to understand the comparison of Family 19 and Family 18 and therefore, a portion of the results from the paper [21] are shown in this chapter.

2.2 Materials and Methods

2.2.1 Two Distinct Families of Carnivorous Plant Chitinases Are Found in *D. capensis*

Gene sequences annotated as coding for chitinases using the MAKER-P (v2.31.8) pipeline [49] and a BLAST search against SwissProt (downloaded 8/30/15) and InterProScan [50]were clustered by sequence similarity, along with chitinases previously identified from *Dionaea muscipula* [6] and various species of *Drosera* and *Nepenthes* [51]. Annotated sequence alignments of the Family 18 and Family 19 chitinases are shown in Figure 3.1. We have identified four fragments ranging from 41%-100% identity to the DcChit1_1 fragment previously found by Renner and Specht in *D. capensis* genomic DNA [51] (Figure 2.4). Several well-characterized reference sequences (e.g. chitinases from Vitis vinifera, Brassica napus, and *Hordeum vulgare*) are also included for comparison. Using the characterization scheme of the carbohydrate-active enzymes (CAZy) database [52, 53], the chitinases investigated here belong to Family 18 (orange) or Family 19 (green). Overall, the sequence identity among the Family 18 chitinases from Caryophylalles carnivorous plants is much higher than that of Family 19, as illustrated in Figure 3.1A and B. These two types of chitinases have different folds and are thought to have evolved independently, [54, 55], consistent with their separation into separate clusters (Figure 3.1C). Family 18 contains types III and V, while types I, II and IV belong to Family 19 [6]. My collaborator Vy Doung worked on Family 19. [1].

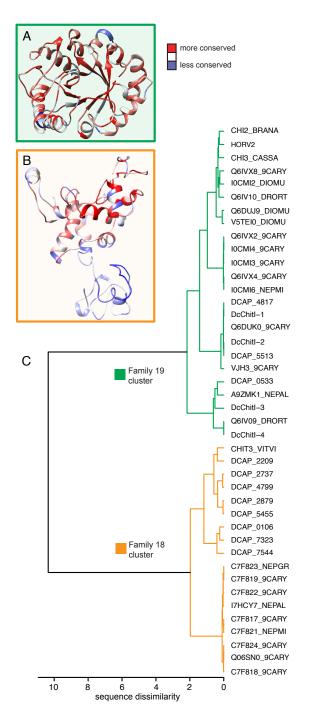


Figure 2.1: Clustering of chitinases identified from the *D. capensis* genome, compared with those from other Caryophylalles carnivorous plants and well-characterized reference sequences. All of the sequences examined belong to GH Families 18 or 19. The sequence dissimilarity used here is the e-distance metric of Székely and Rizzo [4] (with $\alpha = 1$). This parameter is a weighted function of within-cluster similarities and between-cluster differences with respect to a user-specified reference metric, defined here as the raw sequence dissimilarity (1 - (%identity)/100).

2.2.2 Sequence Alignment and Prediction of Putative Protein Structures

Sequences were aligned with ClustalOmega [56] (gap open penalty = 10.0, gap extension penalty = 0.05, hydrophilic residues = GPSNDQERK, weight matrix = BLOSUM). Secretion signal sequences were predicted using Signal 4.1 [26]. Structure prediction was performed as in [47]. In the first stage, the Robetta [28] implementation of Rosetta [27] was used to produce an initial model for each protein. In the second stage, the model was subjected to "in silico maturation." Signal peptides were removed, and disulfide bonds identified by a combination of homology and distance constraints. Protonation states of active site residues were corrected to match literature values where necessary; for Family 18 chitinases, we approximate the sharing of a proton between active site residues D1 and D2 by protonation of D1 (which results in realistic side chain orientations and preserves the attractive interaction between D1 and D2). In the third and final stage, each matured enzyme model was equilibrated in explicit solvent (TIP3P water [57]) under periodic boundary conditions using NAMD [29]. Simulation was performed using the CHARMM36 forcefield [58], with each model being energy-minimized for 10,000 iterations and then simulated at 293K for 500ps; the final protein conformation was retained for subsequent analysis. For the one reference sequence for which a structure was available (HORV2, PDB ID 2BAA, [59]), this was used as the initial starting model (following removal of heteroatoms and protonation using REDUCE [60]). PDB files corresponding to the equilibrated structures for all the proteins discussed in this work available to download and discussed in Table 1 and Table 2.

A sequence alignment for Family 18 chitinases from Caryophylalles carnivorous plants is shown in Figure 2.2. The figure is annotated to highlight specific amino acid properties and important sequence features. The chemical properties of amino acids are color-coded as follows: cysteines are yellow, positively charged residues are blue, negatively charged residues are red, hydrophobic residues are green, and all others are black. Highly conserved residues are indicated with a dot above the sequence position. Cysteine residues involved in structure-stabilizing disulfide bonds are indicated with yellow asterisks, while the active amino acid residues are marked with colored arrows. SignalP 4.1 is used to predict the signal peptide cleavage site, which is specified by underlining the residues on either of the cleavage point. The signal peptide itself is highlighted in light orange. Strikethrough text indicates sequence regions that are absent in the active enzyme, in this case the N-terminal signal peptide that is expressed but removed during maturation. Annotations were performed by homology to a well-characterized acidic endochitinase from *Vitis vinifera* (CHIT3_VITVI, Uniprot ID-P51614).

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DCAP_0106				F PTLNLAGHCD PPSGGCTGLS NDIGTCQSKG
DCAP_7544				S PTLNLAGHCD PPSGGCKSVS NGIRSCQSRG
DCAP_2209				F PVLNLAGHCD PSSGGCTSLT DDINTCQGSN
C7F821_NEPMI C7F817 9CARY				F PVLNLAGHCD PSSNGCTGLS TDITSCQNQG F PVLNLAGHCD PSSNGCTGLS TDITSCQNQG
I7HCY7 NEPAL				PVLNLAGHCD PSSNGCIGLS IDIISCONOG PVLNLAGHCD PSSNGCTGLS TDIISCONOG
C7F818 9CARY				PVLNLAGHCD PSSNGCIGLS IDIISCONOG
Q06SN0 9CARY				PVLNLAGHCD PSSNGCTGLS TDITSCQNQG
C7F824 9CARY				r PVLNLAGHCD PSSNGCTGLS TDITSCQNQG
C7F822 9CARY				F PVLNLAGHCD PSSNGCTGLS TDITSCQNQG
C7F819 9CARY				F PVLNLAGHCD PSSNGCTGLS TDITSCKNQG
C7F823_NEPGR	MKTHYSSAIL PILTLFV FLSINP	HGS GIAVYWGQNG NEGTLSD	CA TGNYNYVLVS FLTTFGNGQ	F PVLNLAGHCD PSSNGCTGLS TDITSCKNQG
CHIT3_VITVI				F PEINLAGHCN PASNGCTSVS TGIRNCQNRG
DCAP_5455				A PQLNLAGHCD PQSGGCVSLA SEIEHCQSIG
DCAP_2879				/ PQLNLAGHCD PQSGGCVSLA SEIEHCQSIG
DCAP_4799				PVLNLAGHCD PSSGGCVALA SEIEYCQSIG
DCAP_2737	MP-MNLNKLF PLILLFPIII PSL-AH	SQG GIAVYWGQNG FEGTLTQ	CN NGTYKYVNLA FLYIFGSGQ	F PVLNLAGHCD PPSGGCVALA SEIKYCQSIG
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DCAP 7544				R LSEIGQQH GKKVYFSAAP QCPFPDQWDD
DCAP 2209				A LNAYGS-VVLSAAP QCPYPDAHLD
C7F821 NEPMI				A LKGYSQSVLVSAAP QCPYPDAHLD
C7F817 9CARY				A LKGYSQSVLVSAAP QCPYPDAHLD
17HCY7 NEPAL				A LKGYSQSVLVSAAP QCPYPDAHLD
C7F818_9CARY	IKVLLSLGGA SGSYSLVSTD DADQVA	YLW NNYLGGQ-SD SRPLGSA	LD GIDFDIESGS DNYWGDLAS	A LKNYSQSVLVSAAP QCPYPDAHLD
Q06SN0_9CARY	IKVLLSLGGA SGSYSLVSTD DADQVA	YLW NNYLGGQ-SD SRPLGSA	LD GIDFDIESGS DNYWGDLAT	A LKNYSQSVLVSAAP QCPYPDAHLD
C7F824_9CARY				A LKNYSQSVLVSAAP QCPYPDAHLD
C7F822_9CARY				A LKGYSQSVLVSAAP QCPYPDAHLD
C7F819_9CARY				A LKGYSQSVLVSAAP QCPYPDAHLD
C7F823_NEPGR				A LKGYSQSVLVSAAP QCPYPDAHLD
CHIT3_VITVI				A LSRIEFQQER GRKVYLTAAP QCPFPDKVPG
DCAP_5455				LARYSAK GKRVHLSAAP QCPFPDAHLG
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DCAP_0106	PVLQTGLIKL VWIQFYNNPE CEYNSG	PSA FQNSWNQWTS SVPASQF	VG LPASPSTGCD GYVDPSDIE VG LPASPSAAGD GYVDPSDVN	S GILPFIKQSE GKYGGIMLWD RGCDIQTGFS
DCAP_0106 DCAP_7544	PVLQTGLIKL VWIQFYNNPE CEYNSG PVLRTGLIDF VWIQFYNNQE CEFKSG	PSA FQNSWNQWTS SVPASQFI PVD FQNSWRKWTS SIPARKFI	VG LPASPSTGCD GYVDPSDIE VG LPASPSAAGD GYVDPSDVN: VG LPASHAAAGD GYVPSALMK:	S GILPFIKQSE GKYGGIMLWD RGCDIQTGFS S QLLPFVQQSG DKYGGVMLWD RGNDIKSGYS
DCAP_0106 DCAP_7544 DCAP_2209	PVLQTGLIKL VWIQFYNNPE CEYNSG PVLRTGLIDF VWIQFYNNQE CEFKSG TAIATGLFNY VWVQFYNNPS CEYVSD	PSA FQNSWNQWTS SVPASQF PVD FQNSWRKWTS SIPARKF -SN LLSSWNQWSP VVKTL	VG LPASPSTGCD GYVDPSDIE VG LPASPSAAGD GYVDPSDVN VG LPASHAAAGD GYVPSALMK LG LPASTDAAGS GYIPPDVLT	S GILPFIKQSE GKYGGIMLWD RGCDIQTGFS S QLLPFVQQSG DKYGGVMLWD RGNDIKSGYS S QVLPSIKGS- SNYGGVMLWN KYYDDGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI	PVLQTGLIKL VWIQFYNNPE CEYNSG PVLRTGLIDF VWIQFYNNQE CEFKSG TAIATGLFNY VWVQFYNNPS CEYVSD KAIATGIFDY VWVQFYNNEQ CEYVND	PSA FQNSWNQWTS SVPASQF PVD FQNSWRKWTS SIPARKF SN LLSSWNQWSP VVKTL -TN LLSAWNQWTS SQ-ANVV	VG LPASPSTGCD GYVDPSDIE VG LPASPSAAGD GYVDPSDVN: VG LPASHAAAGD GYVPSALMK: LG LPASTDAAGS GYIPPDVLT: LG LPASTAAANS GYIPPDVLT:	S GILPFIKQSE GKYGGIMLWD RGCDIQTGFS QLLPFVQQSG DKYGGVMLWD RGNDIKSGYS QVLPSIKAS- SNYGGVMLWS KYYDDGYS QVLPSIKAS- SKYGGVMLWS KYYDDGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY	PVLQTGLIKL VWIQFYNNPE CEYNSG PVLRTGLIDF VWIQFYNNQE CEFKSG TAIATGLFNY VWVQFYNNPS CEYVSD KAIATGIFDY VWVQFYNNEQ CEYVND KAIATGIFDY VWVQFYNNEQ CEYVND	PSA FQNSWNQWTS SVPASQF PVD FQNSWRKWTS SIPARKF SILSSWNQWSP VVKTL TN LLSAWNQWTS SQ-ANVV SQ-ANVV	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAAGD GYVDPSDVN: VG LPASHAAAGD GVVPSALMK: LG LPASTDAAGS GYIPPDVLT: LG LPASTAAANS GYIPPDVLT: LG LPASTAAANS GYIPPDVLT:	S GILPFIKQSE GKYGGIMLMD RGCDIQTGFS S QLLPFVQQSG DKYGGVMLMD RGNDIKSGYS QVLPSIKGS- SNYGGVMLMN KYYDDGYS S QVLPSIKAS- SKYGGVMLMS KYYDNGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY I7HCY7_NEPAL	PULQTGLIKL VWIGFYNNE CEYNSG PVLRTGLIDF VWIGFYNNG CEFKSG TAIATGLENY VWOGFYNNES CEYVSD KAIATGIFDY VWVQFYNNEQ CEYVND KAIATGIFDY VWVQFYNNEQ CEYVSD QAIATGIFDY VWVQFYNNEQ CEYVSD	PSA FQNSWNQWTS SVPASQF PVD FQNSWRKWTS SIPARKF -SN LLSSWNQWSP VVKTL -TN LLSAWNQWTS SQ-ANVV -TN LLSAWNQWTS SQ-ANVV -TN LLSAWNQWTS SQ-ANVV	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDVN: VG LPASHAACD GYVPSDLM: LG LPASTDAACS GYIPPDVLT: LG LPASTAANS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT:	S GLEPFIKQSE GKYGGIMLWD RGCDIQTGFS QLLPFVQQSG DKYGGVMLWD RGNDIKSGYS QVLPSIKRS- SNYGGVMLWN KYYDDGYS S QVLPSIKRS- SKYGGVMLWS KYYDNGYS QVLPSIKRS- SKYGGVMLWS KYYDNGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY 17HCY7_NEPAL C7F818_9CARY	PVLQTGLIKL VWIQFYNNPE CEYNSG PVLRTGLIDF VWIQFYNNPE CEFNSG TAIATGIENY VWQFYNNPS CEFVSD KAIATGIFDY VWVQFYNNPQ CEYVND KAIATGIFDY VWVQFYNNPQ CEYVND QAIATGIFDY VWVQFYNNPQ CEYVND LAIATGIFDY VWVQFYNNPQ CEYVND	PSA FQNSWNQWTS SVPASQF) PVD FQNSWRKWTS SIPARKF) -SN LLSANNQWTS SQ-ANVVI -TN LLSANNQWTS SQ-ANVVI -TN LLSANNQWTS SQ-ANVVI -TN LLSANNQWTS SQ-ANVVI	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAAGD GYVDPSDVN: UG LPASHAAGD GYVPSVNLI LG LPASTDAAGS GYIPPDVLT: LG LPASTDAAGS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYISPDVLT:	S GILPFIKQSE GKYGGIMLMD RGCDIQTGFS QLLPFVQQSG DKYGGVMLMD RGNDIKSGYS QVLPSIKAS- SXYGGVMLMN KYYDDGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS QVLPSIKAS- SKYGGVMLMS KYYDMGYS QVLPSIKAS- SKYGGVMLMS KYYDMGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY I7HCY7_NEPAL C7F818_9CARY Q06SN0_9CARY	PVLQTGLIKL VWIQFYNNPE CEYNSG PVLRTGLIDF VWIQFYNNPE CEFNSG TAIATGLENY VWVQFYNNPS CEFVSD KAIATGIEDY VWVQFYNNPQ CEYVND QAIATGIEDY VWVQFYNNPQ CEYVND LAIATGIEDY VWVQFYNNPQ CEYVND LAIATGIEDY VWVQFYNNPQ CEYVND	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ I-SN LLSSWNQWSP VVKTLJ I-TN LLSANNQWTS SQ-ANVVJ I-TN LLSANNQWTS SQ-ANVVJ I-TN LLSANNQWTS SQ-ANVVJ I-TN LLSANNQWTS SQ-ANVVJ I-TN LLSANNQWTS SQ-ANVVJ	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIM: VG LPASTAAACD GYVPSDIM: LG LPASTDAAGS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYISPDVLT: LG LPASTDAASS GYISPDVLT:	S GILPFIKQSE GKYGGIMLMD RGCDIQTGFS QLLPFVQQSG DKYGGVMLMD RGNDIKSGYS QVLPSIKAS- SKYGGVMLMS KYYDDGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY 17HCY7_NEPAL C7F818_9CARY Q06SN0_9CARY C7F824_9CARY	PULQTGLIKL WWIGFYNNE CEYNSG PVLRTGLIDF VWIGFYNNE CEFKSG TATATGLFNY WWOGFYNNES CEFYSD KATATGIFDY WWOGFYNNES CEFYND KATATGIFDY WWOGFYNNES CEFYND LATATGIFDY WWOGFYNNES CEFYDD LATATGIFDY WWOGFYNNES CEFYDD LATATGIFDY WWOGFYNNES CEFYDD	PEA FONSWNQWTS SVPASOF PVD FONSWRKWTS SIPARKFI ISN LLSSNNQWSP VVKTLI I-TN LLSAWNQWTS SQ-ANVVI I-TN LLSAWNQWTS SQ-ANVVI I-TN LLSAWNQWTS SQ-ANVVI I-TN LLSAWNQWTS SQ-ANVVI I-TN LLSAWNQWTS SQ-ANVVI	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIM: VG LPASHAACD GYVPSDIM: LG LPASTDAACS GYIPPDVLT: LG LPASTDAACS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYISPDVLT: LG LPASTDAASS GYISPDVLT: LG LPASTDAASS GYISPDVLT: LG LPASTDAASS GYISPDVLT:	S GLEPFIKQSE GKYGGIMLWD RGCDIQTGFS QLLPFIKQSE GKYGGVMLWD RGNDIKSGYS QVLPSIKKS- SNYGGVMLWN KYYDDGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY I7HCY7_NEPAL C7F818_9CARY Q06SN0_9CARY	PVLQTGLIKL VWIQFYNNPE CEYNSG PVLRTGLIDF VWIQFYNNPE CEFYSG TALATGLFNY VWQFYNNPG CEFYSD KAIATGIFDY VWVQFYNNPG CEYVND KAIATGIFDY VWVQFYNNPG CEYVND LAIATGIFDY VWVQFYNNPG CEYVND LAIATGIFDY VWVQFYNNPG CEYVND TAIATGIFDY VWVQFYNNPG CEYVND	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -AN LLSAWNQWTS SQ-ANVVJ	VG LPASPSTGCD GYVDPSDUE: VG LPASPSAAGD GYVDPSDVN: UG LPASTAAGD GYVPSVNV: LG LPASTAARS GYIPPDVLT: LG LPASTAANS GYIPPDVLT: LG LPASTAASS GYIPPDVLT: LG LPASTDASS GYIPPDVLT: LG LPASTDAASS GYISPDVLT: LG LPASTDAASS GYISPDVLT: LG LPASTDAASS GYIPPDVLT:	S GILPFIKQSE GKYGGIMLMD RGCDIQTGFS QLLPFVQQSG DKYGGVMLMD RGNDIKSGYS QVLPSIKAS- SXYGGVMLMN KYYDDGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY T7HCY7_NEPAL C7F818_9CARY Q06SN0_9CARY C7F824_9CARY C7F822_9CARY	PVLQTGLIKL VWIQFYNNPE CEYNSG PVLRTGLIDF VWIQFYNNPE CEYNSG TALATGLENY VWVQFYNNPQ CEYVND KAIATGIFDY VWVQFYNNPQ CEYVND QAIATGIFDY VWVQFYNNPQ CEYVND LAIATGIFDY VWVQFYNNPQ CEYVND LAIATGIFDY VWVQFYNNPQ CEYVND TALATGIFDY VWVQFYNNPQ CEYVND TALATGIFDY VWVQFYNNPQ CEYVND	PSA FQNSWNQWTS SVPASQF) PVD FQNSWRKWTS SIPARKF) ISN LLSSWNQWTS SQ-ANVVJ I-TN LLSAWNQWTS SQ-ANVVJ	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIM: VG LPASHAACD GYVPSDIM: LG LPASTDAAGS GYIPPDVLT; LG LPASTDAASG GYIPPDVLT; LG LPASTDAASG GYIPPDVLT; LG LPASTDAASG GYISPDVLT; LG LPASTDAASG GYISPDVLT; LG LPASTDAASG GYISPDVLT; LG LPASTDAASG GYIPPDVLT; LG LPASTDAASG GYIPPDVLT;	S GILPFIKQSE GKYGGIMLMD RGCDIQTGFS QLLPFVQQSG DKYGGVHLMD RGNDIKSGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY I7HCY7_NEPAL C7F818_9CARY Q06SN0_9CARY C7F824_9CARY C7F824_9CARY C7F822_9CARY C7F819_9CARY	PULQTGLIKL VWIQFYNNE CEYNSG PVLRTGLIDF VWIQFYNNE CEYNSG TALATGLENY VWUQFYNNES CEYNSD KALATGIEDY VWUQFYNNEQ CEYNDD KALATGIEDY VWUQFYNNEQ CEYNDD LALATGIEDY VWUQFYNNEQ CEYNDD LALATGIEDY VWUQFYNNEQ CEYNDD TALATGIEDY VWUQFYNNEQ CEYNDD TALATGIEDY VWUQFYNNEQ CEYNDD TALATGIEDY VWUQFYNNEQ CEYNDD	PEA FONSWNQWTS SVPASOF PVD FONSWRKWTS SIPARKFI ISN LLSSNNQWSP VVKTLI I-TN LLSANNQWTS SQ-ANVVI I-TN LLSANNQWTS SQ-ANVVI	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIM: VG LPASHAACD GYVPSDIM: LG LPASTDAACD GYVPSDIM: LG LPASTDAACD GYVPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYISPDVLT: LG LPASTDAASS GYISPDVLT: LG LPASTDAASS GYISPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT:	S GILPFIKQSE GKYGGIMLMD RGCDIQTGFS QLLPFVQQSG DKYGGVMLMD RGNDIKSGYS QVLPSIKAS- SXYGGVMLMN KYYDDGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS QVLPSIKAS- SKYGGVMLMS KYYDNGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY 17HCY7_NEPAL C7F818_9CARY Q06SN0_9CARY C7F824_9CARY C7F822_9CARY C7F822_9CARY C7F819_9CARY C7F823_NEPGR	PVLQTGLIKL VWIQFYNNE CEYNSG PVLRTGLIDF VWIQFYNNEG CEFYSG TALATGLENY VWQFYNNEG CEFYSD KAIATGIFDY VWVQFYNNEG CEYVND KAIATGIFDY VWVQFYNNEG CEYVND LAIATGIFDY VWVQFYNNEG CEYVND LAIATGIFDY VWVQFYNNEG CEYVND TALATGIFDY VWVQFYNNEG CEYVND TALATGIFDY VWVQFYNNEG CEYVND TALATGIFDY VWVQFYNNEG CEYVND TALATGIFDY VWVQFYNNEG CEYVND TALATGIFDY VWVQFYNNEG CEYVND	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ	VG LPASPSTGCD GYVDPSDUE: VG LPASPSAAGD GYVDPSDVN: VG LPASTDAAGD GYVDPSDVN: LG LPASTDAAGS GYIPPDVLT: LG LPASTDAAGS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT:	S GLEPFIKQSE GKYGGIMLWD RGCDIQTGFS QLLPFIKQSE GKYGGVMLWD RGCDIQTGFS QVLPSIKKSS SNYGGVMLWN KYYDDGYS QVLPSIKAS- SKYGGVMLWS KYYDDGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY 17HCY7_NEPAL C7F818_9CARY Q06SN0_9CARY C7F824_9CARY C7F824_9CARY C7F822_9CARY C7F819_9CARY C7F823_NEPGR CHT3_VITVI DCAP_2675 DCAP_2879	PULQTGLIKL WWIQFYNNE CEYNSG PULRGLID WWIQFYNNG CEFKSG RALATGLENY WWQFYNNG CEYVSD KALATGIEDY WWQFYNNEQ CEYVND KALATGIEDY WWQFYNNEQ CEYVND LALATGIEDY WWQFYNNEQ CEYVND LALATGIEDY WWQFYNNEQ CEYVND TALATGIEDY WWQFYNNEQ CEYNND TALATGLEDY WWQFYNNEP CEYLNG TALKTGLEDY WWQFYNNEP CEYLNG	PSA FONSWNQWTS SVPASOF PVD FONSWRKWTS SIPARKFI ISN LLSSNNQWSP VVKTLİ I-TN LLSANNQWTS SQ-ANVVI I-TN LLSANNQWTS SQ-ANVVI ITNN LLSSWRLWDS QRYIRKLİ ITTN LLSSWRLWDS QRYIRKLİ	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIM: VG LPASHAACD GYVPSDIM: LG LPASTDAACD GYVPPDVLT: LG LPASTDAACD GYVPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYISPDVLT: LG LPASTDAASS GYISPDVLT: LG LPASTDAASS GYISPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GFIPANVLT: MG LPAATEAAGS GFIPANVLT: MG LPAATEAAGS GFIPANVLT:	S GLEPFIKQSE GKYGGIMLMD RGCDIQTGFS QLLPFIKQSE GKYGGVMLMD RGCDIQTGFS QVLPSIKKS- SNYGGVMLMN KYYDDGYS QVLPSIKKS- SKYGGVMLMS KYYDDGYS QVLPSIKKS- SKYGGVMLMS KYYDNGYS QVLPSIKKS- SKYGGVMLMS KYYDNGYS QVLPVIKKT- RKYGGVMLYS RYYDTLFGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_SCARY U7HCY7_MEPAL C7F818_9CARY Q06SN0_SCARY C7F824_9CARY C7F824_9CARY C7F824_9CARY C7F823_NEPGR CHIT3_VITVI DCAP_5455 DCAP_4799	PVLQTGLIKL VWIQFYNNPE CEYNSG PVLRTGLIDF VWIQFYNNPE CEYNSG TALATGLENY VWQFYNNPE CEYVSD KAIATGIFDY VWVQFYNNPQ CEYVND KAIATGIFDY VWVQFYNNPQ CEYVND LAIATGIFDY VWVQFYNNPQ CEYVND LAIATGIFDY VWVQFYNNPQ CEYVND TALATGIFDY VWVQFYNNPQ CEYVND TALATGIFDY VWVQFYNNPQ CEYVND TALATGIFDY VWVQFYNNPP CEYLNG TALKTGLEDY VWVQFYNNPP CEYLNG TALKTGLEDY VWVQFYNNPP CEYLNG TALKTGLEDY VWVQFYNNPP CEYLNG	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNLWSS GRYIRKL TTN LLSAWNLWSS GRYIRKL	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIW: VG LPASTAAACD GYVPSDIW: LG LPASTDAACS GYIPPDVLT: LG LPASTDAACS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYISPDVLI LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTAACS GFIPANVLT: MG LPAATEAACS GFIPANVLT: MG LPAATEAACS GFIPTDVLT'	S GILPFIKQSE GKYGGIMLWD RGCDIQTGFS QLLFPVQSG DKYGGVMLWD RGNDIKSGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPVIKKT- RKYGGVMLWS KYYDDQSGYS QVLPVIKKT- RKYGGVMLFS RYYDTLTGYS PVLPVIKKT- RKYGGVMLFS RYYDTLTGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY 17HCY7_NEPAL C7F818_9CARY Q06SN0_9CARY C7F824_9CARY C7F824_9CARY C7F822_9CARY C7F819_9CARY C7F823_NEPGR CHT3_VITVI DCAP_2675 DCAP_2879	PVLQTGLIKL VWIQFYNNPE CEYNSG PVLRTGLIDF VWIQFYNNPE CEYNSG TALATGLENY VWQFYNNPE CEYVSD KAIATGIFDY VWVQFYNNPQ CEYVND KAIATGIFDY VWVQFYNNPQ CEYVND LAIATGIFDY VWVQFYNNPQ CEYVND LAIATGIFDY VWVQFYNNPQ CEYVND TALATGIFDY VWVQFYNNPQ CEYVND TALATGIFDY VWVQFYNNPQ CEYVND TALATGIFDY VWVQFYNNPP CEYLNG TALKTGLEDY VWVQFYNNPP CEYLNG TALKTGLEDY VWVQFYNNPP CEYLNG TALKTGLEDY VWVQFYNNPP CEYLNG	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNLWSS GRYIRKL TTN LLSAWNLWSS GRYIRKL	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIW: VG LPASTAAACD GYVPSDIW: LG LPASTDAACS GYIPPDVLT: LG LPASTDAACS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYISPDVLI LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTAACS GFIPANVLT: MG LPAATEAACS GFIPANVLT: MG LPAATEAACS GFIPTDVLT'	S GLEPFIKQSE GKYGGIMLMD RGCDIQTGFS QLLPFIKQSE GKYGGVMLMD RGCDIQTGFS QVLPSIKKS- SNYGGVMLMN KYYDDGYS QVLPSIKKS- SKYGGVMLMS KYYDDGYS QVLPSIKKS- SKYGGVMLMS KYYDNGYS QVLPSIKKS- SKYGGVMLMS KYYDNGYS QVLPVIKKT- RKYGGVMLYS RYYDTLFGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_SCARY U7HCY7_MEPAL C7F818_9CARY Q06SN0_SCARY C7F824_9CARY C7F824_9CARY C7F824_9CARY C7F823_NEPGR CHIT3_VITVI DCAP_5455 DCAP_4799	PULQTGLIKL WWIQFYNNE CEYNSG PVLRTGLIDF WWIQFYNNG CEFKSG KAIATGIFNY WWQFYNNEG CEFYND KAIATGIFDY WWQFYNNEG CEYVND KAIATGIFDY WWQFYNNEG CEYVND LAIATGIFDY WWQFYNNEG CEYVND LAIATGIFDY WWQFYNNEG CEYVND TAIATGIFDY WWQFYNNEG CEYND TAIATGIFDY WWQFYNNED CEYLNG TALKTGLFDY WWQFYNNEP CEYLNG TALKTGLFDY WWQFYNNEP CEYLNG TALKTGLFDY WWQFYNNEP CEYLNG TALRTGLFDY WWQFYNNEP CEYLNG	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNLWSS GRYIRKL TTN LLSAWNLWSS GRYIRKL	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIW: VG LPASTAAACD GYVPSDIW: LG LPASTDAACS GYIPPDVLT: LG LPASTDAACS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYISPDVLI LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTAACS GFIPANVLT: MG LPAATEAACS GFIPANVLT: MG LPAATEAACS GFIPTDVLT'	S GILPFIKQSE GKYGGIMLWD RGCDIQTGFS QLLFPVQSG DKYGGVMLWD RGNDIKSGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPVIKKT- RKYGGVMLWS KYYDDQSGYS QVLPVIKKT- RKYGGVMLFS RYYDTLTGYS PVLPVIKKT- RKYGGVMLFS RYYDTLTGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F521_NEPMI C7F817_8CARY U7HCY7_NEPAL C7F818_9CARY Q06SN0_9CARY C7F824_9CARY C7F824_9CARY C7F824_9CARY C7F823_NEPGR CHIT3_VITVI DCAP_5455 DCAP_4799 DCAP_2737	PVLQTGLIKL VWIQFYNNPE CEYNSG PVLRTGLIDF VWIQFYNNPE CEYNSG TALATGLENY VWQFYNNPE CEYVSD KAIATGIFDY VWVQFYNNPQ CEYVND KAIATGIFDY VWVQFYNNPQ CEYVND LAIATGIFDY VWVQFYNNPQ CEYVND LAIATGIFDY VWVQFYNNPQ CEYVND TALATGIFDY VWVQFYNNPQ CEYVND TALATGIFDY VWVQFYNNPQ CEYVND TALATGIFDY VWVQFYNNPP CEYLNG TALKTGLEDY VWVQFYNNPP CEYLNG TALKTGLEDY VWVQFYNNPP CEYLNG TALKTGLEDY VWVQFYNNPP CEYLNG	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNLWSS GRYIRKL TTN LLSAWNLWSS GRYIRKL	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIW: VG LPASTAAACD GYVPSDIW: LG LPASTDAACS GYIPPDVLT: LG LPASTDAACS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYISPDVLI LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTAACS GFIPANVLT: MG LPAATEAACS GFIPANVLT: MG LPAATEAACS GFIPTDVLT'	S GILPFIKQSE GKYGGIMLWD RGCDIQTGFS QLLFPVQSG DKYGGVMLWD RGNDIKSGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPVIKKT- RKYGGVMLWS KYYDDQSGYS QVLPVIKKT- RKYGGVMLFS RYYDTLTGYS PVLPVIKKT- RKYGGVMLFS RYYDTLTGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY 17HCY7_NEPAL C7F818_9CARY Q06SN0_9CARY C7F824_9CARY C7F822_9CARY C7F829_9CARY C7F829_9CARY C7F829_9CARY C7F825_DCAP_2879 DCAP_2737	PVLQTGLIKL VWIQFYNNE CEYNSG PVLRTGLIDF VWIQFYNNG CEFKSG TALATGLENY VWQFYNNEG CEYVND KAIATGIEDY VWVQFYNNEG CEYVND QAIATGIEDY VWVQFYNNEG CEYVND LAIATGIEDY VWVQFYNNEG CEYVND LAIATGIEDY VWVQFYNNEG CEYVND TALATGIEDY VWVQFYNNEG CEYVND TALATGIEDY VWVQFYNNEG CEYVND TALATGIEDY VWVQFYNNEG CEYVND TALATGIEDY VWVQFYNNEG CEYVND TALATGIEDY VWVQFYNNEG CEYVND TALATGIEDY VWVQFYNNEG CEYVND TALATGLEDY VWVQFYNNEG CEYVND TALATGLEDY VWVQFYNNEG CEYLNG TALKTGLEDY VWVQFYNNEF CEYLNG TALKTGLEDY VWVQFYNNEF CEYLNG TALKTGLEDY VWVQFYNNEF CEYLNG	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNLWSS GRYIRKL TTN LLSAWNLWSS GRYIRKL	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIW: VG LPASTAAACD GYVPSDIW: LG LPASTDAACS GYIPPDVLT: LG LPASTDAACS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYISPDVLI LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTAACS GFIPANVLT: MG LPAATEAACS GFIPANVLT: MG LPAATEAACS GFIPTDVLT'	S GILPFIKQSE GKYGGIMLWD RGCDIQTGFS QLLFPVQSG DKYGGVMLWD RGNDIKSGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPVIKKT- RKYGGVMLWS KYYDDQSGYS QVLPVIKKT- RKYGGVMLFS RYYDTLTGYS PVLPVIKKT- RKYGGVMLFS RYYDTLTGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY 17HCY7_NEPAL C7F818_9CARY Q06SN0_9CARY C7F824_9CARY C7F824_9CARY C7F824_9CARY C7F823_NEPGR CHIT3_VITVI DCAP_5455 DCAP_2879 DCAP_2737 DCAP_7323 DCAP_7323 DCAP_0106	PVLQTGLIKL VWIQFYNNE CEYNSG PVLRTGLIDF VWIQFYNNG CEFKSG TALATGLENY VWQFYNNEG CEYVND KALATGIEDY VWVQFYNNEG CEYVND QATATGIEDY VWVQFYNNEG CEYVND LALATGIEDY VWVQFYNNEG CEYVND LALATGIEDY VWVQFYNNEG CEYVND TALATGIEDY VWVQFYNNEG CEYVND TALATGIEDY VWVQFYNNEG CEYVND TALATGIEDY VWVQFYNNEG CEYVND TALATGIEDY VWVQFYNNEG CEYVND TALATGIEDY VWVQFYNNEG CEYVND TALATGIEDY VWVQFYNNEG CEYVND TALATGLEDY VWVQFYNNEP CEYLNG TALKTGLEDY VWVQFYNNEP CEYLNG TALKTGLEDY VWVQFYNNEP CEYLNG TALKTGLEDY VWVQFYNNEP CEYLNG TALKTGLEDY VWVQFYNNEP CEYLNG TALKTGLEDY VWVQFYNNEP CEYLNG TALKTGLEDY VWVQFYNNEP CEYLNG	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNLWSS GRYIRKL TTN LLSAWNLWSS GRYIRKL	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIW: VG LPASTAAACD GYVPSDIW: LG LPASTDAACS GYIPPDVLT: LG LPASTDAACS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYISPDVLI LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTAACS GFIPANVLT: MG LPAATEAACS GFIPANVLT: MG LPAATEAACS GFIPTDVLT'	S GILPFIKQSE GKYGGIMLWD RGCDIQTGFS QLLPFVQQSG DKYGGVMLWD RGNDIKSGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPVIKKT- RKYGGVMLYS KYYDDQSGYS QVLPVIKKT- RKYGGVMLFS RYYDTLTGYS PVLPVIKKT- RKYGGVMLFS RYYDTLTGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY 17HCY7_NEPAL C7F818_9CARY Q06SN0_9CARY C7F824_9CARY C7F824_9CARY C7F829_9CARY C7F823_NEPGR CHIT3_VITVI DCAP_5455 DCAP_2879 DCAP_2737 DCAP_7323 DCAP_7323 DCAP_7544	PULQTGLIKL WWIGFYNNE CEYNSG PVLRTGLIDF VWIQFYNNE CEYNSG TAIATGLENY WWQFYNNES CEYNSD KAIATGIENY WWQFYNNES CEYND KAIATGIENY WWQFYNNES CEYND LAIATGIENY WWQFYNNES CEYND LAIATGIENY WWQFYNNES CEYND LAIATGIENY WWQFYNNES CEYND TAIATGIENY WWQFYNNES CEYND TAIATGIENY WWQFYNNES CEYND TAIATGIENY WWQFYNNES CEYND TAIATGIENY WWQFYNNES CEYND TAIATGIENY WWQFYNNES CEYND TAIATGIENY WWQFYNNES CEYND TAIATGLENY WWQFYNNES CEYND	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNLWSS GRYIRKL TTN LLSAWNLWSS GRYIRKL	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIW: VG LPASTAAACD GYVPSDIW: LG LPASTDAACS GYIPPDVLT: LG LPASTDAACS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYISPDVLI LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTAACS GFIPANVLT: MG LPAATEAACS GFIPANVLT: MG LPAATEAACS GFIPTDVLT'	S GILPFIKQSE GKYGGIMLWD RGCDIQTGFS QLLPFVQQSG DKYGGVMLWD RGNDIKSGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPVIKKT- RKYGGVMLYS KYYDDQSGYS QVLPVIKKT- RKYGGVMLFS RYYDTLTGYS PVLPVIKKT- RKYGGVMLFS RYYDTLTGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY 17HCY7_NEPAL C7F818_9CARY Q06SN0_9CARY C7F824_9CARY C7F824_9CARY C7F823_NEPGR CHIT3_VITVI DCAP_9CARY DCAP_4799 DCAP_2737 DCAP_7323 DCAP_7323 DCAP_7544 DCAP_7544 DCAP_7544	PVLQTGLIKL VWIQFYNNDE CEYNSG PVLRTGLIDF VWIQFYNNDE CEYNSG TALATGLENY VWQFYNNDE CEYVND KAIATGIFDY VWQFYNNDE CEYVND KAIATGIFDY VWQFYNNDE CEYVND LAIATGIFDY VWQFYNNDE CEYVND LAIATGIFDY VWQFYNNDE CEYVND TALATGIFDY VWQFYNNDE CEYVND TALATGIFDY VWQFYNNDE CEYVND TALATGIFDY VWQFYNNDE CEYVND TALATGIFDY VWQFYNNDP CEYLNG TALKTGLFDY VWQFYNNDP CEYLNG	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNLWSS GRYIRKL TTN LLSAWNLWSS GRYIRKL	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIW: VG LPASTAAACD GYVPSDIW: LG LPASTDAACS GYIPPDVLT: LG LPASTDAACS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYISPDVLI LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTAACS GFIPANVLT: MG LPAATEAACS GFIPANVLT: MG LPAATEAACS GFIPTDVLT'	S GILPFIKQSE GKYGGIMLWD RGCDIQTGFS QLLPFVQQSG DKYGGVMLWD RGNDIKSGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPSIKAS- SKYGGVMLWS KYYDNGYS QVLPVIKKT- RKYGGVMLYS KYYDDQSGYS QVLPVIKKT- RKYGGVMLFS RYYDTLTGYS PVLPVIKKT- RKYGGVMLFS RYYDTLTGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY 17HCY7_NEPAL C7F818_9CARY Q06SN0_9CARY C7F824_9CARY C7F822_9CARY C7F819_9CARY C7F819_9CARY C7F819_9CARY C7F819_9CARY C7F819_9CARY C7F825_DCAP_8455 DCAP_8455 DCAP_8455 DCAP_7323 DCAP_7323 DCAP_7323 DCAP_7324 DCAP_7244 DCAP_2209	PULQTGLIKL VWIQFYNNE CEYNSG PVLRTGLIDF VWIQFYNNE CEYNSG TAIATGLFNY VWUQFYNNES CEYND KAIATGIFDY VWUQFYNNES CEYND KAIATGIFDY VWUQFYNNES CEYND LAIATGIFDY VWUQFYNNES CEYND LAIATGIFDY VWUQFYNNES CEYND LAIATGIFDY VWUQFYNNES CEYND TAIATGIFDY VWUQFYNNES CEYND	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNLWSS GRYIRKL TTN LLSAWNLWSS GRYIRKL	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIM: VG LPASHAACD GYVPSDIM: LG LPASTDAACD GYVPPDVLT; LG LPASTDAASG GYIPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPASTDAASS GYIPPDVLI; LG LPASTDAASS GYIPPDVLI; LG LPASTDAASS GYIPPDVLI; LG LPASTDAASS GYIPPDVLI; LG LPASTDAASS GYIPPDVLI; LG LPASTDAASS GYIPPDVLI; LG LPASTDAASS GFIPANVLT; MG LPAGPQAAGS GFIPANVLT; LG LPAATQAAGS GFIPTDVLT;	S GLEPFIKQSE GKYGGINLWD RGCDIQTGFS QLLPFIKQSE GKYGGVMLWD RGCDIQTGFS QULPSIK&S- SNYGGVMLWN KYYDDGYS QULPSIK&S- SKYGGVMLWS KYYDNGYS QULPSIK&S- SKYGGVMLWS KYYDNGYS QULPVIKKT- RKYGGVMLWS RYYDDLTGYS QULPVIKKT- RKYGGVMLYS RYYDTLTGYS QULPVIKKT- PKYGGVMLWS RYDDTLTGYS QULPVIKKT- PKYGGVMLWS RYYDTLTGYS
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DCAP_0106 DCAP_7544 DCAP_2209 C7F817_9CARY 17HCY7_NEPAL C7F817_9CARY 006SN0_9CARY C7F824_9CARY C7F822_9CARY C7F822_9CARY C7F822_9CARY C7F823_NEPGR CHIT3_VITVI DCAP_5455 DCAP_2879 DCAP_2737 DCAP_2737 DCAP_7323 DCAP_7323 DCAP_7544 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY I7HCY7_NEPAL C7E919_9CAPY	PULQTGLIKL VWIOFYNNE CEYNSG PVLRTGLIDF VWIOFYNNE CEYNSG TAIATGLENY VWVQFYNNES CEYVSD KAIATGIEDY VWVQFYNNES CEYVND KAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGLEDY VWVQFYNNES CEYVND TAIATGLEDY VWVQFYNNES CEYND TAIATGLEDY VWVQFYNNES CEYND TALKTGLEDY VWVQFYNNES CEYND TALKTGLEDY VWVQFYNNES CEYND MUIGNV SAILGSV SAILSSV SAILSSV SAILSSV SAILSSV	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNLWSS GRYIRKL TTN LLSAWNLWSS GRYIRKL	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIM: VG LPASHAACD GYVPSDIM: LG LPASTDAACD GYVPPDVLT; LG LPASTDAASG GYIPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPASTDAASS GYIPPDVLI; LG LPASTDAASS GYIPPDVLI; LG LPASTDAASS GYIPPDVLI; LG LPASTDAASS GYIPPDVLI; LG LPASTDAASS GYIPPDVLI; LG LPASTDAASS GYIPPDVLI; LG LPASTDAASS GFIPANVLT; MG LPAGPQAAGS GFIPANVLT; LG LPAATQAAGS GFIPTDVLT;	S GLEPFIKQSE GKYGGINLWD RGCDIQTGFS QLLPFIKQSE GKYGGVMLWD RGCDIQTGFS QULPSIK&S- SNYGGVMLWS KYYDDGYS QULPSIK&S- SKYGGVMLWS KYYDNGYS QULPSIK&S- SKYGGVMLWS KYYDNGYS QULPVIK&F- RKYGGVMLWS KYYDDL-GYS QULPVIKKT- RKYGGVMLYS RYYDDLTGYS QULPVIKKT- RKYGGVMLS RYDDLTGYS QULPVIKKT- PKYGGVMLWS RYDDLTGYS QULPVIKKT- PKYGGVMLWS RYDDLTGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F817_9CARY 17HCY7_NEPAL C7F817_9CARY 006SN0_9CARY C7F824_9CARY C7F822_9CARY C7F822_9CARY C7F822_9CARY C7F823_NEPGR CHIT3_VITVI DCAP_5455 DCAP_2879 DCAP_2737 DCAP_2737 DCAP_7323 DCAP_7323 DCAP_7544 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY I7HCY7_NEPAL C7E919_9CAPY	PULQTGLIKL VWIOFYNNE CEYNSG PVLRTGLIDF VWIOFYNNE CEYNSG TAIATGLENY VWVQFYNNES CEYVSD KAIATGIEDY VWVQFYNNES CEYVND KAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGLEDY VWVQFYNNES CEYVND TAIATGLEDY VWVQFYNNES CEYND TAIATGLEDY VWVQFYNNES CEYND TALKTGLEDY VWVQFYNNES CEYND TALKTGLEDY VWVQFYNNES CEYND MUIGNV SAILGSV SAILSSV SAILSSV SAILSSV SAILSSV	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNLWSS GRYIRKL TTN LLSAWNLWSS GRYIRKL	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIVI. VG LPASTAAACD GYVPSDIVI. LG LPASTDAACD GYVPPDVLT: LG LPASTDAASG GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLI: LG LPAATEAACS GFIPANVLT: LG LPAATEAACS GFIPANVLT: LG LPAATQAAGS GFIPTDVLT' LG LPAATQAAGS GFIPTDVLT'	S GLEPFIKQSE GKYGGINLWD RGCDIQTGFS QLLPFIKQSE GKYGGVMLWD RGNDIKSGYS QVLPSIKRS- SNYGGVMLWS KYYDDGYS QVLPSIKRS- SKYGGVMLWS KYYDNGYS QVLPSIKRS- SKYGGVMLWS KYYDNGYS QVLPVIKKT- RKYGGVMLWS KYYDDITGYS QVLPVIKKT- RKYGGVMLYS RYYDTLTGYS QVLPVIKKT- PKYGGVMLWS RYYDTLTGYS QVLPVIKKT- PKYGGVMLWS RYYDTLTGYS QVLPVIKKT- PKYGGVMLWS RYYDTLTGYS QVLPVIKKT- PKYGGVMLWS RYYDTLTGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F817_9CARY 17HCY7_NEPAL C7F817_9CARY 006SN0_9CARY C7F824_9CARY C7F822_9CARY C7F822_9CARY C7F822_9CARY C7F823_NEPGR CHIT3_VITVI DCAP_5455 DCAP_2879 DCAP_2737 DCAP_2737 DCAP_7323 DCAP_7323 DCAP_7544 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY I7HCY7_NEPAL C7E919_9CAPY	PULQTGLIKL VWIOFYNNE CEYNSG PVLRTGLIDF VWIOFYNNE CEYNSG TAIATGLENY VWVQFYNNES CEYVSD KAIATGIEDY VWVQFYNNES CEYVND KAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGLEDY VWVQFYNNES CEYVND TAIATGLEDY VWVQFYNNES CEYND TAIATGLEDY VWVQFYNNES CEYND TALKTGLEDY VWVQFYNNES CEYND TALKTGLEDY VWVQFYNNES CEYND MUIGNV SAILGSV SAILSSV SAILSSV SAILSSV SAILSSV	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNLWSS GRYIRKL TTN LLSAWNLWSS GRYIRKL	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIW: VG LPASHAACD GYVPSDIW: LG LPASTAAANS GYIPPDVLT; LG LPASTAANS GYIPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPASTDAASS GFIPANVLT; MG LPAGPQAAGS GFIPANVLT; LG LPAATQAAGS GFIPTDVLT; LG LPAATQAAGS GFIPTDVLT;	S GLEPFIKQSE GKYGGINLWD RGCDIQTGFS QLLPFIKQSE GKYGGVMLWD RGNDIKSGYS QVLPSIKRS- SNYGGVMLWS KYYDDGYS QVLPSIKRS- SKYGGVMLWS KYYDNGYS QVLPSIKRS- SKYGGVMLWS KYYDNGYS QVLPVIKKT- RKYGGVMLWS KYYDDITGYS QVLPVIKKT- RKYGGVMLYS RYYDTLTGYS QVLPVIKKT- PKYGGVMLWS RYYDTLTGYS QVLPVIKKT- PKYGGVMLWS RYYDTLTGYS QVLPVIKKT- PKYGGVMLWS RYYDTLTGYS QVLPVIKKT- PKYGGVMLWS RYYDTLTGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F817_9CARY 17HCY7_NEPAL C7F817_9CARY 006SN0_9CARY C7F824_9CARY C7F822_9CARY C7F822_9CARY C7F822_9CARY C7F823_NEPGR CHIT3_VITVI DCAP_5455 DCAP_2879 DCAP_2737 DCAP_2737 DCAP_7323 DCAP_7323 DCAP_7544 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY I7HCY7_NEPAL C7E919_9CAPY	PULQTGLIKL VWIOFYNNE CEYNSG PVLRTGLIDF VWIOFYNNE CEYNSG TAIATGLENY VWVQFYNNES CEYVSD KAIATGIEDY VWVQFYNNES CEYVND KAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGLEDY VWVQFYNNES CEYVND TAIATGLEDY VWVQFYNNES CEYND TAIATGLEDY VWVQFYNNES CEYND TALKTGLEDY VWVQFYNNES CEYND TALKTGLEDY VWVQFYNNES CEYND MUIGNV SAILGSV SAILSSV SAILSSV SAILSSV SAILSSV	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNLWSS GRYIRKL TTN LLSAWNLWSS GRYIRKL	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIVI. VG LPASTAAACD GYVPSDIVI. LG LPASTDAACD GYVPPDVLT: LG LPASTDAASG GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLI: LG LPAATEAACS GFIPANVLT: LG LPAATEAACS GFIPANVLT: LG LPAATQAAGS GFIPTDVLT' LG LPAATQAAGS GFIPTDVLT'	S GLEPFIKQSE GKYGGINLWD RGCDIQTGFS QLLPFIKQSE GKYGGVMLWD RGNDIKSGYS QVLPSIKRS- SNYGGVMLWS KYYDDGYS QVLPSIKRS- SKYGGVMLWS KYYDNGYS QVLPSIKRS- SKYGGVMLWS KYYDNGYS QVLPVIKKT- RKYGGVMLWS KYYDDITGYS QVLPVIKKT- RKYGGVMLYS RYYDTLTGYS QVLPVIKKT- PKYGGVMLWS RYYDTLTGYS QVLPVIKKT- PKYGGVMLWS RYYDTLTGYS QVLPVIKKT- PKYGGVMLWS RYYDTLTGYS QVLPVIKKT- PKYGGVMLWS RYYDTLTGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F817_9CARY 17HCY7_NEPAL C7F817_9CARY 006SN0_9CARY C7F824_9CARY C7F822_9CARY C7F822_9CARY C7F822_9CARY C7F823_NEPGR CHIT3_VITVI DCAP_5455 DCAP_2879 DCAP_2737 DCAP_2737 DCAP_7323 DCAP_7323 DCAP_7544 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY I7HCY7_NEPAL C7E919_9CAPY	PULQTGLIKL VWIOFYNNE CEYNSG PVLRTGLIDF VWIOFYNNE CEYNSG TAIATGLENY VWVQFYNNES CEYVSD KAIATGIEDY VWVQFYNNES CEYVND KAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGLEDY VWVQFYNNES CEYVND TAIATGLEDY VWVQFYNNES CEYND TAIATGLEDY VWVQFYNNES CEYND TALKTGLEDY VWVQFYNNES CEYND TALKTGLEDY VWVQFYNNES CEYND MUIGNV SAILGSV SAILSSV SAILSSV SAILSSV SAILSSV	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNLWSS GRYIRKL TTN LLSAWNLWSS GRYIRKL	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIM: VG LPASTAAACD GYVPSDIM: LG LPASTAAACD GYVPPDVLT; LG LPASTAAACD GYVPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPASTDAASS GFIPANVLT; MG LPAGPQAAGS GFIPANVLT; LG LPAATQAAGS GFIPTDVLT; LG LPAATQAGS GFIPTDVLT; LG LPAATQ	S GLEPFIKQSE GKYGGINLWD RGCDIQTGFS QLLPFIKQSE GKYGGVNLWD RGCDIQTGFS QULPSIKSS SNYGGVNLWS KYYDDGYS QULPSIKSS SNYGGVNLWS KYYDDGYS QULPSIKSS SKYGGVNLWS KYYDNGYS QULPSIKSS SKYGGVNLWS KYYDN-GYS QULPYIKKT PKYGGVNLS RYYDTLTGYS QULPVIKKT PKYGGVNLS RYDTLTGYS QULPVIKKT PKYGGVNLS RYYDTLTGYS QULPVIKKT PKYGGVNLWS RYDTLTGYS
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DCAP_0106 DCAP_7544 DCAP_2209 C7F817_9CARY 17HCY7_NEPAL C7F817_9CARY 006SN0_9CARY C7F824_9CARY C7F822_9CARY C7F822_9CARY C7F822_9CARY C7F823_NEPGR CHIT3_VITVI DCAP_5455 DCAP_2879 DCAP_2737 DCAP_2737 DCAP_7323 DCAP_7323 DCAP_7544 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY I7HCY7_NEPAL C7E919_9CAPY	PULQTGLIKL VWIOFYNNE CEYNSG PVLRTGLIDF VWIOFYNNE CEYNSG TAIATGLENY VWVQFYNNES CEYVSD KAIATGIEDY VWVQFYNNES CEYVND KAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND LAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGIEDY VWVQFYNNES CEYVND TAIATGLEDY VWVQFYNNES CEYVND TAIATGLEDY VWVQFYNNES CEYND TAIATGLEDY VWVQFYNNES CEYND TALKTGLEDY VWVQFYNNES CEYND TALKTGLEDY VWVQFYNNES CEYND MUIGNV SAILGSV SAILSSV SAILSSV SAILSSV SAILSSV	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSSWNQWFS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNLWSS GRYIRKL TTN LLSAWNLWSS GRYIRKL	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIE: VG LPASPSAACD GYVDPSDIW: LG LPASTAAACD GYVPSDIW: LG LPASTAAACD GYVPPDVLT: LG LPASTAANS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLT: LG LPASTDAASS GYIPPDVLI: LG LPASTDAASS GYIPPDVLI: LG LPASTDAASS GYIPPDVLI: LG LPASTAASS GYIPPDVLI: LG LPASTAASS GYIPPDVLI: LG LPASTAASG GFIPANVLT: MG LPAATEAACS GFIPANVLT: MG LPAATEAACS GFIPANVLT: LG LPAATQAAGS GFIPTDVLT' LG LPAATQAAGS GFIPTDVLT'	S GLEPFIKQSE GKYGGINLWD RGODIQTGFS QLLPFIKQSE GKYGGVMLWD RGODIKSGYS QULPSIKSS- SNYGGVMLWS KYYDDGYS QULPSIKSS- SNYGGVMLWS KYYDDGYS QULPSIKSS- SKYGGVMLWS KYYDNGYS QULPSIKSS- SKYGGVMLWS KYYDNGYS QULPVIKKT- RKYGGVMLWS KYYDDLTGYS QULPVIKKT- RKYGGVMLYS RYYDDLTGYS QULPVIKKT- RKYGGVMLS RYYDTLTGYS QULPVIKKT- PKYGGVMLWS RYYDTLTGYS QULPVIKKT- PKYGGVMLWS RYYDTLTGYS QULPVIKKT- PKYGGVMLWS RYYDTLTGYS QULPVIKKT- PKYGGVMLWS RYYDTLTGYS
DCAP_0106 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F817_9CARY Q06SN0_9CARY C7F824_9CARY C7F824_9CARY C7F822_9CARY C7F829_9CARY C7F829_9CARY C7F829_9CARY C7F829_9CARY C7F829_9CARY C7F825 DCAP_2879 DCAP_2737 DCAP_7323 DCAP_7323 DCAP_7323 DCAP_7323 DCAP_7544 DCAP_2209 C7F821_NEPMI C7F818_9CARY C7F818_9CARY C7F828_9CARY C7F824_9CARY C7F829_9CARY C7F829_9CARY C7F829_9CARY C7F823_NEPGR CHT3_VITVI DCAP_5455	PULQTGLIKL VWIGFYNNE CEYNSG PVLRTGLIDF VWIGFYNNE CEYNSG TAIATGLENY VWUGFYNNES CEYND KAIATGIFDY VWUGFYNNES CEYND CAIATGIFDY VWUGFYNNES CEYND LAIATGIFDY VWUGFYNNES CEYND LAIATGIFDY VWUGFYNNES CEYND TAIATGIFDY VWUGFYNNES CEYND SAIKTGLEDY VWUGFYNNES CEYND SAIKDSV	PSA FQNSWNQWTS SVPASQFJ PVD FQNSWRKWTS SIPARKFJ -SN LLSSWNQWFS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ -TN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNQWTS SQ-ANVVJ TTNN LLSAWNLWSS GRYIRKL TTN LLSAWNLWSS GRYIRKL	VG LPASPSTGCD GYVDPSDIE: VG LPASPSAACD GYVDPSDIM: VG LPASPSAACD GYVDPSDIM: LG LPASTAAACD GYVPPDVLT; LG LPASTAAACD GYVPPDVLT; LG LPASTDAASS GYIPPDVLT; LG LPAATDAASS GFIPANVLT; LG LPAATDAASS GFIPANVLT; LG LPAATQAAGS GFIPTDVLT; LG LPAATQAGS GFIPTDVLT; LG LPAATQ	S GLEPFIKQSE GKYGGINLWD RGODIQTGFS QLLPFIKQSE GKYGGVMLWD RGODIKSGYS QULPSIKSS- SNYGGVMLWS KYYDDGYS QULPSIKSS- SNYGGVMLWS KYYDDGYS QULPSIKSS- SKYGGVMLWS KYYDNGYS QULPSIKSS- SKYGGVMLWS KYYDNGYS QULPVIKKT- RKYGGVMLWS KYYDDLTGYS QULPVIKKT- RKYGGVMLYS RYYDDLTGYS QULPVIKKT- RKYGGVMLS RYYDTLTGYS QULPVIKKT- PKYGGVMLWS RYYDTLTGYS QULPVIKKT- PKYGGVMLWS RYYDTLTGYS QULPVIKKT- PKYGGVMLWS RYYDTLTGYS QULPVIKKT- PKYGGVMLWS RYYDTLTGYS
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Figure 2.2: Sequence alignment for Family 18 chitinases, annotated by homology to the reference sequence CHIT3_VITVI. The "DXDXE" motif, in which the acidic residues are marked with red arrows, is imperative for the enzyme activity. Orange arrows indicate residues implicated in substrate binding.

Family 19 contains Class I, II, and IV chitinases, all of which are characterized by an anomeric inverting mechanism [61, 62]. Annotations for the Family 19 chitinases are shown in Figure 2.3. Family 19 contains Class I, II, and IV chitinases, all of which are characterized by an anomeric inverting mechanism [61, 62]. The N-terminal chitin-binding domain is present in Class I and absent in Class II, which are otherwise similar in sequence. Family 19 chitinases from plants have in common a catalytic domain with an active glutamic acid residue. The active site motif surrounding the active E is either HETT (type I and II) or HETG (type IV) [51], both of which are observed in this set of proteins. Annotations for the Family 19 chitinases are shown in Figure 2.3. Amino acid and sequence features are indicated as in Figure 2.2, with the following additions, when present: the C-rich domain is highlighted in light green, the P-rich hinge in light blue, and the C-terminal extension (CTE) in light gray. Both the C-rich domain and the P-rich hinge are highly variable in length and are absent in some sequences. Only three chitinases in this set contain the CTE, which targets those sequences to the vacuole. The reference sequences for this cluster are CHI3_CASSA (*Castanea sativa*), CHI2_BRANA (*Brassica napus*), and HORV2 (*Hordeum vulqare*).

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Q6IV09_DRORT										
CHI3_CASSA										
Q6IVX8_9CARY			MNAPCFCFHA	QKMRNHKH	STMRG	WVVLLLLNLP	-FLSAFQC	GQQAGGALCH	SGLCCSQWGW	CGTTSDYCG-
V5TEI0_DIOMU					MRN	WVILLICISE	FERRELAEOC	GSOAGGALCP	NGLCCSOYGW	CGTTSAYCG-
Q6DUJ9_DIOMU					MGN	WVLLLLCISP	-FFSRTLAEQC	GSQAGGALCP	NGLCCSQYGW	CGATSAYCG-
VJH3_9CARY					<mark>MKI</mark> -	SLLLLEVVP	-LLSGTSAQQC	GYQAGGALCP	NGLCCSKYGY	CGTTSAYCG-
DCAP_5513 Q6DUK0 9CARY					MR1-	-PILLEBCVAP	-LLSGTYAVQC	GSEVGGALCP	NGLCCSKYGY	CGTTSAYCG-
DCAP 4817									NGLCCSKYGY	
HORV2								GOLVGGALOT	NGDCCSRTGT	COTTORICO
CHI2_BRANA						CLLLFLIFSF	LISFSLAEOC	GROAGGALCP	NGLCCSEFGW	CGDTEAYCKO
Q6IV10_DRORT										
I0CMI2_DIOMU										
IOCMI3_9CARY										
IOCMI4_9CARY										
IOCMI6_NEPMI										
Q6IVX2_9CARY				M					GGLCCSQYGW	
Q6IVX4_9CARY				M					GGLCCSQYGW	
DCAP_0533	MTIVHAS	TNKAKLDFSF	-FSLPYTSLQT	PKLYNYKNMA	-LSIKIKTH	-FAIIFIIIVV	-FLSSSLAQDC	GCD	SSLCCSQYGY	CGTSDAYCG-
A9ZMK1_NEPAL						-AF-LAGILAGV	-PENTARÓNC	GCA	ANLCCSKWGY	CGTGDAICG-
	estes str						*			
Q6IV09_DRORT	••••	• •••	• •••		•		* ••	••	•	• •••
CHI3 CASSA	AGCOSOCSSP	TT	TTSSP	TASSGGGGDV	GSLISASLFD	OMLKYRNDPR	CKSNGFYTYN	AFIAAAR-SF	NGFGTTGDVT	TRKRELAAFL
Q6IVX8_9CARY			PPPPATPSPP							
v5tei0_diomu			PP							
Q6DUJ9_DIOMU	AGCQSQCGGS	S	PP	SPSGGGDV	GSIVTNEIFN	QMLLHRNDNA	CPANGFYTYN	AFIEAAR-SF	PGFGTTGDVN	TQRKELAAFF
VJH3_9CARY	DGCQSQCGGS	SPPPAPP-	SPP	SPSGGG <mark>D</mark> V	SSIITSQIFN	QMLLHRNDNA	CPANGFYSYQ	AFINAAR-KF	SGFGTTGDTN	TRKNELAAFF
DCAP_5513	PGCQSQCGGS	SPPPAPPS	PTPSPP	SPSGGGDV	SSIITSQIFN	QMLLHRNDNA	CPANGFYSYQ	AFLDAAR-KF	SGFGTTGDIN	TRKKELAAFF
Q6DUK0_9CARY										
DCAP_4817	PGCQSQCGGS	SPPPAPPS	PTPSPP							
HORV2	DCC00000000000000000000000000000000000								PGFGTTGSAD	
CHI2_BRANA	PGCQSQCGGT	r		PGPTGDL	SGIISRSQFD	DMLKHRNDNA	CPARGFYTYD	AFINAAK-SF	rererTGDTA	IRANELAAFF
Q6IV10_DRORT I0CMI2_DIOMU						-MIKHPND2.3	COGRGEVEN	AFTABAK-CT	PQFGTTGSAE	TRKRETAAFT
IOCMI2_DIOMO					FN	OMLKHRNDGG	CPAKGEYTYD	AFIAAAK-SF	PAFAATGDAA	TREETAAFI.
IOCMI4 9CARY										
IOCMI6_NEPMI										
Q6IVX2_9CARY	AGCQSQCSFS			GGDP	SSLVTRDKFN	QMLKHRNDGG	CPAKGFYTYD	AFIAAAK-SF	PAFAATGDAA	TRKREIAAFL
Q6IVX4 9CARY	AGCOSOCSES			GGDP	SSLVTRDKEN	OMLKHENDGG	CPAKGEYTYD	AFTAAAK-SF	PAFAATGDAA	TRKRETAAFL
DCAP_0533	VGCQEGPCKS	AVN		NTKNDVSV	PDVVSD AFFN	GII-DQAAST	CEGIGFYSRA	GFLSAWESNY	TDFGTTGSVE	ESLREIAAFF
A9ZMK1_NEPAL	PGCQEGPCYS	SG		GGGSSV	ADIVTDSFFD	GII-NQASSS	CAGKYFYSRS	AFLDALD-SY	PAFGTSSDAD	TNKQEIAAFF
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Q6IV09_DRORT			GGYCFVRQND			-SDRYYGRGP	IQLTNRNNYE			
CHI3_CASSA	AQTSHETTGG	WATAPDGPYA	WGYCFVMENN	KQTYC	R-S-KSWPCV	-SDRYYGRGP FGKQYYGRGP	IQLTNRNNYE IQLTHNYNYG	QAGKAIGADL	INNPDLVATN	PTISFKTAIW
CHI3_CASSA Q6IVX8_9CARY	AQTSHETTGG GQTSHETTGG	WATAPDGPYA WATAPDGPYA	WGYCFVMENN WGYCFKEEVG	KQTYC QPGSYC	R-S-KSWPCV VPS-TQWPCA	-SDRYYGRGP FGKQYYGRGP AGKSYYGRGP	IQLTNRNNYE IQLTHNYNYG IQLSYNYNYG	QAGKAIGADL PSGQAIGQPL	INNPDLVATN LENPDLVAGD	PTISFKTAIW VIVSFETAIW
CHI3_CASSA Q6IVX8_9CARY V5TEI0_DIOMU	AQTSHETTGG GQTSHETTGG GQTSHETTGG	WATAPDGPYA WATAPDGPYA WPTAPDGPYA	WGYCFVMENN WGYCFKEEVG WGYCFKQEQG	KQTYC QPGSYC NPPSYC	R-S-KSWPCV VPS-TQWPCA EPS-TAYPCA	-SDRYYGRGP FGKQYYGRGP AGKSYYGRGP PGKSYYGRGP	IQLTNRNNYE IQLTHNYNYG IQLSYNYNYG IQISYNYNYG	QAGKAIGADL PSGQAIGQPL QCGDSIGQPL	INNPDLVATN LENPDLVAGD LANPDLVADD	PTISFKTAIW VIVSFETAIW ALISFETAIW
CHI3_CASSA Q6IVX8_9CARY	AQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG	WATAPDGPYA WATAPDGPYA WPTAPDGPYA WPTAPDGPYA	WGYCFVMENN WGYCFKEEVG	KQTYC QPGSYC NPPSYC NPPSYC	R-S-KSWPCV VPS-TQWPCA EPS-TAYPCA EPS-TAYPCA	-SDRYYGRGP FGKQYYGRGP AGKSYYGRGP PGKSYYGRGP PGKSYYGRGP	IQLTNRNNYE IQLTHNYNYG IQLSYNYNYG IQISYNYNYG IQISYNYNYG	QAGKAIGADL PSGQAIGQPL QCGDSIGQPL QCGDSIGQPL	INNPDLVATN LENPDLVAGD LANPDLVADD LANPDLVADD	PTISFKTAIW VIVSFETAIW ALISFETAIW ALISFETAIW
CHI3_CASSA Q6IVX8_9CARY V5TEI0_DIOMU Q6DUJ9_DIOMU	AQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GG	WATAPDGPYA WATAPDGPYA WPTAPDGPYA WSTAPDGPYA WSTAPDGPYA WPTAPDGPYA	WGYCFVMENN WGYCFKEEVG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG	KQTYC QPGSYC NPPSYC NPGDYC NPGDYC	R-S-KSWPCV VPS-TQWPCA EPS-TAYPCA EPS-TAYPCA APS-STYPCA VQS-STYPCA	-SDRYYGRGP FGKQYYGRGP AGKSYYGRGP PGKSYYGRGP PGQKYYGRGP PGQKYYGRGP	IQLTNPNNYE IQLTHNYNYG IQLSYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG	QAGKAIGADL PSGQAIGQPL QCGDSIGQPL QCGDSIGQPL LCGAAINQPL QCGVAINQPL	INNPDLVATN LENPDLVAGD LANPDLVADD LANPDLVADD LSNPGLVASD LSNPDLVASN	PTISFKTAIW VIVSFETAIW ALISFETAIW ADISFETAIW ADISFETAIW ADVSFETAIW
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CHI3_CASSA Q6IVX8_9CARY V5TEI0_DIOMU Q6DU39_DIOMU VJH3_9CARY DCAP_5513 Q6DUK0_9CARY DCAP_4817	AQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GG GQTSHETTGG	WATAPDGPYA WATAPDGPYA WPTAPDGPYA WPTAPDGPYA WSTAPDGPYA WPTAPDGPYA WPTAPDGPYA	WGYCFVMENN WGYCFKEEVG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG	KQTYC QPGSYC NPPSYC NPGDYC NPGDYC NPGDYC NPGDYC	R-S-KSWPCV VPS-TQWPCA EPS-TAYPCA EPS-TAYPCA APS-STYPCA VQS-STYPCA VQS-STYPCA	-SDRYYGRGP FGKQYYGRGP AGKSYYGRGP PGKSYYGRGP PGQKYYGRGP PGKKYYGRGP PGKKYYGRGP	IQLINFNNYE IQLIHNYNYG IQLSYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG	QAGKAIGADL PSGQAIGQPL QCGDSIGQPL QCGDSIGQPL LCGAAINQPL QCGVAINQPL QCGAAINQPL QCGAAINQPL	INNPDLVATN LENPDLVAGD LANPDLVADD LANPDLVADD LSNPGLVASD LSNPDLVASN LSNPDLVASN	PTISFKTAIW VIVSFETAIW ALISFETAIW ADISFETAIW ADVSFETAIW ADVSFETAIW ADVSFETAIW
CHI3_CASSA QGIVX8_9CARY V5TEI0_DIOMU QGDU39_DIOMU VJH3_9CARY DCAP_5513 QGDUK0_9CARY DCAP_4817 HORV2	AQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GG GG GQTSHETTGG AQTSHETTGG	WATAPDGPYA WATAPDGPYA WPTAPDGPYA WSTAPDGPYA WSTAPDGPYA WPTAPDGPYA WPTAPDGPYA WATAPDGAFA	WGYCFVMENN WGYCFKEEVG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG	KQTYC QPGSYC NPPSYC NPGDYC NPGDYC NPGDYC NPGDYC ASSDYC	R-S-KSWPCV VPS-TQWPCA EPS-TAYPCA APS-STYPCA VQS-STYPCA VQS-STYPCA VQS-STYPCA TPS-AQWPCA	-SDRYYGRGP FGKQYYGRGP PGKSYYGRGP PGKSYYGRGP PGQKYYGRGP PGKKYYGRGP PGKKYYGRGP PGKKYYGRGP	IQLINFNNYE IQLINNYNYG IQLSYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQLSYNYNYG IQLSHNYNYG	QAGKAIGADL PSGQAIGQPL QCGDSIGQPL QCGDSIGQPL QCGAAINQPL QCGAAINQPL QCGAAINQPL PAGRAIGVDL	INNPDLVATN LENPDLVAGD LANPDLVADD LSNPGLVADD LSNPDLVASN LSNPDLVASN LSNPDLVASN LANPDLVASN	PTISFKTAIW VIVSFETAIW ALISFETAIW ADISFETAIW ADVSFETAIW ADVSFETAIW ADVSFETAIW ATVGFKTAIW
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CHI3_CASSA QGIVX8_9CARY VSTEI0_DIOMU QGDUJ3_DIOMU VJH3_9CARY DCAP_5513 QGDUK0_9CARY DCAP_4817 HORVZ CH12_BRANA QGIV10_DERORT	AQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GG GG GQTSHETTGG AQTSHETTGG GQTSHETTGG -QTHHETRGG	WATAPDGPYA WATAPDGPYA WPTAPDGPYA WSTAPDGPYA WPTAPDGPYA WPTAPDGPYA WPTAPDGPYA WATAPDGPYS WATAPDGPYS	WGYCFVMENN WGYCFKQEVG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQA	KQTYC QPGSYC NPGSYC NPGDYC NPGDYC NPGDYC ASSDYC PSSDYC SPGDYC	R-S-KSWPCV VPS-TQWPCA EPS-TAYPCA APS-STYPCA VQS-STYPCA VQS-STYPCA VQS-STYPCA SPS-AGWPCA SPS-AGWPCA	- SDRYYGRGP FGRQYYGRGP PGRSYYGRGP PGRSYYGRGP PGKRYYGRGP PGKRYYGRGP PGKRYYGRGP PGKRYYGRGP SGKRYYGRGP	IQLINFNNYE IQLIHNYNYG IQLSYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQLSHNYNYG IQISHNYNYG IQISHNYNYG	QAGKAIGADL PSGQAIGQPL QCGDSIGQPL LCGAAIQQPL QCGVAINQPL QCGVAINQPL QCGAAINQPL PAGRAIGVDL QCGRAIGSDL PCGRAIGSDL	INNPDLVATN LENPDLVAGD LANPDLVADD LSNPGLVASD LSNPDLVASN LSNPDLVASN LSNPDLVASN LANPDLVASN LNNPDLVASN	PTISFKTAIW VIVSFETAIW ALISFETAIW ADISFETAIW ADVSFETAIW ADVSFETAIW ATVGFKTAIW PVIAFKAAIW SVISFKSAFW
CHI3_CRSA QGIVX8_9CARY VSTEID_DIOMU QGDUJ9_DIOMU VJH3_9CARY DCAP_5513 QGDUK0_9CARY DCAP_4817 HORV2 CHI2_BRANA QGIVI0_DRORT IOCM12_DIOMU	AQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG AQTSHETTGG QQTSHETTGG GQTSHETTGG GQTSHETTGG	WATAPDGPYA WATAPDGPYA WPTAPDGPYA WSTAPDGPYA WSTAPDGPYA WPTAPDGPYA WATAPDGPYA WATAPDGAFA WATAPDGPYA WPTAPDGPYA	WGYCFVMENN WGYCFKEEVG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQR WGYCFLTEQG	KQTYC QPGSYC NPPSYC NPGDYC NPGDYC NPGDYC NPGDYC ASSDYC SPSNYC SPPSYC	R-S-KSWPCV VPS-TQWPCA EPS-TAYPCA PS-STYPCA VQS-STYPCA VQS-STYPCA VQS-STYPCA VQS-STYPCA TPS-AQWPCA SPS-AEWPCA TPS-SQWPCA	-SDRYYGRGP FGRQYYGRGP AGRSYYGRGP PGRSYYGRGP PGRSYYGRGP PGRYYGRGP PGRYYGRGP PGRYYGRGP SGRSYYGRGP AGRYYGRGP	IQLTNENNYE IQLZINYNYG IQLSYNYNYG IQLSYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQLSYNYNYG IQLSWNYNYG IQLSWNYNYG IQLSWNYNYG IQLSWNYNYG	QAGKAIGADL PSQQAIGQPL QCGDSIGQPL LCGAAINQPL QCGVAINQPL QCGAAINQPL QCGAAINQPL PAGRAIGVDL PCGRAIGSDL PCGRAIGVDL	INNPDLVATN LENPDLVADD LANPDLVADD LSNPGLVASD LSNPDLVASN LSNPDLVASN LANPDLVASN LNNPDLVASD LNNPDLVATD	PTISFKTAIW VIVSFETAIW ALISFETAIW ADISFETAIW ADVSFETAIW ADVSFETAIW ADVSFETAIW ATVGFKTAIW PVIAFKAAIW SVISFKSAFW PIVSFKTALW
CH12_CASSA Q61VX8_9CARY V5TE10_DTOMU Q6DUJ9_DTOMU VJH3_9CARY DCAP_5513 Q6DUK0_9CARY DCAP_4817 HORV2 CH12_BRANA Q61V10_DRORT 10CM12_PCARY	AQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG AQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG	WATAPDGPYA WATAPDGPYA WPTAPDGPYA WSTAPDGPYA WSTAPDGPYA WPTAPDGPYA WATAPDGPYA WATAPDGPYA WATAPDGPYS WPTAPDGPYA	WGYCFVMENN WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFLFEQG WGYCYLFEQG	KQTYC QPGSYC NPFSYC NPGDYC NPGDYC NPGDYC NPGDYC ASSDYC FSSDYC SPGDYC NPFSYC NPFSYC NPFSYC	R-S-KSWPCV VPS-TQWPCA EPS-TAYPCA APS-STYPCA VQS-STYPCA VQS-STYPCA VQS-STYPCA TPS-AQWPCA SPS-AEWPCA EPS-SQWPCA EPS-SQWPCA VQS-AQWPCV	-SDRYYGRGP FGRQYYGRGP AGRSYYGRGP PGRSYYGRGP PGGRYYGRGP PGKRYYGRGP PGKRYYGRGP PGKRYYGRGP SGRSYYGRGP AGKRYYGRGP	IQLTNENNYE IQLEHNYNYG IQLSYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQLSHNYNYG IQLSHNYNYG IQLSHNYNYG IQLSPNENYG IQLSPNENYG IQISYNENYG	QAGKAIGADL PSGQAIGQPL QCGDSIGQPL QCGDSIGQPL LCGAAINQPL QCGAAINQPL QCGAAINQPL QCGAAINQPL PAGRAIGVDL QCGRAIGSDL PAGQAIGQDL AAGKAIGVDL	INNPDLVATN LENPDLVADD LANPDLVADD LSNPGLVADD LSNPGLVASD LSNPDLVASN LSNPDLVASN LANPDLVASD LNNPDLVASD LNNPDLVATD LNNPDLVATD LNNPDLVATD	PTISFKTAIW VIVSFETAIW ALISFETAIW ADISFETAIW ADVSFETAIW ADVSFETAIW ADVSFETAIW PVIAFKAAIW SVISFKSAFW PVVSFKTAIW PVVSFKTAIW
CH13_CASSA OG1VX8_9CARY V5TE10_DIOMU V5TE10_DIOMU VJH3_9CARY DCAP_5513 OG4DUK0_9CARY DCAP_5513 OG40X0_9CARY CH12_BRANA Q61V10_DRORT IOCM13_9CARY IOCM13_9CARY	AQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG AQTSHETTGG AQTSHETTGG AQTSHETTGG AQTSHETTGG	WATAPDGPYA WATAPDGPYA WPTAPDGPYA WPTAPDGPYA WPTAPDGPYA WPTAPDGPYA WPTAPDGPYA WATAPDGPYA WPSAPDGPYA WPSAPDGPYA WASAPDGPYA	WGYCFVMENN WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFLREQG WGYCFLREQG WGYCFLREQG WGYCFLREQG	R=QTYC QPGSYC NPFSYC NPGYC NPGDYC NPGDYC NPGDYC NPGYC PSSNYC SPGSYC NPGSYC NPGSYC	R-S-KSWPCV VPS-TQWPCA EPS-TAYPCA APS-STYPCA VQS-STYPCA VQS-STYPCA VQS-STYPCA TPS-AQWPCA SPS-AEWPCA EPS-SQWPCA VQS-AQWPCV VQS-AQWPCV	-SDRYYGRGP FGRQYYGRGP PGRSYYGRGP PGRSYYGRGP PGQRYYGRGP PGRYYGRGP PGRYYGRGP PGRYYGRGP PGRYYGRGP AGRYYGRGP AGRYYGRGP	IQLTNENNYE IQLTNENYNYG IQLSYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQLSHNYNYG IQLSHNYNYG IQLSFNFNYG IQISYNFNYG IQISYNFNYG	QAGKAIGADL PSGQAIGQPL QCGDSIGQPL QCGDSIGQPL LCGAAINQPL QCGVAINQPL QCGAAINQPL QCGAAINQPL QCGAAIQVDL QCGRAIGVDL PAGQAIGQDL AAGKAIGVDL	INNPDLVATN LENPDLVAGD LANPDLVADD LSNPGLVADD LSNPGLVASN LSNPDLVASN LSNPDLVASN LANPDLVASN LNNPDLVASN LNNPDLVATD LNNPDLVATD LNNPDLVATD LNNPDLVEKD	PTISFRTAIW VIVSFRTAIW ALISFRTAIW ADISFRTAIW ADVSFRTAIW ADVSFRTAIW ADVSFRTAIW AVVSFRTAIW SVISFKSAFW PIVSFKTAIW PVVSFKTAIW
CH12_CRSSA Q61VX8_QCARY V5TE10_DTOMU Q6DUJ9_DTOMU VJH3_GCARY DCAP_5513 Q6DUK0_9CARY DCAP_4817 HORV2 CH12_BRNAN Q61V10_DRORT 10CM12_DTOMU 10CM13_QCARY 10CM14_SCARY 10CM14_SCARY 10CM14_SCARY	AQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG AQTSHETTGG QQTSHETTGG AQTSHETTGG AQTSHETTGG AQTSHETTGG AQTSHETTGG	WATAPDGPYA WATAPDGPYA WPTAPDGPYA WSTAPDGPYA WPTAPDGPYA WPTAPDGPYA WPTAPDGPYA WATAPDGAFA WATAPDGAFA WATAPDGAFA WASAPDGPYA WASAPDGPYA	WGYCFVMENN WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQEQG WGYCFKQERG WGYCFKQERG WGYCFLFEQG WGYCYLFEQG WGYCYLFEQG WGYCYLFEQG	E	R-S-KSWPCV VPS-TQWPCA EPS-TAYPCA APS-STYPCA VQS-STYPCA VQS-STYPCA TPS-AQWPCA TPS-SQWPCA TPS-SQWPCA VQS-AQWPCV VQS-AQWPCV VQS-AQWPCV	-SDRYYGRGP FGRQYYGRGP PGRSYYGRGP PGRSYYGRGP PGQRYYGRGP PGRYYGRGP PGRYYGRGP PGRYYGRGP PGRYYGRGP AGKYYGRGP AGKYYGRGP	IQLTNENNYE IQLIHNYNYG IQLSYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQISYNYNYG IQLSHNYNYG IQLSHNYNYG IQLSYNENYG IQLSYNENYG IQISYNENYG IQISYNENYG	QAGKAIGADL PSGQAIGQPL QCGDSIGQPL LCGAAINQPL QCGAAINQPL QCGAAINQPL PAGRAIGVDL PCGRAIGVDL PAGRAIGVDL AAGKAIGVDL AAGKAIGVDL	INNPDLVATM LENPDLVAGD LANPDLVADD LSNPGLVADD LSNPGLVASM LSNPDLVASM LSNPDLVASM LANPDLVASM LNNPDLVATD LNNPDLVATD LNNPDLVEKD LNNPDLVEKD	PTISFNTAIM VIVSFETAIM ALISFETAIM ADISFETAIM ADVSFETAIM ADVSFETAIM ADVSFETAIM ADVSFETAIM VVISFNTAIM VVISFNTAIM PVVSFNTAIM PVVSFNTAIM
CH13_CASSA OG1VX8_9CARY V5TE10_DIOMU V5TE10_DIOMU VJH3_9CARY DCAP_5513 OG4DUK0_9CARY DCAP_5513 OG40X0_9CARY CH12_BRANA Q61V10_DRORT IOCM13_9CARY IOCM13_9CARY	AQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG GQTSHETTGG AQTSHETTGG AQTSHETTGG GQTSHETTGG GQTSHETTGG AQTSHETTGG AQTSHETTGG AQTSHETTGG AQTSHETTGG AQTSHETTGG AQTSHETTGG AQTSHETTGG	WATAPDGYYA WATAPDGYYA WPTAPDGYYA WSTAPDGYYA WSTAPDGYYA WPTAPDGYYA WATAPDGYYA WATAPDGYYA WPSAPDGYYA WASAPDGYYA WASAPDGYYA WASAPDGYYA	WGYCFVMENN WGYCFKQ2QG WGYCFKQ2QG WGYCFKQ2QG WGYCFKQ2QG WGYCFKQ2QG WGYCFKQ2QG WGYCFKQ2QG WGYCFKQ2QG WGYCFLEQG WGYCYLE2QG WGYCYLE2QG WGYCYLE2QG WGYCYLE2QG WGYCYLE2QG	E	R-S-KSWPCV R-S-KSWPCV VPS-TQWPCA EPS-TAYPCA APS-STYPCA VQS-STYPCA VQS-STYPCA VQS-STYPCA SPS-ABWPCA SPS-ABWPCA EPS-SQWPCA VQS-AQWPCV	-SD#YG6(CP FGEQYG6(CP FGEQYG6(CP FGESYYG6(CP FGEYYG6(CP FGEYYG6(CP FGEYYG6(CP FGEYYG6(CP FGEYYG6(CP FGEYYG6(CP FGEYYG6(CP AGKYYG6(CP AGKYYG6(CP AGKYYG6(CP AGKYYG6(CP	IQLIMENNYE IQLIMENNYE IQLIYNYNYG IQIIYNYNYG IQIIYNYNYG IQIIYNYNYG IQIIYNYNYG IQLIYNYNYG IQLIYNYNYG IQIIYNENYG IQIIYNENYG IQIIYNENYG IQIIYNENYG IQIIYNENYG	QAGKAIGADL PSGQAIGQPL QCGDSIGQPL QCGDSIGQPL QCGAINQPL QCGAAINQPL QCGAAINQPL PAGRAIGVDL PAGRAIGVDL PAGQAIGQDL AAGKAIGVDL AAGKAIGVDL AAGKAIGVDL	INNPDLVATM LENPDLVADD LANPDLVADD LANPDLVADD LSNPGLVASD LSNPDLVASN LSNPDLVASN LSNPDLVASN LANPDLVATD LNNPDLVATD LNNPDLVATD LNNPDLVEKD LNNPDLVEKD LNNPDLVEKD	PTISFNTAIM VIVSFETAIM ALISFETAIM ADISFETAIM ADISFETAIM ADVSFETAIM ADVSFETAIM ATVGFKTAIM PVISFKTAIM PVVSFKTAIM PVVSFKTAIM PVVSFKTAIM PVVSFKTAIM
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Figure 2.3: Sequence alignment and annotation for Family 19 chitinases. Many sequences in this cluster contain a chitin-binding C-rich domain (light green) that is connected to the active region by a P-rich hinge (light blue). Three sequences in this cluster contain a C-terminal extension (CTE) that causes the proteins to be targeted to the vacuole.

Four Family 19 chitinase fragments were identified from the *D. capensis* genome by performing a BLAST search for DcChit_1, a chitinase fragment previously identified from genomic DNA of the same organism [51]. Their sequences range from 41%-100% identity to Dc-Chit1_1. These fragments contain part of the N-terminal region, including the C-rich domain and the P-rich hinge, neither of which was observed in the original fragment, along with part of the catalytic domain (Figure 2.4). However, these sequences are all truncated before the catalytic residues. Sequencing of the *D. capensis* transcriptome will clarify whether these are fragments of active genes containing one or more introns, or inactive pseudogenes, which are relatively common in gene families undergoing rapid evolution [63] (as is the case for many proteins associated with pathogen defense) [64].

DcChitI_2 IOCMI1 DROCA	
DcChitI_1 DcChitI_3 DcChitI_4	
DcChitI_2 I0CMI1_DROCA DcChitI_1 DcChitI_3 DcChitI_4	SPTPSPPSPS GGGDVSSIIT SQIFNQMLLH RNDNACPANG FYSYQAFLDA ARKFSGFGTT GDINTRKKEL AAFFGQTSHE TTG PSPS GGGDVSSIIT SQIFNQMLLH RNDNACPAHG FYSYQAFLDA ARKFSGFGTT GDINTRKKEL AAFFGQTSHE TT
DcChitI_2 I0CMI1_DROCA DcChitI_1 DcChitI_3 DcChitI_4	IILYAWIKDE AID

Figure 2.4: Chitinase 1 fragments discovered using a BLAST search of the *D. capensis* genome against the DcChitI_1 fragment previously identified by Renner and Specht from *D. capensis* genomic DNA.

2.2.3 Preliminary Structural Models and In silico Maturation

Preliminary models for both Family 18 and Family 19 chitinases were produced using Rosetta [28], implemented in the online Robetta server [27]. The Rosetta structures contain the full sequences, including the N-terminal signal peptides, and in some cases, C-terminal targeting peptides that are also cleaved during maturation. These Rosetta models then underwent the *in silico* maturation process [48], and the process is illustrated in Figure 2.5 for a represen-

tative family 18 chitinase, DCAP_2209. The initial Rosetta sequence, including the signal peptide and lacking post-translational modifications, is shown in Figure 2.5. In order to generate the equilibrated structure Figure 2.5b, which more closely approximates the active form of the enzyme in solution, the signal sequence is removed, disulfide bonds are added using homology to a reference sequence (in this case CHIT3_VITVI), and the structure is equilibrated in explicit solvent. Many Family 18 chitinases from plants contain three disulfide bonds [65, 66], although examples without any disulfide bonds also exist [67]. Three are found in all the Family 18 chitinases in this set, as in CHIT3_VITVI [36], and hevamine from *Hevea brasiliensis* (PDB ID: 2HVM) [68]. The functionally important cis peptide bonds are captured by the molecular models for all the Family 18 chitinases examined here except for DCAP_7323, which unlikely to be active in any case because it is truncated at the N-terminal end.

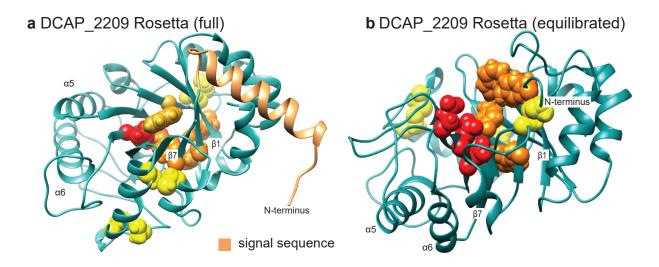


Figure 2.5: DCAP_2209 (a) before and (b) after *in silico* maturation. The light orange helix in part a is the N-terminal signal sequence. Important residues are color-coded as follows: Red: catalytically active residues of the "DXDXE" motif. Orange: aromatic substrate-binding residues. Yellow: Cysteines in disulfide bonds.

Figure 2.6 shows full-length structures for Q6IVX8_9CARY and Q6IVX2_9CARY (Family 19) from *Drosera spatulata*. The N-terminal and C-terminal targeting sequences are exposed on the surface of the protein, as expected. The P-rich hinge in these proteins is variable

in length, and highly flexible, as illustrated by the different relative conformations of the catalytic and C-rich chitin binding domains observed here.

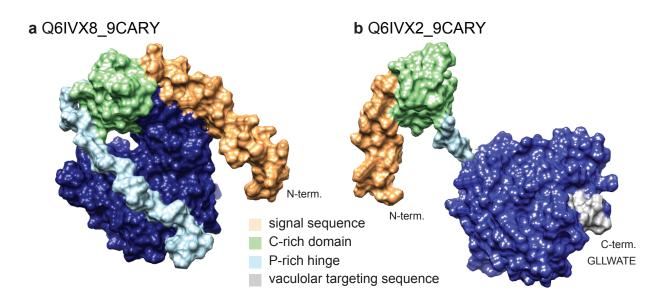


Figure 2.6: Initial Rosetta structures for two class I chitinases from *Drosera spatulata*, Q6IVX8_9CARY and Q6IVX2_9CARY, illustrating positioning of the N-terminal and C-terminal targeting sequences and the variability in length and conformation for the P-rich hinge.

All initial and equilibrated structures are available for download as PDB files from our paper online [1]. The available structures for Families 18 and 19 are tabulated in Tables 1 and 2, respectively.

Organism	Sequence Elements included	File Name
Vitis vinifera	signal, active region	$\rm CHIT3_VITVI_m1.pdb$
Vitis vinifera	active region	$\rm CHIT3_VITVI_mature_m1.pdb$
D. capensis	active region	DCAP_7323_m1.pdb
D. capensis	active region	$DCAP_7323_mature_m1.pdb$
D. capensis	signal, active region	DCAP_0106_m1.pdb
D. capensis	active region	$DCAP_0106_mature_m1.pdb$
D. capensis	signal, active region	DCAP_7544_m1.pdb
D. capensis	active region	$DCAP_7544_mature_m1.pdb$
D. capensis	signal, active region	$DCAP_2209_m1.pdb$
D. capensis	active region	$DCAP_2209_mature_m1.pdb$
N. mirabilis	signal, active region	$C7F821_NEPMI_m1.pdb$
N. mirabilis	active region	$\rm C7F821_NEPMI_mature_m1.pdb$
D. spatulata	signal, active region	$\rm C7F817_9CARY_m1.pdb$
D. spatulata	active region	$C7F817_9CARY_mature_m1.pdb$
N. alata	signal, active region	I7HCY7_NEPAL_m1.pdb
N. alata	active region	$I7HCY7_NEPAL_mature_m1.pdb$
D. spatulata	signal, active region	$C7F818_9CARY_m1.pdb$
D. spatulata	active region	$C7F818_9CARY_mature_m1.pdb$
D. spatulata	signal, active region	$Q06SN0_9CARY_m1.pdb$
D. spatulata	active region	$Q06SN0_9CARY_mature_m1.pdb$
D. spatulata	signal, active region	$\rm C7F824_9CARY_m1.pdb$
D. spatulata	active region	$C7F824_9CARY_mature_m1.pdb$
D. spatulata	signal, active region	$\rm C7F822_9CARY_m1.pdb$
D. spatulata	active region	$C7F822_9CARY_mature_m1.pdb$
D. spatulata	signal, active region	$\rm C7F819_9CARY_m1.pdb$
D. spatulata	active region	$C7F819_9CARY_mature_m1.pdb$
N. gracilis	signal, active region	$\rm C7F823_NEPGR_m1.pdb$
N. gracilis	active region	$\rm C7F823_NEPGR_mature_m1.pdb$
D. capensis	signal, active region	$DCAP_5455_m1.pdb$
D. capensis	active region	$DCAP_5455_mature_m1.pdb$
D. capensis	signal, active region	$DCAP_{2879}m1.pdb$
D. capensis	active region	$DCAP_{2879}mature_m1.pdb$
D. capensis	signal, active region	$DCAP_4799_m1.pdb$
D. capensis	active region	$DCAP_4799_mature_m1.pdb$
D. capensis	signal, active region	$DCAP_2737_m1.pdb$
D. capensis	active region	$DCAP_2737_mature_m1.pdb$
	Vitis vinifera Vitis vinifera D. capensis D. capensis D. capensis D. capensis D. capensis D. capensis D. capensis D. capensis D. capensis N. mirabilis N. mirabilis D. spatulata D. spatulata	Vitis viniferasignal, active regionVitis viniferaactive regionD. capensisactive regionD. capensissignal, active regionN. mirabilissignal, active regionN. mirabilissignal, active regionD. spatulatasignal, active regionD. capensissignal, active regionD. capensissignal, active regionD. capensis

Protein HORV2 HORV2 Q6IV09_DRORT Q6IV09_DRORT CHI3_CASSA CHI3_CASSA Q6IVX8_9CARY Q6IVX8_9CARY V5TEI0_DIOMU V5TEI0_DIOMU Q6DUJ9_DIOMU Q6DUJ9_DIOMU VJH3_9CARY VJH3_9CARY DCAP_5513 DCAP_5513 Q6DUKO_9CARY Q6DUKO_9CARY DCAP_4817 DCAP_4817 CHI2_BRANA CHI2_BRANA Q6IV10_DRORT Q6IV10_DRORT I0CMI2_DIOMU I0CMI2_DIOMU I0CMI3_9CARY I0CMI3_9CARY I0CMI4_9CARY I0CMI4 9CABY I0CMI6_NEPMI I0CMI6_NEPMI Q6IVX2_9CARY Q6IVX2_9CARY Q6IVX4_9CARY Q6IVX4_9CARY $DCAP_0533$ DCAP_0533 A9ZMK1_NEPAL A9ZMK1_NEPAL Organism

H. vulgare

H. vulgare

D. rotundifolia

D. rotundifolia

 $Castanea\ sativa$

Castanea sativa

D. spatulata

D. spatulata

D. muscipula

D. muscipula

D. muscipula

D. muscipula

D. spatulata

D. spatulata

D. capensis

D. capensis

D. spatulata

D. spatulata

D. capensis

D. capensis

B. napus

B. napus

D. rotundifolia

D. rotundifolia

 $D. \ muscipula$

D. muscipula

D. spatulata

D. spatulata

D. spatulata

D. spatulata

N. mirabilis

N. mirabilis

D. spatulata

D. spatulata

D. spatulata

D. spatulata

D. capensis

D. capensis

N. alata

N. alata

Sequence Elements included active region active region active region active region C-rich domain, P-rich hinge, active region C-rich domain, P-rich hinge, active region signal, C-rich domain, P-rich hinge, active region C-rich domain, P-rich hinge, active region signal, C-rich domain, P-rich hinge, active region C-rich domain, P-rich hinge, active region signal, C-rich domain, P-rich hinge, active region C-rich domain, P-rich hinge, active region signal, C-rich domain, P-rich hinge, active region C-rich domain, P-rich hinge, active region signal, C-rich domain, P-rich hinge, active region C-rich domain, P-rich hinge, active region active region active region signal, C-rich domain, P-rich hinge, active region C-rich domain, P-rich hinge, active region signal, C-rich domain, P-rich hinge, active region, CTE C-rich domain, P-rich hinge, active region active region active region active region active region active region active region active region active region active region active region signal, C-rich domain, P-rich hinge, active region, CTE C-rich domain, P-rich hinge, active region signal, C-rich domain, P-rich hinge, active region, CTE C-rich domain, P-rich hinge, active region signal, C-rich domain, P-rich hinge, active region, C-terminal domain C-rich domain, P-rich hinge, active region, C-terminal domain signal, C-rich domain, P-rich hinge, active region C-rich domain, P-rich hinge, active region

File Name HORV2 PDBID: 2BAA HORV2_crystal_struc_mature_m1.pdb Q6IV09_DRORT_m1.pdb Q6IV09_DRORT_mature_m1.pdb CHI3_CASSA_m1.pdb CHI3_CASSA_mature_m1.pdb Q6IVX8_9CARY_m1.pdb Q6IVX8_9CARY_mature_m1.pdb V5TEI0_DIOMU_m1.pdb V5TEI0_DIOMU_mature_m1.pdb Q6DUJ9_DIOMU_m1.pdb 6DUJ9_DIOMU_mature_m1.pdb VJH3_9CARY_m1.pdb VJH3_9CARY_mature_m1.pdb DCAP_5513_m1.pdb DCAP_5513_mature_m1.pdb Q6DUKO_9CARY_m1.pdb $Q6DUKO_9CARY_mature_m1.pdb$ DCAP_4817_m1.pdb DCAP_4817_mature_m1.pdb CHI2_BRANA_m1.pdb CHI2_BRANA_mature_m1.pdb Q6IV10_DRORT_m1.pdb Q6IV10_DRORT_mature_m1.pdb I0CMI2_DIOMU_m1.pdb I0CMI2_DIOMU_mature_m1.pdb I0CMI3_9CARY_m1.pdb I0CMI3_9CARY_mature_m1.pdb I0CMI4_9CARY_m1.pdb I0CMI4_9CARY_mature_m1.pdb I0CMI6_NEPMI_m1.pdb I0CMI6_NEPMI_mature_m1.pdb Q6IVX2_9CARY_m1.pdb $Q6IVX2_9CARY_mature_m1.pdb$ Q6IVX4_9CARY_m1.pdb Q6IVX4_9CARY_mature_m1.pdb DCAP_0533_m1.pdb DCAP_0533_mature_m1.pdb A9ZMK1_NEPAL_m1.pdb A9ZMK1_NEPAL_mature_m1.pdb

2.2.4 Network Modeling and Analysis

In collaboration with Prof. Carter T. Butts, we mapped each equilibrated protein structure to a protein structure network (PSN) as defined by the representation of [69] using software tools from [47]; these in turn make use of VMD [70] and the **statnet** toolkit [71, 72] within the R statistical computing system [73]. To compare PSNs, we use the structural distance approach of [74], which defines a metric on graph pairs that is in our case equal to the number of edges in one graph that would need to be altered in order to make it isomorphic to the other. (Isolate addition was performed when comparing graphs with differing numbers of vertices.) To remove size effects, the raw distance between each pair of PSNs was normalized by the number of vertices, yielding a metric corresponding to edge changes per vertex. These normalized structural distances were analyzed using hierarchical clustering using R. Additional network analysis and visualization was performed using the **network** and **sna** libraries within **statnet** [72, 75].

2.3 Results

This work reports molecular models and functional predictions from protein structure networks for eleven new chitinases from D. capensis, including a novel class IV chitinase with two active domains. This architecture has previously been observed in microorganisms but not in plants. This work used a combination of comparative and de novo structure prediction followed by molecular dynamics simulation to produce models of the mature forms of these proteins in aqueous solution. Protein structure network analysis of these and other plant chitinases reveal characteristic features of the two major chitinase families. Vy Duong worked mostly on Family 19 and my work was mostly concentrated on Family 18. It is important to understand the comparison of Family 19 and Family 18 and therefore, a portion of the results from the paper [21] are shown in this chapter.

2.3.1 D. capensis Chitinases are Predicted to Adopt Folds Consistent with Active Enzymes

The catalytic action of family 18 chitinases, which retains the β -anomeric carbon stereochemistry from the substrate to the product, is based on substrate-assisted hydrolysis of the glycosidic bond [76, 77, 78]. Catalysis is initiated by distorting the -1 sugar ring subsite adjacent to the glycosidic bond. Next, Asp 123 rotates to form hydrogen bonds with both Glu 127 and the N-acetyl group of the +1 sugar. This step protonates Glu 127. Then, the anomeric carbon is subjected to a nucleophilic attack by the oxygen from the N-acetyl group, forming an oxazolinium ion as an intermediate, followed by cleavage of the glycosidic bond by hydrolysis to generate smaller fragments. The DXDXE motif is essential for activity, hence fragments that were lacking this sequence due to truncation were excluded from the protein set.

Family 18 chitinases, which retain the β -anomeric carbon stereochemistry from the substrate to the product, adopt the $(\alpha$ - β)₈ triosephosphateisomerase (TIM)-barrel fold [66, 76], shown for DCAP_0106 in Figure 2.7A. The active site (Figure 2.7B), consists of a characteristic DXXDXDXE motif [66, 76]. The "tunnel" containing the active site is shaped by an unusual structural feature, two non-proline cis peptide bonds that are highly conserved, although the particular residues involved are somewhat variable [68, 33]. The cis peptide bonds (shown in black in Figure 2.7C), are captured by the molecular models for all full-length Family 18 chitinases examined here. The shape of the tunnel and the surface formed by the aromatic rings opposite the catalytic D and E residues acts to guide the chitin polymer chains into the active site, leading to processive activity [79]. The ability of Family 18 chitinases to keep the strand that is currently being degraded from re-encountering solid substrate is thought to be a key determinant of their ability to hydrolyze crystalline polysaccharides [80].

The Family 19 chitinases, all of which are characterized by an anomeric inverting mechanism

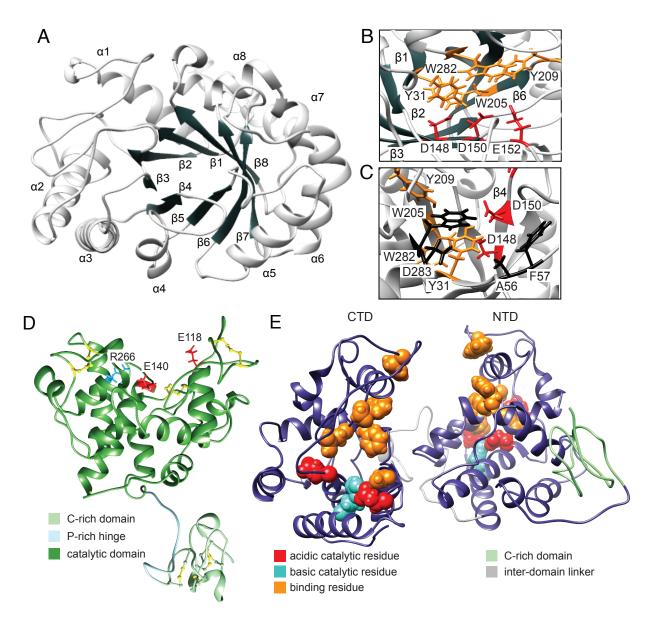


Figure 2.7: Equilibrated structures of the mature sequences of chitinases from carnivorous plants. A. DCAP_0106, a representative Family 18 chitinase, after *in silico* maturation. Numbering of secondary structure elements follows the convention of Si et al. [5]. B. Notably, the tunnel containing the active site has two surfaces with different chemical properties; the aromatic rings (orange) hold the more hydrophobic face of the chitin polymer in place, while the acidic residues (red) perform hydrolysis of the glycosidic linkages. C. Two conserved non-proline cis peptide bonds (black) are critical to shaping the active site tunnel in Family 18 chitinases. D. Chitinase VF-1 from *Dionaea muscipula* V5TEI0_DIOMU [6], with important sequence features and active site residues labeled (red: acidic active residue. blue: basic active residue. yellow: disulfide bond). E. The two-domain chitinase DCAP_0533. Color coding is as in D, with the addition of substrate-binding residues in orange.[1]

[61], have diverse structural features. Much of the structural and functional diversity results from two highly variable regions, the C-rich chitin-binding domain and the P-rich hinge [81, 82], each of which may vary in length or be absent altogether. We have identified two class I chitinases (DCAP_4817 and DCAP_5513) and one class IV chitinase (DCAP_0533) from the D. capensis genome. Most of the sequences in this set contain N-terminal secretion signals, however two D. spatulata sequences (Q6IVX2_9CARY and Q6IVX4_9CARY) and the reference sequence CHI2_BRANA contain short C-terminal extensions indicating targeting to the vacuole, consistent with their playing a purely defensive role. One sequence each from D. capensis (DCAP_5513), D. rotundifolia (Q6IV09_DRORT), and D. spatulata (Q6DUK0_9CARY) is missing one or more critical active site residues; in other organisms, enzymatically non-functional chitinase homologs are often present and can serve as chitinbinding proteins [83]. The predicted structure after *in silico* maturation for a representative chitinase, VF-1 from *D. muscipula* (Figure 2.7 is in good agreement overall with the homology model of Paszota et al. [6], with the active site residues positioned in a shallow cleft on the surface of the active domain. The two models do differ in the relative orientations of the domains; however examination of the other models in this set suggests that the P-rich hinge is highly flexible as seen in Figure 2.6.

2.3.2 The Novel Class IV Chitinase DCAP_0533 Has Two Functional Domains

This work identified a new class IV chitinase from *D. capensis*, DCAP_0533. A class IV chitinase has previously been described as one of the most abundant proteins in the pitcher fluid *N. alata* [45], where it preferentially hydrolyzes small GlcNAc oligomers over larger polymeric substrates [84]. Unlike other known plant chitinases, DCAP_0533 contains two class IV catalytic domains. The N-terminal domain appears to be fully active, while the C-terminal domain lacks one of the active residues but contains a full complement of substrate-

binding residues (Figure 2.7E, Figures 2.9, Figure 2.10). Multidomain chitinases containing dedicated substrate-binding domains have previously been observed in microbes [85]. For example, ChiA from the thermophilic archeon *Pyrococcus kodakaraensis*, has two chitinase domains and three catalytically inactive substrate binding domains, allowing separate optimizaton of substrate binding and catalytic function [86]. AFM data suggests the binding is mostly determined by interaction of the aromatic residues in the binding site (orange in Figure 2.7E) with the pyranose rings of the substrate [87]. This type of functionality has not been previously observed in plants; we hypothesize that it is an adaptation associated with carnivory, perhaps related to more effective breakdown of small oligosaccharides to components that can be used as a nitrogen source.

Structurally, each domain consists of two lobes with eight helices each, separated by a large active site cleft Figure 2.10(a). In Figure 2.10(b), the two domains of this protein are shown overlaid with the crystal structures of class IV chitinases from Zea mays (PDBID: 4MCK, 60% identity with the NTD) and *Picea abies* (PDBID: 3HBE, 64% identity with the CTD). The NTD Figure 2.10(c) has an N-terminal signal peptide, a conserved C-rich binding domain, and a catalytic domain that appears to be functional. In its homolog CHIA_MAIZE, Chaudet et. al. characterized four catalytic residues (E62, E71, E165, and R171) [88], all of which have counterparts in the NTD of DCAP_0533 (E173, E182, E278, R290) Figures 2.9 and 2.10. Previous modeling studies of well-characterized class I chitinases from barley, mustard, and chestnut seed homologs (barley: E67, mustard: E212, chestnut: E124) suggest the necessity of E62 in CHIA_MAIZE and E173 in the NTD of DCAP_0533 as a proton donor [89, 90, 91]. Overall, mutagenesis studies highlight the significance of E62 as an essential residue of the catalytic triad (E62, E165, R171 in CHIA_MAIZE) which we use to infer an equivalent catalytic triad in the NTD of DCAP_0533 (E173, E278, and R290). It has also been hypothesized that purpose of the triad is to alter the surrounding environment to induce activation of the glutamic acid in the HETG/I (class IV) or HETT (class I/II) motif by changing its pKa [91].

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Q6WSR8_PICAB				MGSII-	-IDKSVMALVL-	VLLLV-GVSV-	NAQNCGCATG	VCCSQYGYCG	TTSAYCGKGC	KSGPCYSSGG
N_term_DCAP_0533	MTIVHASTNK-	-AKLDFSFFSL	PYTSLQTPKL	YNYKNMALSI	KIKTHFAIIF	-IIIVVFLSSS	-LAQDCGCDSS	LCCSQYGYCG	TSDAYCGVGC	QEGPCKSAVN
C_term_DCAP_0533							-AQSCGCAAG	LCCSKYGYCG	TTSSYCGDGC	QAGPCSSTPT
CHIA_MAIZE				MANAP	-RILALGLL-	-ALLCAAAGPA-	-AAQNCGCQPN	FCCSKFGYCG	TTDAYCGDGC	QSGPCRSGGG
C5YBE7_SORBI				MENAP	RILTVLALGL-	-ALLCAGAGPA-	-AAQNCGCQPG	YCCSQYGYCG	KGDAYCGKGC	RSGPCQGGGG
		•		• *•		• •	•		¥ • *••	¥ •*
Q6WSR8_PICAB	GSPS	-AGGGSVGGI	ISQSFFNGLA	GGAASSCEGK	GFYTYNAFIA	AAN-AYSGFG	TTGSNDVKKR	ELAAFFANVM	HETGGLCYIN	EKNP-PINYC
N_term_DCAP_0533	NT	-KNDVSVPDV	VSDAFFNGII	DQAASTCEGI	GFYSRAGFLS	AWESNYTDFG	TTGSVEESLR	${\color{blue}{\textbf{E}}}{\color{blue}{\textbf{IAAFFAHVT}}}$	HETGHFCYIN	EINGSSKDYC
C_term_DCAP_0533		-SSGVSVPAV	VTDAFFNGII	NQAGSGCPGK	GFYSRSAFLS	AIG-SYPSFG	TTGTTDASKQ	EIAAFFA	HENGGA	SQPKSQYC
CHIA_MAIZE	GGGGGGGGGG	GSGGANVANV	VTDAFFNGIK	NQAGSGCEGK	NFYTRSAFLS	AVN-AYPGFA	HGGTEVEGKR	EIAAFFAHVT	HETGHFCYIS	EINK-SNAYC
C5YBE7_SORBI		SGANVGSV	VTDAFFNGIK	NQAPNWCEGK	NFYTRSAFLN	AAN-AYPGFA	HGGSEVEGKR	${\color{blue}{\textbf{E}}}{\color{blue}{\textbf{IAAFFAHVT}}}$	HETGHFCYIN	EINGASRNYC
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Q6WSR8_PICAB	-				LNNPEKVGQD			-		CNGGNSGEVS
N_term_DCAP_0533	DETNTENDON	DEVEVVEDED						LITSCOCECE		CDGGNTPEVN
					LNSPETVAND					
					LNSPETVAND					
CHIA_MAIZE	DASYTQYPCN	PNKGYYGRGP	LQLSWNYNYG	AAGSSIGF <mark>D</mark> G		AVISFKTALW	FWMNNGI-HS	AIVSGQGFGA	TIRAINS-GE	
	DASYTQYPCN DASNRQWPCA	PNKGYYGRGP AGQKYYGRGP	LQLSWNYNYG LQISWNYNYG	AAGSSIGFDG PAGRDIGFNG	LNSPETVANN	AVISFKTALW AVIAFKTALW	FWMNNGI-HS FWMNNVH	AIVSGQGFGA -GVMPQGFGA	TIRAINS-GE TIRAINGALE	CNGGNLGAVN
CHIA_MAIZE	DASYTQYPCN DASNRQWPCA	PNKGYYGRGP AGQKYYGRGP	LQLSWNYNYG LQISWNYNYG	AAGSSIGFDG PAGRDIGFNG	LNSPETVANN LADPNRVAQD	AVISFKTALW AVIAFKTALW	FWMNNGI-HS FWMNNVH	AIVSGQGFGA -GVMPQGFGA	TIRAINS-GE TIRAINGALE	CNGGNLGAVN CNGNNPAQMN
CHIA_MAIZE C5YBE7_SORBI	DASYTQYPCN DASNRQWPCA DANNRQWPCA	PNKGYYGRGP AGQKYYGRGP PGKKYYGRGP	LQLSWNYNYG LQISWNYNYG LQISWNYNYG	AAGSSIGFDG PAGRDIGFNG PAGKAIGFDG	LNSPETVANN LADPNRVAQD LGNPDKVAQD	AVISFKTALW AVIAFKTALW PVISFKTALW	FWMNNGI-HS FWMNNVH FWMNNVH	AIVSGQGFGA -GVMPQGFGA -QVMPQGFGA	TIRAINS-GE TIRAINGALE TIRAINGALE	CNGGNLGAVN CNGNNPAQMN
CHIA_MAIZE C5YBE7_SORBI	DASYTQYPCN DASNRQWPCA DANNRQWPCA	PNKGYYGRGP AGQKYYGRGP PGKKYYGRGP SQLGVDPGAN	LQLSWNYNYG LQISWNYNYG LQISWNYNYG VSC XX	AAGSSIGFDG PAGRDIGFNG	LNSPETVANN LADPNRVAQD LGNPDKVAQD	AVISFKTALW AVIAFKTALW	FWMNNGI-HS FWMNNVH FWMNNVH	AIVSGQGFGA -GVMPQGFGA -QVMPQGFGA	TIRAINS-GE TIRAINGALE	CNGGNLGAVN CNGNNPAQMN
CHIA_MAIZE C5YBE7_SORBI Q6WSR8_PICAB N_term_DCAP_0533	DASYTQYPCN DASNRQWPCA DANNRQWPCA SRVNYYKKIC DRVKYYKQYC	PNKGYYGRGP AGQKYYGRGP PGKKYYGRGP SQLGVDPGAN DEL	LQLSWNYNYG LQISWNYNYG LQISWNYNYG VSC XX	AAGSSIGFDG PAGRDIGFNG PAGKAIGFDG	LNSPETVANN LADPNRVAQD LGNPDKVAQD	AVISFKTALW AVIAFKTALW PVISFKTALW acidic catal	FWMNNGI-HS FWMNNVH FWMNNVH	AIVSGQGFGA -GVMPQGFGA -QVMPQGFGA C-rich	TIRAINS-GE TIRAINGALE TIRAINGALE	CNGGNLGAVN CNGNNPAQMN
CHIA_MAIZE C5YBE7_SORBI Q6WSR8_PICAB N_term_DCAP_0533 C_term_DCAP_0533	DASYTQYPCN DASNRQWPCA DANNRQWPCA SRVNYYKKIC DRVKYYKQYC ARVQYYKQYC	PNKGYYGRGP AGQKYYGRGP PGKKYYGRGP SQLGVDPGAN DEL	LQLSWNYNYG LQISWNYNYG LQISWNYNYG VSC XX LTC •	AAGSSIGFDG PAGRDIGFNG PAGKAIGFDG	LNSPETVANN LADPNRVAQD LGNPDKVAQD	AVISFKTALW AVIAFKTALW PVISFKTALW	FWMNNGI-HS FWMNNVH FWMNNVH	AIVSGQGFGA -GVMPQGFGA -QVMPQGFGA C-rich	TIRAINS-GE TIRAINGALE TIRAINGALE	CNGGNLGAVN CNGNNPAQMN
CHIA_MAIZE C5YBE7_SORBI Q6WSR8_PICAB N_term_DCAP_0533	DASYTQYPCN DASNRQWPCA DANNRQWPCA SRVNYYKKIC DRVKYYKQYC ARVQYYKQYC ARVQYYKQYC	PNKGYYGRGP AGQKYYGRGP PGKKYYGRGP SQLGVDPGAN DEL	LQLSWNYNYG LQISWNYNYG LQISWNYNYG VSC XX LTC •	AAGSSIGFDG PAGRDIGFNG PAGKAIGFDG	LNSPETVANN LADPNRVAQD LGNPDKVAQD vage site	AVISFKTALW AVIAFKTALW PVISFKTALW acidic catal	FWMNNGI-HS FWMNNVH FWMNNVH ytic residue	AIVSGQGFGA -GVMPQGFGA -QVMPQGFGA C-rich	TIRAINS-GE TIRAINGALE TIRAINGALE	CNGGNLGAVN CNGNNPAQMN

Figure 2.8: Sequence alignment and annotation of Q6WSR8_PICAB, CHIA_MAIZE, and the N-terminal domain (NTD) and C-terminal domain (CTD) of DCAP_0533. For the purpose of comparison, the sequence is manually separated above. We observe high sequence conservation regarding: the signal cleavage site, C-rich domain length and location, cysteines composing disulfide bonds, other binding site residues surrounding the main binding site residues (orange arrows), and catalytic residues except Glu407 of the CTD which is unaligned with Glu113 of Q6WSR8_PICAB

Class IV chitinases exhibit an amino acid substitution in the first active site region relative to Class I chitinases, resulting in a HETG/I motif instead of the HETT motif [51]. A deletion of four amino acids in the Cys-rich binding domain is also observed in class IV chitinases, as shown for a class IV chitinase from *Nepenthes alata* (A9ZMK1_NEPAL) [84] and DCAP_0533 in 2.3. Figure 2.9 shows a sequence alignment of the N- and C-terminal domains of the Class IV chitinase DCAP_0533 with single domain class IV chitinases from *Picea abies* (Q6WSR8_PICAB), *Zea mays* (CHIA_MAIZE), and *Sorghum bicolor* (C5YBE7_SORBI). The two domains of DCAP_0533 were aligned with the most closely related annotated class IV chitinases, those from *Picea abies* (EC: 3.2.1.14, Uniprot: Q6WSR8_PICAB), *Zea mays* (EC: 3.2.1.14, Uniprot: CHIA_MAIZE), and *Sorghum bicolor* (Uniprot: C5YBE7_SORBI) [92, 51, 88] (Figure 2.10).

Linked to the NTD by a cysteine and glycine-rich linker sequence, the CTD of DCAP_0533

(Figure 2.10(d) potentially houses a second catalytic domain or binding domain whose closest structural homolog is Q6WSR8_PICAB from Norway spruce (*Picea abies*) (Figure 2.9). Binding site residues and cysteines involved in disulfide bond formation are conserved in both chitinases. Comparing this sequence with the catalytic triad of Q6WSR8_PICAB (E113, R230, E218), we observe a potentially equivalent triad in the CTD (E407, E507, R519) (Figure 2.10). Ubhayasekera et. al. describe the flexibility of E113 and demonstrate two conformations that it can adopt during catalysis [92]. Although E407 is not located in the equivalent sequence position to E113, the flexibility of this residue in Q6WSR8_PICAB suggests that Glu407 may be at an appropriate distance to function as part of the CTD triad. Alternatively, the CTD may lack catalytic activity and act as a binding domain as in multidomain chitinases from archaea and bacteria.

2.3.3 Description of a Novel Two-Domain Class IV Chitinase

Class IV chitinases exhibit an amino acid substitution in the first active site region relative to Class I chitinases, resulting in a HETG/I motif instead of the HETT motif [51]. A deletion of four amino acids in the Cys-rich binding domain is also observed in class IV chitinases, as shown for a class IV chitinase from *Nepenthes alata* (A9ZMK1_NEPAL) [84] and DCAP_0533 in 2.3. Figure 2.9 shows a sequence alignment of the N- and C-terminal domains of the Class IV chitinase DCAP_0533 with single domain class IV chitinases from *Picea abies* (Q6WSR8_PICAB), *Zea mays* (CHIA_MAIZE), and *Sorghum bicolor* (C5YBE7_SORBI). The two domains of DCAP_0533 were aligned with the most closely related annotated class IV chitinases, those from *Picea abies* (EC: 3.2.1.14, Uniprot: Q6WSR8_PICAB), *Zea mays* (EC: 3.2.1.14, Uniprot: CHIA_MAIZE), and *Sorghum bicolor* (Uniprot: C5YBE7_SORBI) [92, 51, 88] (Figure 2.10). Structurally, each domain consists of two lobes with eight helices each, separated by a large active site cleft (Figure 2.10(a). In Figure 2.10(b), the two domains of this protein are shown overlaid with the crystal structures of class IV chitinases from Zea mays (PDBID: 4MCK, 60% identity with the NTD) and *Picea abies* (PDBID: 3HBE, 64% identity with the CTD). The NTD Figure 2.10(c) has an N-terminal signal peptide, a conserved C-rich binding domain, and a catalytic domain that appears to be functional. In its homolog CHIA_MAIZE, Chaudet et. al. characterized four catalytic residues (E62, E71, E165, and R171) [88], all of which have counterparts in the NTD of DCAP_0533 (E173, E182, E278, R290) (Figures 2.9, 2.10. Previous modeling studies of well-characterized class I chitinases from barley, mustard, and chestnut seed homologs (barley: E67, mustard: E212, chestnut: E124) suggest the necessity of E62 in CHIA_MAIZE and E173 in the NTD of DCAP_0533 as a proton donor [89, 90, 91]. Overall, mutagenesis studies highlight the significance of E62 as an essential residue of the catalytic triad (E62, E165, R171 in CHIA_MAIZE) which we use to infer an equivalent catalytic triad in the NTD of DCAP_0533 (E173, E278, and R290). It has also been hypothesized that purpose of the triad is to alter the surrounding environment to induce activation of the glutamic acid in the HETG/I (class IV) or HETT (class I/II) motif by changing its pKa [91].

Linked to the NTD by a cysteine and glycine-rich linker sequence, the CTD of DCAP_0533 (Figure 2.10(d) potentially houses a second catalytic domain or binding domain whose closest structural homolog is Q6WSR8_PICAB from Norway spruce (*Picea abies*) (Figure 2.9). Binding site residues and cysteines involved in disulfide bond formation are conserved in both chitinases. Comparing this sequence with the catalytic triad of Q6WSR8_PICAB (E113, R230, E218), we observe a potentially equivalent triad in the CTD (E407, E507, R519) (Figure 2.10). Ubhayasekera et. al. describe the flexibility of E113 and demonstrate two conformations that it can adopt during catalysis [92]. Although E407 is not located in the equivalent sequence position to E113, the flexibility of this residue in Q6WSR8_PICAB

suggests that Glu407 may be at an appropriate distance to function as part of the CTD triad. Alternatively, the CTD may lack catalytic activity and act as a binding domain as in multidomain chitinases from archaea and bacteria.

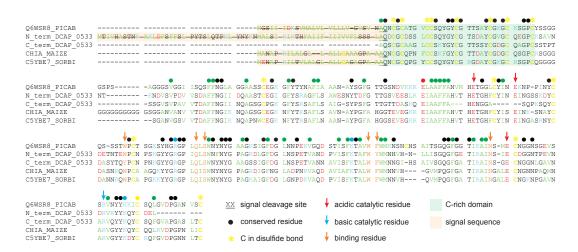


Figure 2.9: Sequence alignment and annotation of Q6WSR8_PICAB, CHIA_MAIZE, and the N-terminal domain (NTD) and C-terminal domain (CTD) of DCAP_0533. For the purpose of comparison, the sequence is manually separated above. We observe high sequence conservation regarding: the signal cleavage site, C-rich domain length and location, cysteines composing disulfide bonds, other binding site residues surrounding the main binding site residues (orange arrows), and catalytic residues except Glu407 of the CTD which is unaligned with Glu113 of Q6WSR8_PICAB

2.3.4 Network Analysis Shows Substantial Topological Differences by Family and within Proteins

When selecting potential targets for biophysical characterization, it is useful to consider general patterns of structural similarity or difference within and between families that may correlate with functional differences. Protein structure networks are useful for this purpose, as they directly encode the potential for direct physical interaction between functional groups (rather than representing detailed structure through properties such as side chain dihedral angles that can often vary substantially and dynamically without impacting protein function). In collaboration with Prof. Carter T. Butts, we employ the PSN representation of

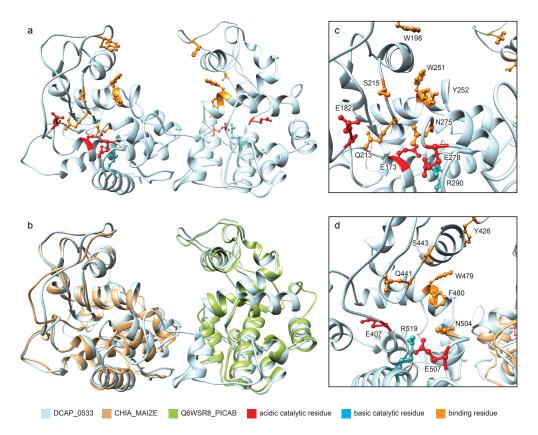


Figure 2.10: DCAP_0533 comparison with CHIA_MAIZE (4MCK) and Q6WSR8_PICAB (3HBE) and close up of catalytic residues and binding residues: (a) Robetta generated predicted structure with highlighted catalytic residues and binding residues. (b) Superimposition of CHIA_MAIZE and Q6WSR8_PICAB against DCAP_0533. (c) Catalytic site of NTD with 1-letter residue code and specifier. Catalytic triad consists of E173, E278, R290. (d) Catalytic site of CTD with 1-letter residue code and specifier. Catalytic triad consists of E407, E507, R519.

[69], where vertices represent small moieties and edges represent the potential for direct interaction (as determined by moiety-specific proximity constraints). Given two or more such PSNs, we may compare their topology by the structural distance method of [74], identifying the smallest number of edge changes (i.e. altered inter-moiety interactions) needed to make one PSN isomorphic to the other. Figure 2.11 depicts respective hierarchical clusterings of the Family 18 (panel A) and Family 19 (panel B) chitinases based on this notion of structural similarity, with distances normalized by the number of vertices to yield a metric with units of average changed interactions per moiety. For Family 18, the pattern of topological similarity is strikingly close to the pattern of sequence similarity, although somewhat more diversity can be seen among structures than among sequences (compare with Figure 4.9). By contrast, topological clustering of Family 19 chitinases shows substantial differences from the equivalent sequence-based clustering. For instance, while DCAP_0533, A9ZMK1_NEPAL, and Q6IV09_DRORT belong to an outlying but internally cohesive cluster with respect to sequence similarity, the three show markedly different topologies (and, indeed, are split between the two large structural clusters characterizing the family). More broadly, we find that the Family 19 chitinases divide structurally into two primary clusters (rather than the four obtained from sequence similarity), both of which are internally heterogeneous and neither of which maps cleanly onto the clusters found by sequence similarity. The relationship between sequence and structure is thus much more tightly coupled for Family 18 than Family 19.

Further insight into the structural differences between the two families can be obtained by considering variation in the properties of their respective PSNs. Here, we examine four basic graph-level indices (GLIs) related to protein network organization. Transitivity [93] is defined as the fraction of (i, j, k) two-paths for which there exists an (i, k) edge, and is a standard measure of triadic closure; in the PSN context, higher levels of transitivity are associated with structures that are closely and uniformly packed, with few cavities or extended regions. *Degree* is defined as the number of edges incident on a given vertex; for a PSN, this corresponds to the number of other moleties with which a given chemical group is in contact. The standard deviation of the degree distribution within a PSN then provides a measure of the level of heterogeneity in local packing around chemical groups, and we employ it here as a second GLI. At a somewhat less local level, the (degree) core number of a given vertex [94] provides a measure of the extent to which that vertex is embedded in a region of high cohesion within the graph. More precisely, the k-th core (or k-core) of a graph is defined as the maximum set of vertices having at least k neighbors within the set. The core number of a vertex is then the number of the highest-order k-core to which it belongs. Although each k-core is not necessarily cohesive as a whole, cores with $k \ge 2$ are composed of unions of cohesive subgraphs, such that all vertices with high core numbers necessarily belong to highly cohesive subgroups. In a PSN context, cohesive subgroups of moieties are joined by multiple, redundant paths and cannot be pulled apart without severing large numbers of edges. At the level of the entire PSN, then, the standard deviation of the core number serves as an indicator of the degree of heterogeneity in structural cohesion, and distinguishes between highly organized structures and structures that combine rigidly and loosely bound regions. Finally, we consider an indicator of the global path structure within the PSN, which we call *M-eccentricity*. The *eccentricity* of a vertex is the maximum geodesic distance from that vertex to any other vertex in the graph [95]; we here refer to the corresponding mean geodesic distance as the M-eccentricity. Vertices with high M-eccentricity are on average peripheral to the graph structure, while those with low M-eccentricity are relatively centrally located. At the level of the PSN as a whole, the standard deviation of the M-eccentricity distinguishes between uniformly globular structures and structures with deformations or other elongations, and we employ it as our fourth GLI.

Panel C of Figure 2.11 shows the distribution of the above GLI values for both chitinase families. All GLIs were calculated using the sna library [75]; to facilitate visualization, each GLI was standardized across the combined set of PSNs by subtracting the mean and dividing by the standard deviation prior to analysis. As is clear from Figure 2.11, the two families differ markedly on these four characteristics. On average, the Family 18 structures are substantially more homogeneous with respect to extended structure, local packing, and cohesion, while also being less transitive (p < 0.001 for all measures, two-tailed *t*-test). With respect to variation within family, the Family 18 structures show significantly less variability in eccentricity heterogeneity and transitivity (permutation test of logged IQR ratios, respective p values < 1e - 5 and 0.007), but comparable variability with respect to heterogeneity in local packing and cohesion (respectively p = 0.146 and p = 0.064).

To provide an intuition for how these patterns play out in specific cases, Figure 2.12 shows vertex-level core numbers and M-eccentricity scores for the structures of CF821_NEPMI

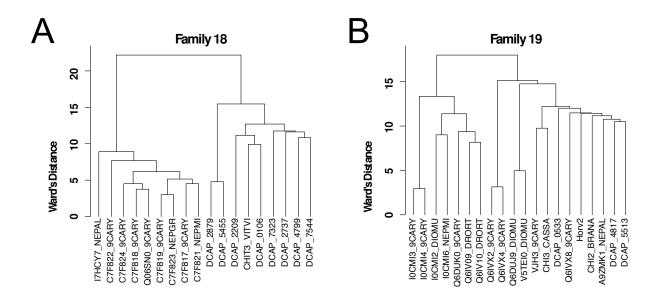


Figure 2.11: (a)-(b) Within-family clustering of chitinases by normalized structural distances. Ward's method (in the generalization of [4]) was employed to construct a hierarchical clustering of Family 18 (a) and Family 19 (b) chitinases based on topological dissimilarity. Sequence similarity is broadly recapitulated by the structural distances in Family 18, while Family 19 shows distinct patterns of variation.

(Family 18) and DCAP_5513 (Family 19). These structures were chosen by finding the PSN in each family with the smallest median distance to each other structure in the family, and are hence broadly representative of the classes in question. The core number visualizations of panels (a) and (b) clearly show that CF821_NEPMI is dominated be a large and uniformly cohesive core region, with few vertices in the outer region (i.e., lower cores). By contrast, the highly irregular structure of DCAP_5513 has numerous areas of low cohesion (including much of the C-rich domain) as well as the highly cohesive region associated with the central helices (compare with Figure 2.7). Differences in global structure are brought into sharp relief by the M-eccentricity visualizations of panels (c) and (d). The uniform and tightly connected topology of CF821_NEPMI results in a large number of vertices with short path distances to nearly all other chemical groups in the protein, and relatively little overall variation. Moieties in DCAP_5513, on the other hand, may be at an average distance of more than 9 steps from the rest of the protein, with large differences between the relatively central vertices in the helical region and those in the outer portions of the C-rich domain or the P-rich hinge.

Taken together with the findings above and Prof. Carter T. Butt's PSN analysis of chitinases from our paper [1], findings suggest substantial structural differences in the basic organization of the Family 18 and Family 19 chitinases, with the former having more internally homogeneous structures, and with structural differences being more closely related to differences in sequence. Family 19 is on the whole more diverse, and contains members that are on average less internally homogeneous. The presence of a higher volume of lowcohesion regions in the Family 19 chitinases suggests that these enzymes may be more prone to thermal denaturation than those in Family 18 (since low-cohesion regions require fewer disrupted edges to pull apart), but may also have functional significance (e.g., by allowing enhanced flexibility). Such structural insights from PSN topology complement those gained by studying specific features, and are more easily extended to analyzing large numbers of sequences.

2.4 Conclusion

Modeling and analysis of Family 18 and 19 chitinases from *D. capensis* and several related species reveal a number of novel enzymes that present promising targets for subsequent expression and biophysical characterization. These include what is to our knowledge the first plant chitinase found with multiple active domains, as well as several proteins that differ in more conventional ways from others in their class. Comparative network analysis of these structures reveals within- and between-family differences in structural properties, with Family 18 chitinases tending to be substantially more homogeneous in internal structure and Family 19 chitinases showing variation in cohesion and packing with possible implications for both function and thermal stability. These results also demonstrate the potential of *in silico* pipelines to move rapidly from genomic DNA to predictions of tertiary structure and

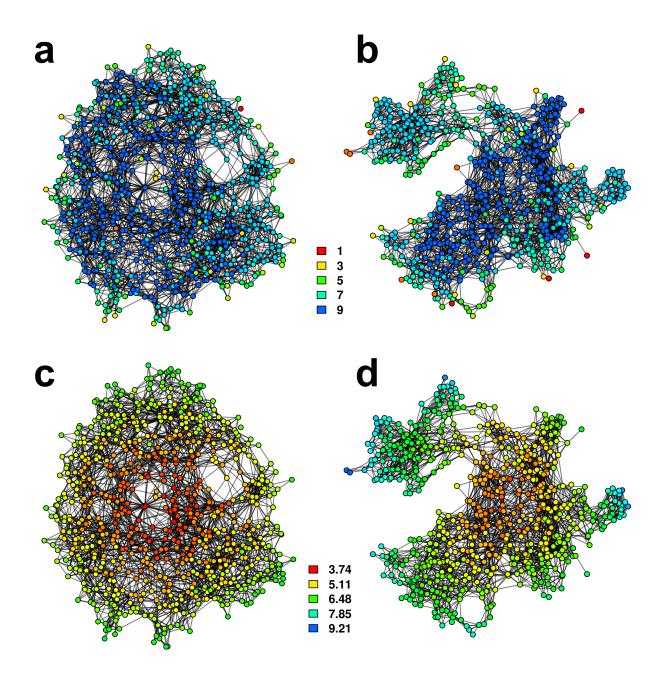


Figure 2.12: PSN Visualizations for family-representative structures C7F821_NEPMI (Family 18, (a) and (c)) and DCAP_5513 (Family 19, (b) and (d)). In panels (a) and (b), vertices are colored by k-core number; vertices with higher core numbers are embedded in more strongly cohesive local structures. Panels (c) and (d) show vertices by M-eccentricity (with higher values indicating a higher mean distance to other vertices in the network). The much higher level of internal heterogeneity in DCAP_5513 versus C7F821_NEPMI is immediately evident, with the former containing complex and irregular structure that subjects some vertices to higher levels of both cohesion and proximity than others.

comparative analysis thereof. As the "genomic revolution" makes such data available at an ever-increasing rate, such pipelines will become critical to our ability to exploit this scientific resource.

Chapter 3

Insights into esterase/lipases, phospholipases and nucleases from the carnivorous plant *Drosera capensis*

Drosera capensis represents a so far underexploited reservoir of novel enzymes with potentially useful activities. In this chapter I present the results from estarase/lipases, phospholipases and nucleases found in *Drosera capensis*.

3.1 Protein structure networks provide insight into active site flexibility in esterase/lipases from the carnivorous plant *Drosera capensis*

Esterase/lipases play important roles in plant defense, stress responses, and drought tolerance. Plant genomes and transcriptomes have provided a wealth of data about expression patterns and the circumstances under which these enzymes are upregulated, e.g. pathogen defense and response to drought; however predicting the function of these enzymes from genomic or transcriptome data is challenging due to weak sequence conservation among the diverse members of this group. Functional blocks mediating enzyme activity have been identified; however progress to date has been hampered by the paucity of information on the structural relationships among these sequence regions and their relationships with substrate specificity and enzymatic activity. We have developed a methodology for efficient target selection based on molecular modeling and analysis of protein structure networks. Here this approach is demonstrated for 26 previously uncharacterized esterase/lipases from the genome of the carnivorous plant *Drosera capensis*. Analysis of the network relationships among functional blocks and among the chemical moieties making up the catalytic triad reveals potentially functionally significant differences that are not apparent from sequence analysis alone.

I worked on choosing the protein set, determining the functional regions of interest, generating the predicted structures, analyzing sequence and structure data, performing sequence annotation and comparisons and wrote the manuscript with my co-authors on this paper. A portion of the paper [2] is reproduced in this chapter to understand the importance of the results found in this study. For more details and in-depth analysis, please refer the paper [2].

3.1.1 Background

In land plants, tissues that are exposed to air are protected by the cuticle, a composite biomaterial comprising a cross-linked polyester scaffold interpenetrated by wax components [96]. The cuticle provides a barrier that minimizes water loss and protects the plant from pathogen infection. The relative quantities of hydrophilic and hydrophobic components must be appropriately balanced and spatially located to adhere to the underlying cell walls while presenting a hydrophobic surface to the air interface [97]. Numerous enzymes are involved in producing the polymer components of this material, including esterases, lipases, and GDSL esterase/lipases. Esterase/lipases belong to the large α/β hydrolase enzyme superfamily, in which the catalytic triad consists of a nucleophile, an acid, and a stabilizing histidine (in this case Ser-Asp-His). This study focusses on the GDSL esterase/lipases, characterized by the proximity of the active serine residue to the N-terminus, as well as by its surrounding residues (canonically GDSL). Numerous plant GDSL esterase/lipases have been discovered from genome and transcriptome data [98, 99]; however their specific functions and substrate preferences remain relatively unexplored despite their potential commercial and technological importance. Much of what is known about the specific enzymatic activities of proteins in this family comes from studies of crop plants that produce large fruits. [100]. Esterase/lipases present attractive targets for biotechnology applications because of their potential for producing robust yet ultimately biodegradable polyester materials and hydrophobic surface coatings.

This study presents molecular modeling and functional analyses of 26 esterase/lipases recently discovered from the genome of the Cape sundew (*Drosera capensis*) [47]. The conservation of active site residues, key functional sequence blocks, and overall protein folds suggests that many of the *D. capensis* esterase/lipase sequences form functional enzymes; however the diversity of sequence and structural features indicates a range of potential molecular targets and enzymatic activities. The study uses sequence analysis, comparative modeling with all-atom refinement followed by *in silico* maturation and comparison of protein structure networks to identify distinct subgroups of proteins as a first step toward target selection for subsequent expression and biochemical characterization. To enable analysis of structural features with potential functional relevance, the study defines two novel types of *functionally-targeted protein structure networks* (FT-PSNs) generated using functional information specific to this protein class. Clustering of FT-PSNs based on connectivity among functional blocks reveals several classes with distinct structural characteristics, which we hypothesize are related to enzyme flexibility and activity. Network connectivity among functional sequence blocks acts as a useful descriptor of protein structure and a predictor of global flexibility, while FT-PSNs based on connectivity among chemical moieties in the neighborhood of the active site are used to construct measures hypothesized to correlate with active site flexibility and hence enzyme promiscuity. Comparison with well-characterized reference sequences suggests that most of the D. capensis esterase/lipases have relatively rigid active sites, consistent with the specific functionality of the tomato GDSL1 enzyme, the best-characterized member of this class so far.

3.1.2 Methods

Sequence Alignments

Sequence alignments for the esterase/lipases from D. capensis are shown along with annotation reference sequences from other plants. Cluster 1 (Figure 3.1) contains enzymes with the traditional GDSL motif, including GDL1_CARPA from *Carica papaya*. Cluster 2 (Figure 3.2) contains only sequences from D. capensis, while Cluster 3 (Figure 3.3) contains two reference sequences from *Arabidopsis thaliana*. Cluster 4 is split into two figures for legibility (Figures 3.4 and 3.5). The alignment figures are annotated the same as chitinases (Chapter 2). Additionally, strikethrough text indicates sequence regions that are absent in the active enzyme, in this case the N-terminal signal peptide that is expressed but removed during maturation. Functional blocks I-IV are highlighted with colored boxes. Annotations were performed by homology to the annotations reference sequences from *C. papaya* and *A. thaliana* found in the UniProt database and identified by their UniProt IDs.

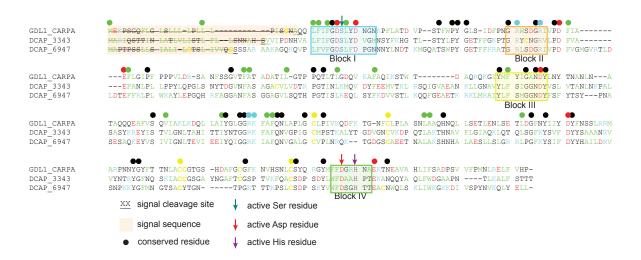


Figure 3.1: Sequence alignment for Cluster 1 esterase/lipases, annotated by homology to the reference sequence GDL1_CARPA. The four functional blocks that are critical for enzyme function are highlighted using outlined colored boxes. The N-terminal signal peptide is highlighted in light orange. Colored arrows indicate the catalytic triad residues. Conserved residues are marked using colored dots: acidic (red), basic (blue), hydrophobic (green), and hydrophilic (black) residues.

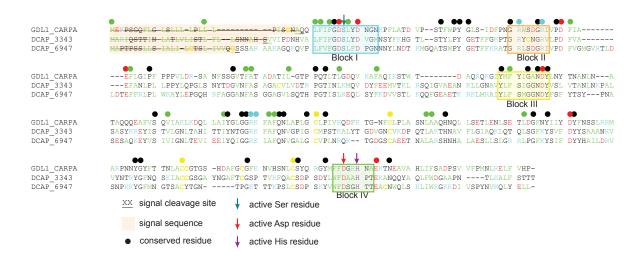


Figure 3.2: Sequence alignment and annotation for Cluster 2. The four block regions are determined by sequence conservation and outlined with colored boxes. Three *D. capensis* esterase/lipases contain the N-terminal signal sequence (highlighted in light orange) and three lack it. The catalytic triad is indicated using colored arrows. Colored dots denote conserved residues.

GLIP6 ARATH	MSSSSSMD-LIMCLLL-LI	
GDL77 ARATH	MTIS TVIAEMSMEL VEV	
DCAP 1840		MMGEG TDVNKRLGAS
DCAP 1460	MR_MARVDDHPHR_RLFMVQATFV_SM	
DCAP 1380	METVGPLGQD YDWAEEDDDD DMNGMEHACM GYISLLHHAT LIRRTALPFE IQQSAAYF HIVKLKINIS FVIERKSNG	A GVPAPHDVAD AAGSLOVPSM
DCAP 0405	MON HSLKWMALYL SIV	
DCAP 4465		AA VVTDPYFPAM
-		
		••••
GLIP6_ARATH	FTFGDSIFDA GNNHYNKNCT AQADFPPYGS SFFHRPT GRFTNGRTVA DFISEFVGLP LQ-KPFLELQ IQILNGTSN	F SNGINFASAG SGLLLDTNKF
GDL77_ARATH	FVFGDSLVDS GNNNYL-VTT ARADSPPYGI DFPTRRPT GRFSNGLNIP DLISEAIGNE EPPLPYLSPELRGRS	L LNGANFASAG IGILNDTGFQ
DCAP_1840	FIFGDSLVDA GNNNFL-PTL SRANVTPNGI DFKASGGTPT GRYSNGRTMS DIIGEELGQS NYAVPFLAPNSTGKA	I LHGVNYASGG GGIINSTGSI
DCAP_1460	FIFGDSLVDN GNNNNI-ASL ARSNYLPYGI DFPQGPT GRFSNGKTTV DVITQLLGFD DY-IP-PHATASGEQ	I LKGVNFASAA AGIREETGQQ
DCAP_1380	FVFGDSIIDV GNNNFL-NSV AKSNFWPYGC DFSRGPT GRFSNGKTVV DFIGELLGIS NI-PAFADPATAGTK	V VTGINYASAA AGILDETGRH
DCAP_0405	FVFGDSVIDS GNNNYL-NSA AKANYFPYGI EFEQGPT GRFCNGRTFM DYLAEMLGLP RI-PAFANPLETGHG	
DCAP_4465	FVFGDSVIDS GNNNYL-NSV AKANYFPYGI DFEQGPT GRFCNGRTFI DYLAEMLGLR RI-PAFANPFETGHG	I LHGINYASAS AGILEETGLA
	Block I Block II	
	• • •• •••	
GLIP6_ARATH	MGVTPIQTQL QQFQTLVEQN LIEKSIIQES L <mark>FLLETGSND I</mark> F-NYFLPFR APTLS	
GDL77_ARATH	FINIIRMYQQL DYFQQYQQRV SRLIGKPQTQRLVSQA I <mark>VLITVGGND F</mark> VNNYFLFPY SAR-SRQFT	
DCAP_1840	FIASVVIHWQ INRLGMDVQV DYFNITRKQC DRLLGASNTRNYIMHES I <mark>FSITIGSND FL</mark> NNYLLPFS SVGSRITQS	
DCAP_1460	LGGRISFNGQV NNYKNTVSQI VNILGDEDSAASYLSKC MYTIGLGNND YLNNYFMPLY YST-SNRYT	
DCAP_1380	WGDRYSLSQQV LNFESTLGQL KNTMATANPT FNMSQHLAKS MVFMSFGSND YLNNYLLPSL YTS-SFTYT	
DCAP_0405	LGDRFSLRRQV QNFEITLTQL RGPMTETEMKDYLPNV LMMMVLGSND YLNNYLLPTL YTS-PKTSQ	
DCAP_4465	LGDRFSLRRQV QNFEITLTQL RGQITETEMKDYLPNV LMMMVLGSND YLNNYLLPTL YPS-SNLYK	P ENFADILISE YNRQIVTLHS
	Block III	
CLID(ADAMU	LGARRIAFFS LGPVGCVPAR AMLPNAPTNK CFGKMNVMAK MYNKRLEDIV NIIPTKYPGA IAVFGAVYGI THRFOTYPA	
GLIP6_ARATH	LGARRIARYS LGPVGCVPAR AMLPNAPINK CFGAMNVMAR MINKRLEDIV NIIPTKIPGA IAVFGAVIGI THRPQTIPA LGVGRVLVTG AGPLGCAPAE LARSGISNGR CSAELQRAAS LYDPOLLOMI NELNKKIGRN VFIAANINOM QEDFLSIPR	
GDL77_ARATH DCAP 1840	LDARKFSVGS VGPIGCIPYQ KIINOINADO CADLPNKMAL AYNSKIKDLI IOLNKNIPGA TFVYANVYDI VMELISNYK	
DCAP_1840 DCAP_1460	FGARKMALIG IGOIGCSPNE LANNSPDGKT CDGKINSANO MFNGKLKSLV DOLNSOFSDA HFIYINAYDI FODMLDNPA	
DCAP_1480 DCAP_1380	IGLRKFFLAG IAPLGCIPNQ RASGLAPAGR CVDSVNQMLG SFNEGLRSLV DQLNSQFSDA HFITINAIDI FQDMLDMFA	
DCAP 0405	ASI TSRPWLEKLA DOLNSNHPDA KFVYGDTYAA SMDMIANGS	
DCAP 4465	LGLRKFFMAG IGPLGCIPNO RASGAGPPGK CVSAVNDMVL MENLRLDKLA DOLNSNHPDA KFVYGDTIAA SMDMIANGS	
GLIP6 ARATH	MOCGREGYKI CNNPNEFLEW DFYHPTEHTY RLMSKALWNG N-KNHIRPFN LMALATNKIT F	
GDL77 ARATH	GLCTVL-SNL CPNRELYVW DAFHPTEKAN RMIVRHILTG T-TKYMNPMN LSSALAL XX signal cleavage s	site 🚽 active Ser residue
DCAP 1840	VPCGPT-SSL CODRHKHVEW DPYHPSEAAN LILAKOLMDG D-TKYVSPIN LROLRDL	,
DCAP 1460	ITCLPM-QQP CPNRNEYLFW DAYHPTEAAN IVVGTRSYRA QSASDAYPYD IQHLAQL signal sequence	🖌 active Asp residue
DCAP 1380	ITCLPY-AVP CANRTQYVFW DAFHVTEAVN SLLARRAFFG P-PTDCYPIN IQQLALL	The second secon
DCAP 0405	VSCLPL-LPP CANRDEYVFW DAFHPTQAAN KTLAAEAYKI I-LSKF • conserved residu	Je 🚽 active His residue
DCAP_4465	VSCLPL-LPP CANRDEYVFW DAFHPTQAAN KILAAEAYKI I-LSKF	Ŧ
-	Block IV	

Figure 3.3: Sequence alignment and annotation for Cluster 3. Reference sequences are GLIP6_ARATH and GDL77_ARATH. All but three Cluster 3 esterase/lipases contain a N-terminal signal peptide (highlighted in light orange). Functional block regions are outlined using colored boxes. Colored dots indicate conserved residues.

Preliminary Structural Models and In Silico Maturation

The study performs *in silico* maturation, which I have previously described for chitinases [21], for each protein. Figure 3.6A shows the workflow of the overall enzyme discovery process as described in Chapter 1. Panels (B) and (C) show an example of a Cluster 2 esterase/lipase, DCAP_8086, before (B) and after (C) the *in silico* maturation process. Further comparison of a Cluster 3 esterase/lipase (DCAP_1460) to Cluster 4 enzymes and a cutin synthase from *Solanum lycopersicum* (tomato), G1DEX3_SOLLC, is shown in Figure 3.7. Functional sequence blocks DCAP_1460 and G1DEX3_SOLLC are highlighted by color (Figure S3.7). DCAP_4076, has an additional C-terminal domain. A PSI-BLAST search for the sequence of this domain indicated that it is related to the negative regulator of systemic acquired

	• <u>• • • • • • • • • • • • • • • • • • </u>
DCAP_6218	
DCAP_6260	MIYSGKGRMH QKNPSPT KIS AISIIHVUV LHRGTHSQSL PQNVTVPALF FFGDSVVDPG NBDWILSVCR ADHPPYGRDF EGGVATGRFS
EXL3_ARATH DCAP 1761	MKDNS-SW-SCSCSWSSWKIC-LLSVLF-LTETITAVKL PPKLIIPAVI AFGDSIVDTG MNNNKTVVK CDFLPYGINF QSCVATGRFC MQ RLYSSMN-QK SLAAKLVVFQ -FIATLHMLSAY KLGRRVPAVF VFGDSIVDTG NNNYLQTIGK CDFPPYGCDF PTGLPTGRYS
DCAP_1701 DCAP_6217	
DCAP 5461	
DCAP 0158	
DCAP 2088	PINATVPGLF VFGDSVDFG NNYNSTLCK SNFPPYGRDF PGGQATGRFS
DCAP 2089	
	Block I Block II
	•••••••••••
DCAP_6218	NAKIPTDYLA ESLGIKDLLP AYLDPTIETE DLLTGVSFAF ACCGYDPLTP QFFVSIHFHF DFYSSSTCIA LVVVHGWILS VTERVPSLQD QLEYFKEYKK
DCAP_6260	NAKIPSDYTA QEFGVKELLP AYLDISLRTE DLLTGVSFAF TCSGYDPMTS L-IF
EXL3_ARATH	DGRVPADLLA EELGIKSIVP AYLDPNLKSK DLLTGVSFAS GGSGYDPITP K-LVAVISLED QLSYFEEYIE
DCAP_1761	NGKVPADLIV EKLGIKEYLP PYLNQSLEFQ DLVTGVNFAT GATGYDPVSA Q-LA
DCAP_6217	NGKIPTDLFA ELLGIKELLP AYLDPTLTTQ DLLTGVSFGS GVAGYDPVSA A-LL
DCAP_5461	NGYIPTDILA QELGIKEMVP SYLDEGLSPN DMLTGVSFAC GCSGWDPSTS RARY
DCAP_0158	NGKVPSDLFA EAFGVKELVP PYLDPSLTMD DLLTGVNFAS ACSGYLPATA LHLSPSLSLED QLDLFKEYIS
DCAP_2088	DGIVPSDIVA QAFGVKKFVP AYLDPSLSTD EMLTGVNFAS ACSGYLPLTA TYKC
DCAP_2089	DGIVPSDIVG NSERKLSNESSNHLV LFQYSSNHLV LFQY
DCAP 6218	KLIAAVGEER TSFIVSKS <mark>IY VVVAGSND</mark> T FTYNLFKL RKEMNMSSYT DLMISQASII LQALYDLG ARRIGVLQLL
DCAP 6260	KVKTAVGGKK ASYVISESYV LIVTGNDFT FNYYGS-LFR SLQYNVSSYC DLLTFASTF L-QELYNLG ARRIGAIGLP PQGCLPAMRT SAEGHSRPCN
EXL3 ARATH	KVKNIVGEAR KDFIVANSLE LLVAGSDDLA NTYTL-A RPEVDVDSYT TIMSDSASEF VTKLYGYG VRVAVFGAP PIGCVPSORT LGGGILRDCA
DCAP 1761	RMTARIGNSK ASKLISKSAY LIFAGSNDIT NTYFIJ-NIR RPQ10/DSYT DLUSFASKF Y-QALYAEG VRMGVMNIP PIGCLPSQHT FAGGLQRSCV
DCAP 6217	KVVAAVGONE TAELISKSWY LVCAGSDD/T DTYFAT-PFR KPFVMTAYA DLLKGFANFF VKELYSMG ARKIGVFSVP KSCLPSART LFGGLFYTCV
DCAP 5461	NMKLEVGDEN VSSILSOAFY LVGTGVNDFL NNFDDPSSER RRNHDLASYT DLLIRFASNS LKALYSLG ARRIGIFGIP PYGHIPPSRT TAGGVFRAPV
DCAP 0158	KLTAEVGEQK TSSLLSQCIL LLATGSNDFN YFYDTQKNGNITAFL DILVNYTSGF LTPIVI
DCAP 2088	KPLYH NNKORLYDTG ARRVVLGDSA POGCVPAART NYGGLLRFIV
DCAP 2089	KVKAAVGEER TTRIISOS <mark>IF IICTGSND</mark> FL YYYETQKSGNMSAYT DSVVNYASGF LKVRRLYDIG ARRIVLGGAP POGCLPAART NYGGLLRFIV
-	Block III
	· · · · · · · · · · · · · · · · · · ·
DCAP_6218	TLY LRGKNPDSRF VYLDLYNPVL RLVQNPTQSG FQISNVG CCATGTSETS IFCNSLFD LFSTCKNESE YLFWDAYHPT
DCAP_6260	KQYNQNAMLF NSKLESIMGS IGKNLTGAKI VYLDLYGPLL QLVKNPENFV IPESPVVTKA RKGDTNREMV -FCALRLISR ETLRCTECSS YKASISISKM
EXL3_ARATH	DNYNEAAKLF NSKLSPKLDS LRKTLPGIKP IYINIYDPLF DIIQNPANYG FEVSNKG CCGTGAIEVA VLCNKIT S-SVCPDVST HVFWDSYHPT
DCAP_1761	DEYNKAALLF NSKLNTEIES LNRNLSGVAM FFLDVYAPLL DLINNPSQAG FEVVDKG CCGTGNIEVS FLCNRLE NLLTCKDATK YIFWDSFHPT
DCAP_6217	PQFDQLALLF NSKLQETVVD LNKNLTGAKL AYIDLYQPLA HLINNGSEYG FQVVNRG CCGTGLFEAS ILCNPFDITCKNDSQ YIFWDAFHPT
DCAP_5461	TEFNNAATLF NFKLQTLIDS INRNFPGAKF GYLDIYSKLM YVIENAADFG CKVNDRG CCGTGLVEMG VACNGLV DIFSCKNNSE YVEWDAAHPT
DCAP_0158	
DCAP_2088	ESFNQDSLDF NLKLQAMLKS LKNTLQGSRF AYFDLYYTVL DLIQKPHEYG FEVVGKG CCGTGFFEEG PLCNIFS SLISCPNASK YVEWDASHPT
DCAP_2089	ESFNQDTLNF NLKLQTMLKS LQNTLQGSRF IYFDFYYTVL DLVQKPHDYG FEVVDKG CCGTGLFEEG PLCNIVS TLISCPNASK YVFWDASHET
	Block IV
DCAP 6218	DRANKIIIEE LFGKKMVSMA CINFGCPEGL SVELAGRTGN RTLASITRAF VQSQRGDIV
DCAP_6218 DCAP_6260	MINUTIE II CARAVOR CIN COLECE SEBREREN KINSTRA VYSTADIV
EXL3 ARATH	BRTYKVLVSL LINKFVNOFV XX signal cleavage site 🗸 active Ser residue
DCAP 1761	BATYRITVDA AVKONINFY
DCAP 6217	QRTQLLVNG LVNTTINDLY N signal sequence dative Asp residue
DCAP 5461	
DCAP 0158	QAAYHVUDR ILINV™PPEF ● conserved residue ↓ active His residue
DCAP 2088	OAAYNIVLAK NFNOTMSKFF
DCAP 2089	QAAYNIIVAR NINQTMSQFF

Figure 3.4: Sequence alignment and annotation of Cluster 4a (first set), annotated by homology to EXL3_ARATH. Cluster 4 is separated into two parts (4a and 4b) for clarity. Block regions I-IV are shown in colored boxes with active site residues marked by colored arrows. Colored dots indicate conserved residues. When present, the N-terminal signal peptide is highlighted in light orange.

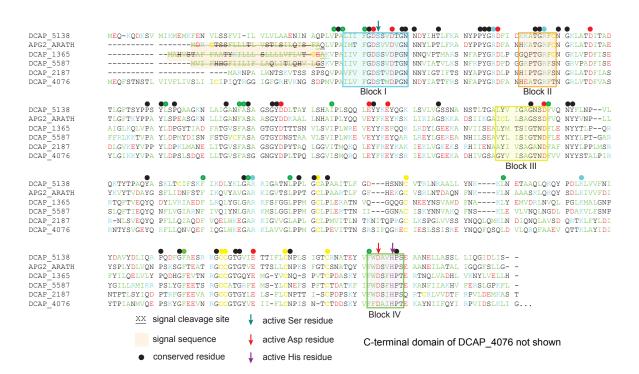


Figure 3.5: Sequence alignment and annotation of Cluster 4b (second set), annotated by homology to APG2_ARATH. Cluster 4 is separated into two parts (4a and 4b) for clarity. Block regions I-IV are shown in colored boxes with active site residues marked by colored arrows. Colored dots indicate conserved residues. When present, the N-terminal signal peptide is highlighted in light orange. DCAP_4076 has an additional C-terminal domain (shown in Figure S3.8).

resistance proteins previously discovered in other plants [2], with approximately 36% sequence identity to the SNI1 proteins from *Arabidopsis thaliana* (Uniprot ID: SNI1_ARATH) and *Glycine max* (Uniprot ID: Q0ZFU8_SOYBN). The *Arabidopsis* protein negatively regulates DNA recombination and gene expression during short-term stress responses. It has been suggested that SNI1_ARATH provides a scaffold for other proteins involved in regulation of transcription to bind; it is possible that this domain is playing a similar role here. DCAP_4076 lacks the N-terminal secretion signal common to many of the esterase/lipases, suggesting an intracellular function (Figure 3.8). The above results were mostly collected by Vy, Prof. Martin and Prof. Butts and are reproduced here to illustrate the importance of estarase/lipases.

The template structures used by Rosetta to calculate the predicted structures for a representative esterase / lipase, DCAP_0434, are tabulated in Table 1.

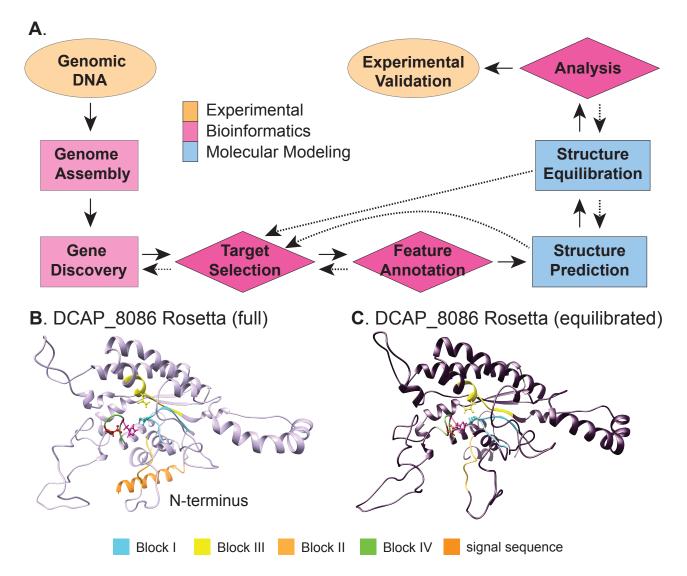


Figure 3.6: (A) Flow chart, made by me, illustrating the overall strategy for identifying enzymatic targets from genomic DNA. The workflow is indicated with solid arrows, while dotted arrows represent steps where information from a later stage of the pipeline enables refinement of earlier stages in an iterative manner. After genome sequencing, assembly, and gene discovery, target proteins are identified based on putative enzymatic activity. Functional sequence features are identified by analogy to annotation reference sequences found in the UniProt database. Structures are predicted using the Rosetta software, and equilibrated in explicit solvent after removal of sequence regions not present in the mature enzyme. Structures are compared using network analytic methods, enabling strategic selection of enzymes for experimental characterization in a future study. (B) DCAP_8086 before and (C) after *in silico* maturation. The light orange helix in part A is the N-terminal signal sequence, which is cleaved upon maturation. Important residues are color-coded as follows: dark cyan (catalytically active serine), red (active site aspartic acid), purple (active site histidine).

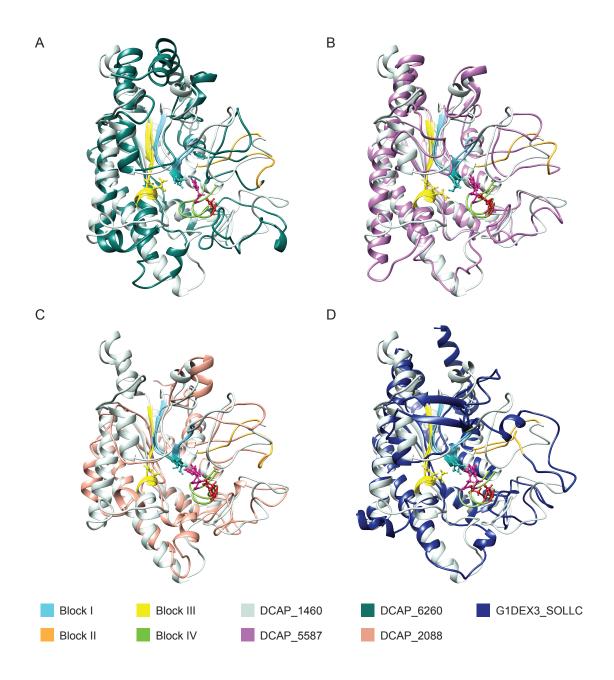


Figure 3.7: Comparison of DCAP_1460 (Cluster 3) to *D. capensis* esterase/lipases from each of the other clusters. These pairwise alignments of structural models provide an indication of the type and magnitude of structural differences between clusters: in general, the overall fold and secondary structural elements is conserved, although considerable variation can be observed in their relative positions and the conformations of loops and termini. Alignment was performed using the matchmaker feature of Chimera with default settings. Functional block regions I-IV are colored accordingly while the catalytic triad (Ser-His-Asp) residues are colored dark cyan, red, and purple. Active site residues are located in block I and IV, binding residues in block II-III. A. Comparison of DCAP_1460 to esterase/lipase DCAP_6260 (Cluster 4a). B. Comparison of DCAP_1460 to DCAP_5587 (Cluster 4b). C. Comparison of DCAP_1460 to DCAP_2088 (Cluster 4a). D. Comparison of DCAP_1460 to model esterase/lipase, G1DEX3_SOLLC, from *Solanum lycopersicum* (tomato).

Protein	Organism	Sequence Elements included	File Name
GDL1_CARPA	Carica papaya	signal, active region	$GDL1_CARPA_m1.pdb$
DCAP_3343	D. capensis	signal, active region	DCAP_3343_m1.pdb
$DCAP_{-}6947$	D. capensis	signal, active region	$DCAP_{6947}m1.pdb$
DCAP_0448	$D. \ capens is$	signal, active region	DCAP_0448_m1.pdb
DCAP_8086	D. capensis	signal, active region	$DCAP_{8086_m1.pdb}$
DCAP_0434	D. capensis	active region	DCAP_0434_m1.pdb
DCAP_4098	D. capensis	active region	$DCAP_4098_m1.pdb$
DCAP_5529	D. capensis	signal, active region	$DCAP_5529_m1.pdb$
DCAP_5165	D. capensis	active region	$DCAP_5165_m1.pdb$
GLIP6_ARATH	A. thaliana	signal, active region	GLIP6_ARATH_m1.pdb
GDL77_ARATH	A. thaliana	signal, active region	$GDL77_ARATH_m1.pdb$
DCAP_1840	D. capensis	active region	$DCAP_{1840_m1.pdb}$
DCAP_1460	D. capensis	signal, active region	$DCAP_1460_m1.pdb$
DCAP_1380	D. capensis	active region	DCAP_1380_m1.pdb
DCAP_0405	D. capensis	signal, active region	DCAP_0405_m1.pdb
$DCAP_{-}4465$	D. capensis	active region	DCAP_4465_m1.pdb
DCAP_6218	D. capensis	active region	DCAP_6218_m1.pdb
DCAP_6260	D. capensis	active region	DCAP_6260_m1.pdb
EXL3_ARATH	A. thaliana	signal, active region	EXL3_ARATH _m1.pdb
DCAP_1761	D. capensis	active region	$DCAP_1761_m1.pdb$
DCAP_6217	D. capensis	signal, active region	$DCAP_6217_m1.pdb$
DCAP_5461	D. capensis	signal, active region	DCAP_5461_m1.pdb
DCAP_0158	D. capensis	signal, active region	DCAP_0158_m1.pdb
DCAP_2088	D. capensis	active region	DCAP_2088_m1.pdb
DCAP_2089	D. capensis	active region	DCAP_2089_m1.pdb
DCAP_5138	D. capensis	active region	DCAP_5138_m1.pdb
APG2_ARATH	A. thaliana	signal, active region	APG2_ARATH _m1.pdb
DCAP_1365	D. capensis	signal, active region	DCAP_1365_m1.pdb
$DCAP_{5587}$	D. capensis	signal, active region	DCAP_5587_m1.pdb
DCAP_2187	D. capensis	active region	DCAP_2187_m1.pdb
DCAP_4076	D. capensis	active region	DCAP_4076_m1.pdb

Table 1: Rosetta structures for esterase / lipases (PDB files available for download)

All initial and equilibrated structures available for download as PDB files are tabulated in Tables 1 and 2, respectively.

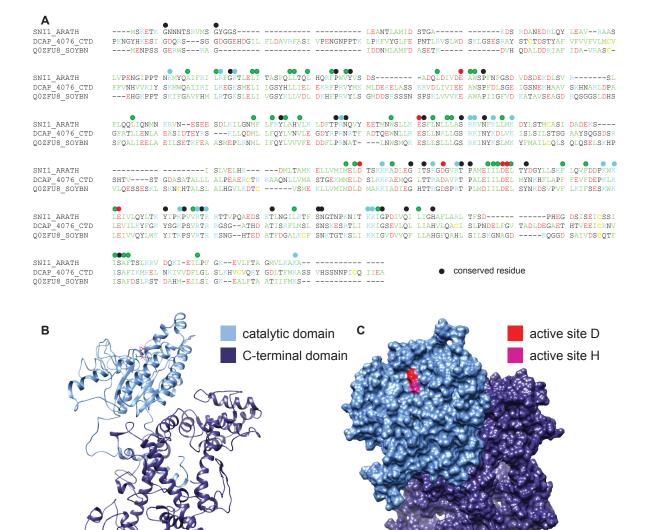


Figure 3.8: A. Sequence alignment of the C-terminal domain of DCAP_4076 with the SNI1 proteins from *Arabidopsis thaliana* (Uniprot ID: SNI1_ARATH) and *Glycine max* (Uniprot ID: Q0ZFU8_SOYBN). B. Ribbon structure of DCAP_4076, with the catalytic domain in light blue and the C-terminal domain in dark blue. C. Structural model of DCAP_4076 showing the surface representation. The active site D (red) and H (magenta) residues are visible at the top of the model.

Protein	Organism	Sequence Elements included	File Name
GDL1_CARPA	Carica papaya	active region	GDL1_CARPA_mature_m1.pdb
DCAP_3343	D. capensis	active region	DCAP_3343_mature_m1.pdb
DCAP_6947	D. capensis	active region	$DCAP_{6947}mature_m1.pdb$
DCAP_0448	D. capensis	active region	$DCAP_0448_mature_m1.pdb$
DCAP_8086	D. capensis	active region	$DCAP_{8086_mature_m1.pdb}$
DCAP_0434	D. capensis	active region	$DCAP_0434_mature_m1.pdb$
DCAP_4098	D. capensis	active region	$DCAP_{4098}mature_m1.pdb$
$DCAP_{-5529}$	D. capensis	active region	$DCAP_5529_mature_m1.pdb$
$DCAP_{-5165}$	D. capensis	active region	$DCAP_5165_mature_m1.pdb$
GLIP6_ARATH	A. thaliana	active region	$GLIP6_ARATH_mature_m1.pdb$
$GDL77_ARATH$	A. thaliana	active region	$GDL77_ARATH_mature_m1.pdb$
DCAP_1840	D. capensis	active region	$DCAP_{1840}mature_m1.pdb$
$DCAP_{-}1460$	D. capensis	active region	$DCAP_{1460_mature_m1.pdb}$
DCAP_1380	D. capensis	active region	$DCAP_{1380}mature_m1.pdb$
DCAP_0405	D. capensis	active region	$DCAP_0405_mature_m1.pdb$
$DCAP_4465$	D. capensis	active region	$DCAP_4465_mature_m1.pdb$
DCAP_6218	D. capensis	active region	$DCAP_6218_mature_m1.pdb$
DCAP_6260	D. capensis	active region	$DCAP_{6260}mature_m1.pdb$
EXL3_ARATH	A. thaliana	active region	$EXL3_ARATH _mature_m1.pdb$
$DCAP_{-1761}$	D. capensis	active region	$DCAP_1761_mature_m1.pdb$
$DCAP_{-6217}$	D. capensis	active region	$DCAP_6217_mature_m1.pdb$
$DCAP_5461$	D. capensis	active region	$DCAP_5461_mature_m1.pdb$
DCAP_0158	D. capensis	active region	$DCAP_0158_mature_m1.pdb$
DCAP_2088	D. capensis	active region	$DCAP_{2088}mature_m1.pdb$
DCAP_2089	D. capensis	active region	$DCAP_{2089}mature_m1.pdb$
$DCAP_{-5138}$	D. capensis	active region	$DCAP_5138_mature_m1.pdb$
APG2_ARATH	A. thaliana	active region	APG2_ARATH _mature_m1.pdb
$DCAP_{-1365}$	D. capensis	active region	$DCAP_{1365}mature_m1.pdb$
$DCAP_{-}5587$	D. capensis	active region	$DCAP_5587_mature_m1.pdb$
DCAP_2187	D. capensis	active region	$DCAP_{2187}mature_m1.pdb$
DCAP_4076	D. capensis	active region	$DCAP_4076_mature_m1.pdb$

Table 2: Mature structures for esterase / lipases (PDB files available for download)

Network Modeling and Analysis

A protein structure network for each protein was calculated as described in chaitinases (Chapter 2) by Dr. Carter Butts. These structures were then secondarily processed to construct functionally targeted PSNs (FT-PSNs) using the sna library [75] within statnet. For further details, please look at the paper [2].

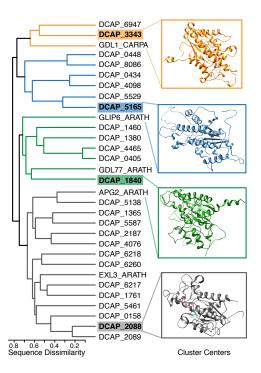


Figure 3.9: Clustering of esterase/lipase sequences identified from the D. capensis genome along with reference sequences from other plants.

3.1.3 Results and Discussion

D. capensis Esterase/Lipases Cluster Into Distinct Subfamilies Based on Sequence Features

Cluster 1 (Figure 4.9) contains sequences that have the canonical GDSL motif, as found in the reference sequence GDL1_CARPA, which was isolated from papaya latex [101] and has been proposed as a "naturally immobilized" biocatalyst for performing regioselective esterification and transesterification reactions [102]. The enzymes in cluster 2 instead have GDSN in the first functional block. Clusters 3 and 4 contain the motif GDSX, where X is usually a hydrophobic residue, but is Ser or Thr in some cases. Overall, the presence of the three active site residues in 24 of the 25 *D. capensis* esterase/lipases suggests they are functionally active enzymes.

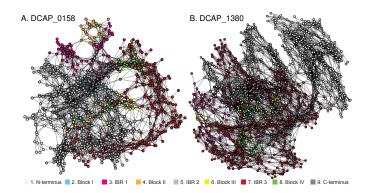


Figure 3.10: A. The sequences of the four conserved blocks. The sizes of the residue labels correlate with the fraction of sequences in the cluster having that residue in the indicated position. Amino acid properties are color coded as follows: hydrophobic-green, positive-blue, negative-red, cysteine-yellow, other-black. B. A representative molecular model of a *D. capensis* esterase/lipase (DCAP_0434) with the four functional blocks highlighted. C. The active site catalytic triad for a typical esterase/lipase (DCAP_0434).

Conserved Active Site Residues Suggest Functional Enzymes

In general, esterase/lipases are characterized by four moderately conserved sequence blocks of length 8-13 residues that contain the cataytic triad, the oxyanion hole proton donors, and other functionally important residues [103]. Functional sequence blocks I-IV are highlighted in the sequence alignments in the methods section. In Figure 3.10A, these functional blocks are represented as sequence logos, where the size of each residue label correlates with the number of instances at that sequence position within each cluster. The Ser-Asp-His catalytic triad is located within two block regions: block I (Ser) and block IV (Asp-His). The remaining two blocks contain conserved oxyanion hole residues, Gly in block II and Asn in block III [100]. Most of the proteins in this set contain the expected functional residues, as exemplified by the reference sequences GDL1_CARPA, GLIP6_ARATH, and GDL7_ARATH, as well as the well-characterized tomato GDSL esterase/lipase G1DEX3_SOLLC. Other results, including but not limited to PSN's and molecular modelling can be found in [2]; I have only included the results that I was directly involved in.

3.1.4 Conclusion

In summary, molecular modeling and protein structure network analysis of 26 esterase/lipases identified from the genomic DNA of *Drosera capensis* suggest that—with the exception of one protein, DCAP_3343—the active site regions of these enzymes are less flexible than those of related microbial proteins. The four clusters produced by the initial sequence analysis and clustering provides a stark contrast to the more limited esterase/lipase inventory typical of microbes (as evidenced by Uniprot searches). Subsequent principal component analysis of active site moieties generated from PSNs further categorized the *D. capensis* and reference sequences from decreasing to increasing active site rigidity. Together, these findings from comparative sequence and structural analyses demonstrate the diverse, *Drosera capensis* esterase/lipase landscape employed in carnivory, defense, and a plethora of functionalities with potentially significant, biotechnological applications.

3.2 The Phospholipases Found in *D. capensis* Form Four Clusters with Homology to Known Sequences

Phospholipases are a diverse set of enzymes that hydrolyze phospholipids. In plants, phospholipase D, phospholipase C, phospholipase A1 (PLA1), and phospholipase A2 (PLA2) have been characterized, that hydrolyze glycerophospholipids at different ester bonds as seen in Figure 3.12 [104, 105]. These enzymes are involved in a broad range of functions in cellular regulation and development, lipid metabolism, abiotic and biotic stress responses and membrane remodeling [104, 105, 106]. Within each type of phospholipase family, there are different families or subfamilies of enzymes that can differ in substrate specificity, cofactor requirement, and/or reaction conditions. These differences provide insights into determining the cellular function of specific phospholipases in plants, and they can be explored for

different industrial applications [104, 105, 106].

With Prof. Rachel Martin, I choose the protein set, generated the predicted structures, and analyzed the sequence and structure data as seen in Figure 3.11. The other team members include Shanon Zhuang, Michelle Xu, and Dr. John E. Kelly with Prof. Carter T. Butts. I performed sequence annotation and comparisons with the team as seen in Figure 3.14 and Figure 3.15. We are currently in the process of analyzing the structures and writing the manuscript.

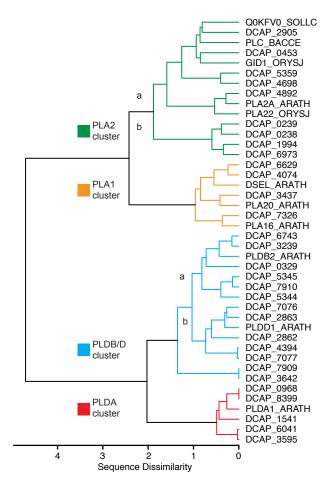


Figure 3.11: The current chosen set for phospholipases is seen in the Figure 3.11 with four different families, PLA2 (shown in green), PLA1 (shown in orange), PLDB/D (shown in blue) and PLDA (shown in red) found in D. capensis

The current chosen set for phospholipases is seen in the Figure 3.11 with four different families, PLA2 (shown in green), PLA1 (shown in orange), PLDB/D (shown in blue) and PLDA (shown in red) in *D. capensis*. An example of the phospholipases found in *D. capensis* is seen in Figure 3.12 where the active site residues are highlighted and lablelled, and the propeptide, C2 and a PLD domain are highlighted in salmon, green and aqua colors respectively. The figure also shows the cut sites of different enzymes on a phospholipase.

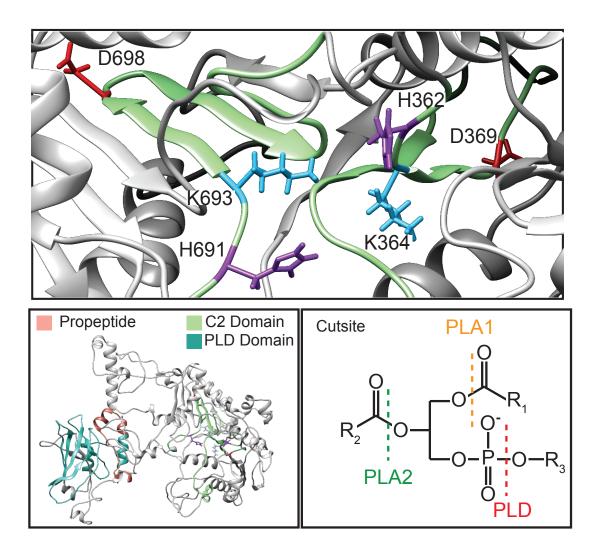


Figure 3.12: An example of the phospholipases found in *D. capensis* is seen in Figure 3.12 where the active site residues are highlighted and labelled, and the propertide, C2 and a PLD domain are highlighted in salmon, green and aqua colors respectively. The figure also shows the cut sites of different enzymes on a phospholipid.

Using the target selection pipeline described in Chapter 1, we have the equilibrated enzyme structures using the *in silico* maturation. Figure 3.13 shows cluster PLA_1 representative DCAP_7326 as an example in (a) and (b). The active site residues are histidine, serine and as-

partic acid (shown in purple, aqua and red respectively). PLA_2 representative DCAP_2905 as an example in (c) and (d). The active site residue is histidine (highlighted in red) while the disulfide bonds are shown in yellow. The structures will be available to download when the paper is published.

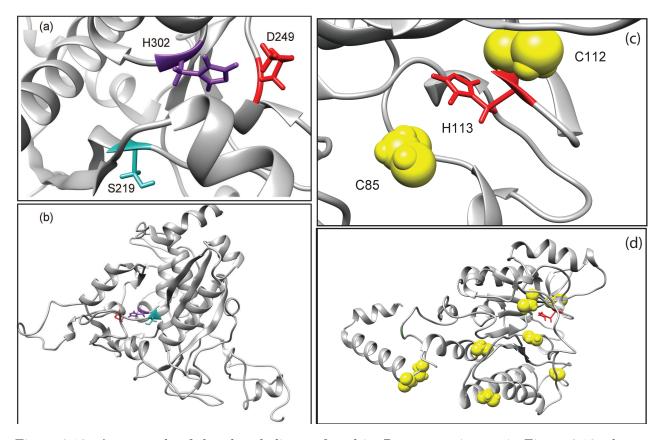


Figure 3.13: An example of the phospholipases found in *D. capensis* is seen in Figure 3.12 where the active site residues are highlighted and labelled, and the propertide, C2 and a PLD domain are highlighted in salmon, green and aqua colors respectively. The figure also shows the cut sites of different enzymes on a phospholipid.

3.3 Nucleases from *Drosera capensis*

Nucleases are enzymes capable of cleaving the phosphodiester bonds between nucleotides of nucleic acids. We found 37 novel nucleases in *D. capensis*. An example of the nucleases found in *D. capensis* is seen in Figure 3.16 where the active site residues are highlighted and lablelled, and the metal ions are highlighted.

With Prof. Rachel Martin and Prof. Carter Butts, I chose the protein set and generated the predicted structures. Manuscript in preparation; next steps include *in silico* maturation, molecular modelling and analysis of the results.

DCAP 7326										DDDKR
PLA16 ARATH	MAATPSHNNL	LTINHKNSIT	GSSSLNTNES	EINFPAKERV	ATRALSETDE	SSLSAVISEL	ERERREROGL	LIEEAEGAGE		
DCAP 3437										
PLA20 ARATH										
DSEL ARATH									MKRKKKEE	EEEKLIVTRE
DCAP 4074								MAYSC	KECEGKEKTE	NKMTODNNKD
DCAP 6629										MTY
	-	• • •••			-	•• •		• •		
DCAP 7326	LADCWR-ETH	GEDDWSRLLD					DREFOSLOME			KLPN-FEKKS
PLA16 ARATH		GEDDWAGLMD		-			-			
DCAP 3437		TTSSWAHLLD								
PLA20 ARATH										
DSEL ARATH	FAKRWR-DLS	GONHWKGMLO	PLDODLREYI	IHYGEMAQAG	YDTFNINTES	OFAGASIYSR	KDFFAKVGLE	IAHPYTKYKV	TKFIYATSDI	HVPESFLLFP
DCAP 4074	IAKKWR-LLG	GQNDWDGLLD	PLDIDLCRYI	LFYGDMAQAT	YDTFDREKAS	KYAGSSLYSK	KDLFAKVGLE	KGNPY-KYRV	TKFLYATSGL	SLPDAFIFKS
DCAP 6629		GONDWDDLLD								
-	•	• • •	•	• • • • • • • • • • • • • • • • • • • •		• -			•	• •
DCAP 7326	RRPKVCSNDA	HWIGYVAVSN	DETS-THLGR	RDIVIAWRGT	VTSLEWISDL	MDILKPISSN	E		IPSHDPTMKV	ESGFVNLYTD
PLA16 ARATH	RWSKVWSKNA	NWMGYVAVSD	DETSRNRLGR	RDIAIAWRGT	VTKLEWIADL	KDYLKPVTEN	К		IRCPDPAVKV	ESGFLDLYTD
DCAP_3437	RSREAWDRES	NWIGYIAVSN	DSVS-EKLGR	REVYVAWRGT	TRDYEWVDIL	GAKLASVKRL	MKDGDEDGD-	E	DEDEDDKEEV	MLGWLTMYVS
pla20 arath	QSRDSWDRES	NWFGYIAVTS	DERS-KALGR	REIYIALRGT	SRNYEWVNVL	GARPTSADPL	LHGP <mark>EQD</mark> GSG	GVVEGTTFDS	DSEDEEGCKV	MLGWLTIYTS
DSEL ARATH	ISREGWSKES	NWMGYVAVTD	DQGT-ALLGR	RDIVVSWRGS	VQPLEWVEDF	EFGLVNAIKI	FGERNDQ		VQI	HQGWYSIYMS
DCAP 4074	LSREAWSKES	NFMGFVAVGT	DESV-AVLGR	RDIVISWRGT	IESLEWVNDL	E FGLVSAS D I	LGKGNVHGV-		EFVNPMV	QEGWYSVYTS
DCAP 6629	VLVEAWSKES	NFMGFVVVGT	DESM-AVLER	RDIVILWRGT	IESLEWVNDL	EFSLVSPSNI	LSKGNVHDV-		EFVNPMV	QGWWYSVYTS
_										
			• •	• •	00 000		• •			••
DCAP_7326	GNESCKYCRF	SAREQ							GPR	
DCAP_7326 PLA16_ARATH		SAREQ SAREQ	VIYEATRL	MDMYKDE	VLSITVTGHS	L			GPR	VGNFRFKERL
_	KDTTCKFARF DDPKSEFTKV	SAREQ SARKQ	VIYEATRL ILTEVKRL LLKKIGKL	MDMYKDE VEEHGDDDDS IEEYKHE	VLSITVTGHS DLSITVTGHS KLSIVFTGHS	L LGGALAILSA LGASLSVISA	YDIAEMRLN- FDVVENLT	SEIP	GPR VTVLTYGGPR VSAFVFGCPK	VGNFRFKERL VGNVRFRERM VGNKQFNDRL
PLA16_ARATH	KDTTCKFARF DDPKSEFTKV	SAREQ	VIYEATRL ILTEVKRL LLKKIGKL	MDMYKDE VEEHGDDDDS IEEYKHE	VLSITVTGHS DLSITVTGHS KLSIVFTGHS	L LGGALAILSA LGASLSVISA	YDIAEMRLN- FDVVENLT	SEIP	GPR VTVLTYGGPR VSAFVFGCPK	VGNFRFKERL VGNVRFRERM VGNKQFNDRL
pla16_arath DCAP_3437	KDTTCKFARF DDPKSEFTKV NHPESKFTKL	SAREQ SARKQ	VIYEATRL ILTEVKRL LLKKIGKL LLAKIKEL	MDMYKDE VEEHGDDDDS IEEYKHE LLKYKDE	VLŠITVTGHŠ DLSITVTGHS KLSIVFTGHS KPSIVLTGHS	L LGGALAILSA LGASLSVISA LGATEAVLAA	YDIAEMRLN- FDVVENLT YDIAENGSS-	-RSKKGKVIP SEIP DDVP	GPR VTVLTYGGPR VSAFVFGCPK VTAIVFGCPQ	VGNFRFKERL VGNVRFRERM VGNKQFNDRL VGNKEFRDEV
PLA16_ARATH DCAP_3437 PLA20_ARATH DSEL_ARATH DCAP_4074	KDTTCKFARF DDPKSEFTKV NHPESKFTKL QDERSPFTKT EDPKSPFNKT	SAREQ SARKQ SLRSQ NARDQ SARQQLKFAV	VIYEATRL ILTEVKRL LLKKIGKL LLAKIKEL VLREVGRL IQVLAEVRNL	MDMYKDE VEEHGDDDDS IEEYKHE LLKYKDE LEKYKDE VEKYKNE	VLŠITVTGHŠ DLSITVTGHS KLSIVFTGHS KPSIVLTGHS EVSITICGHS EVSITVTGHS	L LGGALAILSA LGASLSVISA LGATEAVLAA LGAALATLSA LGAALATLNA	YDIAEMRLN- FDVVENLT YDIAENGSS- TDIVANGYNR ADIVANNCSK	SEIP DDVP PKSRPDKSCP PRSMPNKSCL	VTVLTYGGPR VSAFVFGCPK VTAIVFGCPQ VTAFVFASPR VTAIVFASPR	VGNFRFKERL VGNVRFRERM VGNKQFNDRL VGNKEFRDEV VGDSDFRKLF VGDANFKKVF
PLA16_ARATH DCAP_3437 PLA20_ARATH DSEL_ARATH	KDTTCKFARF DDPKSEFTKV NHPESKFTKL QDERSPFTKT EDPKSPFNKT	SAREQ SARKQ SLRSQ NARDQ	VIYEATRL ILTEVKRL LLKKIGKL LLAKIKEL VLREVGRL IQVLAEVRNL	MDMYKDE VEEHGDDDDS IEEYKHE LLKYKDE LEKYKDE VEKYKNE	VLŠITVTGHŠ DLSITVTGHS KLSIVFTGHS KPSIVLTGHS EVSITICGHS EVSITVTGHS	L LGGALAILSA LGASLSVISA LGATEAVLAA LGAALATLSA LGAALATLNA	YDIAEMRLN- FDVVENLT YDIAENGSS- TDIVANGYNR ADIVANNCSK	SEIP DDVP PKSRPDKSCP PRSMPNKSCL	VTVLTYGGPR VSAFVFGCPK VTAIVFGCPQ VTAFVFASPR VTAIVFASPR	VGNFRFKERL VGNVRFRERM VGNKQFNDRL VGNKEFRDEV VGDSDFRKLF VGDANFKKVF
PLAIG_ARATH DCAP_3437 PLA20_ARATH DSEL_ARATH DCAP_4074 DCAP_6629	KDTTCKFARF DDPKSEFTKV NHPESKFTKL QDERSPFTKT EDPKSPFNKT EDPKSPFNKT	SAREQ SARKQ SLRSQ NARDQ SARQQLKFAV STRQQ	VIYEATRL ILTEVKRL LLKKIGKL VLREVGRL IQVLAEVRNL VLVEVRNL	MDMYKDE VEEHGDDDDS IEEYKHE LLKYKDE LEKYKDE VEKYKNE VERYKNE	VLŠITVTGHŠ DLSITVTGHS KLSIVFTGHS KPSIVLTGHS EVSITICGHS EVSITVTGHS EVSITVTSHS	L LGGALAILSA LGASLSVISA LGATEAVLAA LGAALATLSA LGAALATLNA LGAALATLNA	YDIAEMRLN- FDVVENLT YDIAENGSS- TDIVANGYNR ADIVANNCSK ADIVANNCNK	-RSKKGKVIP SEIP DDVP PKSRPDKSCP PRSMPNKSCL PRSMPNKSCL	GPR VTVLTYGGPR VSAFVFGCPK VTAIVFGCPQ VTAFVFASPR VTAIVFASPR VTAIVFASPR	VGNFRFKERL VGNVRFRERM VGNKQFNDRL VGNKEFRDEV VGDSDFRKLF VGDANFKKVF VGDANVF
PLA16_ARATH DCAP_3437 PLA20_ARATH DSEL_ARATH DCAP_4074 DCAP_6629 DCAP_7326	KDTTCKFARF DDPKSEFTKV NHPESKFTKL QDERSPFTKT EDPKSPFNKT EDPKSPFNKT EGL-GVKVLR	SAREQ SARKQ SLRSQ NARDQ SARQQLKFAV STRQQ VVNVHDMVPK	VIYEATRI ILTEVKRL LLKKIGKL VLREVGRL IQVLAEVRNL VLVEVRNL OTPGFFVNEHT	MDMYKDE VEEHGDDDDS IEEYKHE LLKYKDE VEKYKDE VEKYKNE VERYKNE TRVEQNV-GG	VLŠĪTVĪGHŠ DLSITVTGHS KLSIVFTGHS KPSIVLTGHS EVSITVTGHS EVSITVTGHS LSM-ELLPCE	LLISA LGGALAILSA LGASLSVISA LGATEAVLAA LGAALATLSA LGAALATLNA LGAALATLNA ELVLDHKNSP	YDIAEMRLN- FDVVENLT YDIAENGSS- TDIVANGYNR ADIVANNCSK ADIVANNCNK FLKNTNDPVC	-RSKKGKVIP SEIP PKSRPDKSCP PRSMPNKSCL PRSMPNKSCL PRSMPNKSCL AHNLEALLHL	GPR VTVLTYGGPR VSAFVFGCPQ VTAFVFASPR VTAIVFASPR VTAIVFASPR LAGYHGRG	VGNFRFKERL VGNVRFRERM VGNKQFNDRL VGDSDFRKLF VGDANFKKVF VGDANVF RRFGLSSERD
PLA16_ARATH DCAP_3437 PLA20_ARATH DSEL_ARATH DCAP_4074 DCAP_6629 DCAP_7326 PLA16_ARATH	KDTTCKFARF DDPKSEFTKV NHPESKFTKL QDERSFFTKT EDPKSPFNKT EDPKSPFNKT EGL-GVKVLR EEL-GVKVMR	SAREQ SARKQ SLRSQ SARQQLKFAV STRQQ VVNVHDMVPK VVNVHDVVPK	VIYEATRI ILTEVKRL LLKKIGKL ULAKIKEL VLREVGRL IQVLAEVRNL VLVEVRNL TPGFFVNEHT SPGLFLNESR	MDMYKDE VEEHGDDDDS IEEYKHE LLKYKDE LEKYKDE VEKYKNE VERYKNE TRVEQNV-GG PHALMKIAEG	VLŠITVTGHŠ DLSITVTGHS KLSIVFTGHS EVSITLCGHS EVSITUTGHS EVSITVTSHS LSM-ELLPCE LPWCYSHVGE	LGGALAILSA LGASLSVISA LGATEAVLAA LGAALATLSA LGAALATLNA LGAALATLNA ELVLDHKNSP ELALDHQNSP	YDIAEMRLN- FDVVENLT YDIAENGSS- TDIVANGYNR ADIVANNCSK ADIVANNCK FLKNTNDPVC FLKPSVDVST	-RSKKGKVIP SEIP PKSRPDKSCP PRSMPNKSCL PRSMPNKSCL AHNLEALLHL AHNLEALLHL	GPR VTVLTYGGPR VSAFVFGCPK VTAIVFGCPQ VTAFVFASPR VTAIVFASPR LAGYHGRG LDGYHGKG	VGNFRFKERL VGNVPRRERM VGNKQFNDRL VGDSDFRKLF VGDSDFRKLF VGDANFKVF VGDANVF RFGLSSERD ERFVLSSGRD
PLA16_ARATH DCAP_3437 PLA20_ARATH DSEL_ARATH DCAP_4074 DCAP_6629 DCAP_7326 PLA16_ARATH DCAP_3437	KDTTCKFARF DDPKSEFTKV NHPESKFTKU QDERSPFNKT EDPKSPFNKT EGL-GVKVLR EEL-GVKVLR NSHHNLKILH	SAREQ SARKQ SLRSQ SARQQLKFAV STRQQ VUNVHDMVPK VVNVHDMVPK VVNVHDVVPK IRNIIDVIPH	VIYEATRI ILTEVKRL LLKKIGKL VLREVGRL IQVLAEVRNL VLVEVRNL • TPGFFVNEHT SPGLFLNESR YPVRVLG	MDMYRDE VEEHGDDDDS IEEYKHE LLKYKDE LEKYKDE VEKYKNE VERYKNE VERYKNE TRVEQNV-GG PHALMKIAEG	VLŠĪTVĪGHŠ DLSITVĪGHS KLSIVFĪGHS KPSIVLĪGHS EVSITICGHS EVSITVĪGHS EVSITVĪSHS LSM-ELLPCE LPWCYSHVGE YVNTGI	LL- LGGALAILSA LGASLSVISA LGATEAVLAA LGAALATLSA LGAALATLNA LGAALATLNA ELVLDHKNSP ELALDHQNSP ELHIDTRKSP	YDIAEMRLN- FDVVENLT YDIAENGSS- TDIVANGYNR ADIVANNCSK ADIVANNCNK FLKNTNDPVC FLKPSVDVST YLKDSKNPSD	-RSKKGKVIP SEIP PKSRPDKSCP PRSMPNKSCL PRSMPNKSCL PRSMPNKSCL AHNLEALLHL AHNLEALLHL WHNLQTMLHV	UTVLTYGGPR VSAFVFGCPQ VTAIVFGCPQ VTAIVFGCPQ VTAIVFASPR VTAIVFASPR VTAIVFASPR LAGYHGRG LDGYHGKG VNGWNGSN	VÖNFREKERL VGNVGFRERM VGNKGFNDRL VGNKEFRDEV VGDADFKKVF VGDANFKVF VGDANVF RRFGLSSERD ERFVLSSGRD GEFKVKIKRS
PLA16_ARATH DCAP_3437 PLA20_ARATH DSEL_ARATH DCAP_4074 DCAP_6629 DCAP_7326 PLA16_ARATH DCAP_3437 PLA20_ARATH	KDTTCKFARF DDPKSEFTKV NHPESKFTKL QDERSPFTKT EDPKSPFNKT EDPKSPFNKT EGL-GVKVMR NSHHNLKILH MSHKNLKILH	SAREQ SARKQ SIRSQ SARQQLKFAV STRQQ VVNVHDVVPK VVNVHDVVPK IRNIIDVIPH VKNTIDLLTR	VIYEATRI ULTEVKRL LLKKIGKL VLREVGRL IQVLAEVRNL O TPGFFVNEHT SPGLFLNESR YPVRVLG YPGGLG	MDMYRDE VEEHGDDDDS IEEYRHE LEKYKDE LEKYKDE VEKYKNE VERYKNE TRVEQNV-GG PHALMKIAEG	VLŠITVTGHŠ LLSITVTGHS KLSIVFTGHS KVSIVLTGHS EVSITUTGHS EVSITVTGHS EVSITVTGHS LSM-ELLPCE LPWCYSHVGE YVNTGI YVDIGI	L LGALAILSA LGASLSVISA LGATEAVLAA LGAALATLSA LGAALATLNA LGAALATLNA ELVLDHKNSP ELALDHQNSP ELHIDTRKSP NFVIDTKKSP	YDIAEMRLN- FDVVENLT YDIAENGSS- TDIVANGYNR ADIVANNCNK FLKNTNDPVC FLKPSVDVST YLKDSKNPSD FLSDSRNPGD	-RSKKGKVIP SEIP DDVP PKSRPDKSCP PRSMPNKSCL PRSMPNKSCL AHNLEALLHL AHNLEALLHL WHNLQTMLHV WHNLQAMLHV	VTVLTYGGPR VSAFVFGCPK VTAIVFGCPQ VTAIVFGCPQ VTAIVFASPR VTAIVFASPR VTAIVFASPR LAGYHGRG LDGYHGKG VNGWNGSN VAGWNGKK	VGNFRFKERL VGNVQFRDEN VGNKQFNDRL VGNKBFRDEV VGDSDFRKLF VGDANFKKVF VGDANVF RRFGLSSERD ERFVLSSGRD GEFKLWKRS
PLA16_ARATH DCAP_3437 PLA20_ARATH DSEL_ARATH DCAP_4074 DCAP_6629 DCAP_7326 PLA16_ARATH DCAP_3437 PLA20_ARATH DSEL_ARATH	KDTTCKFARF DDFXSEFTKV NHPESKFTKL QDERSFFTKT EDFKSPFNKT EDFKSPFNKT EGL-GVKVLR EEL-GVKVLR EEL-GVKVLR NSHHNLKILH MSHKNLKILH	SAREQ SARKQ SLRSQ SARQQLKFAV STFQQ VUNVHDMVPK VVNVHDVVPK IRNIIDVIPH VRNTIDLLTR TENLPDVIPI	VIYEATRI LITEVKRL LLKKIGKL VLREVGRL IQVLAEVENL IQVLAEVENL TPGFFVNEHT SPGJFLNESR YPVRUG YP-GIG	MDMYKDE VEEHdDDDDS IEEYKDE LLKYKDE LEKYKDE VEKYKNE VERYKNE TRVEQNV-GG PHALMKIAEG	VLŠITVTĠHŠ DLSITVTGHS KLSIVFTGHS KVSIVITGHS EVSITICGHS EVSITVTGHS EVSITVTGHS LSM-ELLPCE LPWCYSHVGE YVNTGI YVDIGI YVDIGI	L- LIGGALAILSA LGASLSVISA LGAZEAVLAA LGAALATLSA LGAALATLNA LGAALATLNA ELVLDHKNSP ELALDHQNSP ELALDHQNSP ELALDTKKSP NFVIDTKKSP	YDIAEMRLN- FDVVENLT YDIAENGSS- TDIVANGSYN ADIVANNCSK ADIVANNCNK FLKNTNDPVC FLKPSVDVST YLKDSKNPSD FLSDSRNPGD	-RSKKGKVIP SEIP PSNPNKSCL PRSMPNKSCL PRSMPNKSCL AHNLEALLHL AHNLEALLHL AHNLEALLHL WHNLQAMLHV WHNLQAMLHV	VTVLTYGOPR VTALVFGOPQ VTALVFGOPQ VTALVFGOPQ VTALVFASPR VTALVFASPR LAGYHGRG LDGYHGRG VNGWNGSN VAGWNGKK	VGNFRFKERL VGNVUFFRERM VGNKQFNDRL VGNKEFRDEV VGDSDFRKLF VGDANFKKVF VGDANVF RRFGLSSERD ERFVLSSGRD GEFKVRIKRS GEFKLMVKRS DLFRLDVERA
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PLA16_ARATH DCAP_3437 PLA20_ARATH DSEL_ARATH DCAP_4074 DCAP_6629 DCAP_7326 PLA16_ARATH DCAP_3437 PLA20_ARATH DSEL_ARATH	KDTTCKFARF DDPKSEFTKV QDERSFTKT EDPKSPFNKT EDPKSPFNKT EGL-GVKVLR EEL-GVKVLR NSHHNLKILH MSHKNLKILH MSHKNLKILH CSYNELKALR	SAREQ SARKQ SLRSQ SARQQLKFAV STFQQ VUNVHDMVPK VVNVHDVVPK IRNIIDVIPH VRNTIDLLTR TENLPDVIPI	VIYEATRI LITEVKRL LLAKIKEL VLREVGRL IQVLAEVENL TPGFFVNEHT SPGLFLNESR YPVRVLG YP-FIG YP-FIG	MDMYKDĒ VEEHdDDDDS IEEYKHE LLKYKDE VEKYKNE VERYKNE TRVEQNV-GG PHALMKIAEG	VLŠITVTĠĤŠ DLSITVTGHS KLSIVFTGHS EVSITICGHS EVSITVTGHS EVSITVTGHS EVSITVTSHS LSM-ELLPCE LPWCYSHVGE YVNTGI YVDTGI YSEVGD YSDVGK	L-LILLSA LGAELAILSA LGAELAVLAA LGAELAVLAA LGAALATLSA LGAALATLNA LGAALATLNA ELVLDHKNSP ELALDHQNSP ELALDHQNSP ELALDTKKSP NFVIDTKKSP ELCIDTSKSK	YDIAEMRLN- FDVVENLT YDIAENGSS- TDIVANGYNR ADIVANNCSK ADIVANNCNK FLKNTNDPVC FLKPSVDVST YLKDSKNPSD FLSDSRNPGD FLSDSRNPGD FLSDSRNPGD		VTVLTYGOPR VTALVFGOPQ VTALVFGOPQ VTALVFGOPQ VTALVFASPR VTALVFASPR VTALVFASPR LAGYHGRG LDGYHGKG VNGWNGSN VAGWNGKK VAGTQGTMKA VAGTQGSK	VGNFRFKERL VGNVUFFRERM VGNKQFNDRL VGNKEFRDEV VGDSDFRKLF VGDANFKKVF VGDANVF RRFGLSSERD ERFVLSSGRD GEFKVRIKRS GEFKLMVKRS GGFKLVINRD
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Figure 3.14: The current chosen set for PLA1 from D. capensis with PLA16_ARATH, PLA20_ARATH and DESL_ARATH from Arabidopsis thaliana as references sequences.

DCAP 1541		
DCAP 6041		ATGSVLFGN LISLQYRKQR IDPHVQMLGI EDVVGGGKG- DRLYATIDLE RARVGRTRLL
DCAP 3595		I EDVVGGGKG- DRLYATIDLE RARVGRTRLL
DCAP_0968		
PLDA1_ARATH	MAQHLLHGTL HATIYEVDAL HGGGVRQGFL GKI	LANV EETIGVGKGE TQLYATIDLQ KARVGRTRKI
DCAP_1541		AYLLVEDII NGHSVDRWLD ILDEEGRPTRSRIHVRLQ FFSVSHDKNW SQGIGSPQFA
DCAP_6041		AYLPVTELL ENEEIDRWLE LLDKEHKPIH KHSKIHVKVQ FLEVTREVNW DRGIKTPKFP
DCAP_3595 DCAP 0968		AYLPVTELL ENEEIDRWLE LLDKEHEPIH KNSKIHVKVQ FLEVTREINW DRGIKTPKFP AYVPVRDLL DGEEVDDWFR ICDEDRNPLD NGAKIHVKLQ FFDVSRERNW DRGIRSAKYP
PLDA1 ARATH		AYIPVDQVI NGEEVDQWVE ILDNDRNPIQ GGSKIHVKLQ YFHVEEDRNW NMGIKSAKFP
_		
DCAP_1541		RRCWENIFD AISNAKHLVY IAGWSVYTPI TLVRDPRRPK QGGGLTLGEL LIKKANENVK
DCAP_6041		HRAWEDVFD AINNAKHLIY ITGWSVYTEI TLIRDNRRPK PGGDITLGEL LKKKANEGVR
DCAP_3595		HRAWEDVFD AINNAKHLIY ITGWSVYTEI TLIRDNRRPK PGGDITLGEL LKKKANEGVR
DCAP_0968 PLDA1 ARATH		HRCWEDVFD AISNAKHLIY ITGWSVYTEI ALVRDQKRPK SGGETTLGEL LKRKANEGVK ORCWEDIFD AISNAKHLIY ITGWSVYAEI ALVRDSRRPK PGGDVTIGEL LKKKASEGVR
	GALLER COCKAPTED INTERVENTION CONTRACT	Accepting How with Light And Applied House Acception
DCAP 1541		CPRNPDDGK SIVQGFEVKY MFTHHQKTVV VDSEGFNAAA GSMVMPKRRI IGFVGGIDLC
DCAP_6041		CPRNPDGGH SLVQDLEIST MFTHHQKIVI ADSALPDGNEDKRRI VSFVGGIDLC
DCAP_3595		CPRNPDGGR SLVQNLEIST MFTHHQKIVI ADSALPDGNEDKRRI VSFVGGIDLC
DCAP_0968		CPRNPDNGS SIVQDLQIST MFTHHQKIVV VDSAMPGGGSHNRRI VSFVGGIDLC
PLDA1_ARATH	VLLLVWDDRT SVDVLKKDGL MATHDEETEN FFRGSDVHCI LO	CPRNPDDGG SIVQSLQIST MFTHHQKIVV VDSEMPSRGGSEMRRI VSFVGGIDLC
DCAP 1541		WHDIHCCLE GPVAWDVLYN FEORWRKOVG DKHIYSMNEI NOFVMPPSET TSSEDTNTWN
DCAP 6041		WHDINCCLE GPVAWDVLIN FEQRWRRQVG DARIISMAEI NGFVMFFSEI ISSEDINIWN WHDINSRLE GPIAWDVLFN FEQRWRRQGG KDLLLNLREL DSVIIPPSPV TYSDDQETWN
DCAP 3595		WHDIHSRLE GPIAWDVLFN FEORWRKOGG KDLLLNLGEL DSVIIPPSPV TYPDDRETWN
DCAP_0968	DGRYDTQFHS LFRTLDTAHH DDFHQPNFPG AAITKGGPRE PU	WHDIHSRLE GPIAWDVLFN FEQRWRKQGG KDILVPLREL DDL-IAPSPV TFREDPETWN
PLDA1_ARATH	DGRYDTPFHS LFRTLDTVHH DDFHQPNFTG AAITKGGPRE PU	WHDIHSRLE GPIAWDVMYN FEQRWSKQGG KDILVKLRDL SDIIITPSPV MFQEDHDVWN
DO3 D 1541		
DCAP_1541 DCAP_6041		AYICAIRRA KNFIYIENQY FIGSSFGWNN -EEIKVEDVG ALNLIPKELS LKIVKKIEAG AYINAIRRA KRFIYIENQY FLGSSFAWKA GDDIKIEDVG CIHLIPKELS LKIVSKIEKG
DCAP 3595		AYINAIRRA RRFIYIENQY FLGSSFAWKA GDDIKIEDVG CLHLIPKELS LKIVSKIEKG
DCAP 0968		AYIHAIRRA KNFIYIENQY FLGSCFSWDH SDDIKLEDIG ALHLIPKELS LKIVSKIEAR
PLDA1_ARATH	VQLFRSIDGG AAAGFPESPE AAAEAGLVSG KDNIIDRSIQ DA	AYIHAIRRA KDFIYVENQY FLGSSFAWAA -DGITPEDIN ALHLIPKELS LKIVSKIEKG
	• • •• • • •••••••• ••••••• •• ••• •	• • • • • • • • • • • • • • • • • • •
DCAP_1541 DCAP 6041		NAIRRARIN THPRDYLSFF CLGNREVKRP GEYLPPEKPE PDSDYSRAQS SRRFMIYVHA QALHAKGIE ANPREYLSFF CLANREVKKS GEYEPSEKPE PDTDYIRAQE SRRFMIYVHA
DCAP 3595		QALHANGIE ANPREYLSFF CLANREVKKS GEYEPSEKPE PDDDIRAGE SRRFMITVHA
DCAP 0968		GALRAKGLQ EDPRNYLTFF CLANREVKRS GEYEPAEQPE PDSDYERAQH ARRFMIYVHS
plda1_arath	EKFRVYVVVP MWPEGLPESG SVQAILDWQR RTMEMMYKDV I	QALRAQGLE EDPRNYLTFF CLGNREVKKD GEYEPAEKPD PDTDYMRAQE ARRFMIYVHT
	++++++++++++++++++++++++++++++++++++++	• ••• ••• ••• • • • • • • • •
DCAP_1541		IRKPARGQI YGFRMALWYE HLRQIQPSFA HPETLECMRD LNKIADDYWK LYSSELLNQD
DCAP_6041 DCAP 3595		HHQPPRGQI HGFRMSLWYE HLGLLHNTFL NPEDPECVKK VNEIALKYWD LYSSDNLEHD HHQPPRGQI HGFRMSLWYE HLGLLDNTFL NPEDPECVKK VNEIALKYWD LYSSESLEHD
DCAP 0968		THQPARGQV HGFRMALWYE HLGHLDDSFN HPESVHCIKK VNQVADKYWD LYSSESLECD
PLDA1_ARATH		HRQPARGQI HGFRMSLWYE HLGMLDETFL DPSSLECIEK VNRISDKYWD FYSSESLEHD
	•••• •• ••• ••• •• ••	
DCAP_1541	LPGHLLRYPI EVTGYGGVLN LPGWKQFPDT KAPVLGTKSD	propeptide
DCAP_6041 DCAP_3595	LPGHLLHYPI DVTAEGEVVE LPGFENFPDT KAKVLGTKSD LPGHLLHYPI DVTAEGEVVE LPGFENFPDT KAKVLGTKSD	C2 domain active His residue
DCAP_3595 DCAP_0968	IPGHLLRYPV GITSEGDVTE LPGFEFFPDT KAKVLGKSD	
PLDA1_ARATH	LPGHLLRYPI GVASEGDITE LPGFEFFPDT KARILGTKSD	PLD domain
—		active Lys residue

Figure 3.15: The current chosen set for PLDA from D. capensis with PLDA1_ARATH from Arabidopsis thaliana as references sequences.

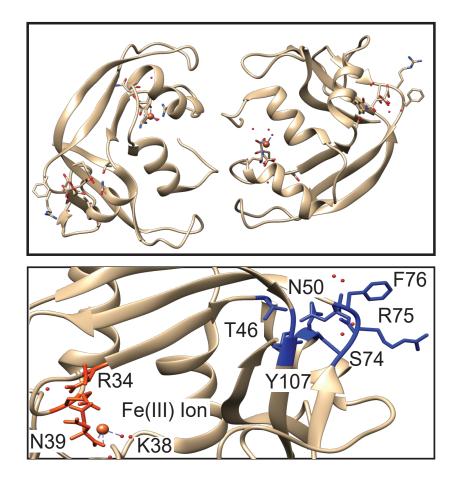


Figure 3.16: An example of the nucleases found in D. capensis where the active site residues are highlighted and labelled, and the metal ions are highlighted.

Chapter 4

Sequence comparison, molecular modeling, and network analysis predict structural diversity in cysteine proteases from the Cape sundew, *Drosera capensis*

Characterization of carnivorous plant digestive enzymes could lead to their use in a variety of laboratory and applications contexts, including analytical use in proteomics studies as well as preventing fouling on the surface of medical devices that cannot be treated under harsh conditions. The proteases of carnivorous plants present attractive targets for exploitation in chemical biology and biotechnology contexts. New proteases may also prove useful for cleaving amyloid fibrils, such as those responsible for the transmission of prion diseases or the formation of biofilms by pathogenic bacteria. The characterization of aspartic proteases from the tropical pitcher plants (*Nepenthes* sp.) [107, 108, 109], has already led to useful advances in mass spectrometry-based proteomics applications, where the ability to digest proteins using a variety of cut sites is essential for identifying proteins and peptides from complex mixtures. Proteases from plant and animal sources are also important components of pharmaceutical preparations for gluten intolerance, arthritis, and pancreatic disease [110]. Therefore, characterizing proteases from *D. capensis* has the potential to diversify the toolbox of proteases with different functional properties that are available for these and other applications.

I contributed to choosing the protein set, determining the functional regions of interest, generating the predicted structures, analyzing sequence and structure data, performing sequence annotation and comparisons and wrote the manuscript with my co-authors on this paper. A portion of the paper [2] is reproduced in this chapter to understand the importance of the results found in this study. For more details and in-depth analysis, please refer the paper [2]

4.1 Background

Plant cysteine proteases form a large and diverse family of proteins that perform cellular housekeeping tasks, fulfill defensive functions, and, in carnivorous plants, digest proteins from prey. It is typical for plants to contain many different cysteine protease isoforms; for instance, in the case of tobacco (*Nicotiana tabacum*), more than 60 cysteine protease genes have been identified [111]. Many of the cysteine proteases of interest are classified by the MEROPS database as family C1 [112], a broad class of enzymes including cathepsins and viral proteases as well as plant enzymes that function to deter herbivory. C1 proteases can operate as endopeptidases, dipeptidyl peptidases, and aminopeptidases [113]. In plants, many C1 enzymes are used to degrade proteins in the vacuole, playing many of the same roles as their lysosomal counterparts in animals [114]. They are also found in fruits, particularly unripe ones; this protease activity impedes insect feeding and also serves to cleave endogenous proteins during fruit ripening. Some families of cysteine proteases in plants have been subject to diversifying selection due to a molecular arms race between these plants and their pathogens; as plants produce proteases that suppress fungal growth, fungi evolve inhibitors specific to these proteases, driving the diversification of plant proteases involved in the immune response [64].

The plethora of paralogs found in a typical plant is indicative of the need for a range of different substrate specificities; this is particularly important in the case of carnivorous plants, which must digest prey proteins to their component amino acids. Aspartic proteases have long been implicated in *Nepenthes* pitcher plant digestion [107, 115], and more recently the cysteine protease dionain 1 has been confirmed as a major digestive enzyme in the Venus flytrap (*Dionaea muscipula*) [116]. In *Drosera capensis*, proteins from prey constitute the major nitrogen source for producing new plant tissue [40]. Given that plant carnivory appears to have evolved from defensive systems in general [117], and that the feeding responses are triggered by the same signaling pathway as is implicated in response to wounding [118], one would expect cysteine proteases to play a major role; here we investigate some of the many cysteine protease genes in *D. capensis* with the objective of adding to the portfolio of cleavage activities available for chemical biology applications. The *D. capensis* enzymes are particularly appealing for mass spectrometry-based proteomics applications, due to their ability to operate under relatively mild conditions, i.e. at room temperature and pH 5.

This study focuses on the C1 cysteine proteases from the Cape sundew (D. capensis), where it uses the pipeline from Chapter 1 to identify structurally distinct subgroups of proteins for subsequent expression and biochemical characterization. C1 cysteine proteases share a common papain-like fold, a property also predicted for the proteins studied here. Despite this conservation of the papain fold and critical active and structural residues, sequence analysis of the D. capensis cysteine proteases indicates that they represent a highly diverse group of proteins, some of which appear to be specific to the Droseraceae. In particular, a large cluster of proteases containing dionains 1 and 3 as well as many homologs from D. capensis has particular sequence features not seen in papain or other reference enzymes. Finally, a new class of granulin domain-containing cysteine proteases is identified, based on clustering of the granulin domains themselves.

Molecular modeling was performed (Prof. Carter Butts and Dr. Xuhong Zhang with the team) in order to translate this sequence diversity into predicted structural diversity, which is more informative for guiding future experimental studies. Examination of the predicted enzyme structures potentially suggests diversity that may imply a variety of substrate preferences and cleavage patterns. Further, the study uses Rosetta [28, 27] to perform comparative modeling with all-atom refinement, described in detail in Chapter 1, combining local homology modeling based on short fragments with de novo structure prediction. The study then employs atomistic MD simulation of these initial structures in explicit solvent to produce equilibrated structures with corrected active site protonation states; these equilibrated structures serve as the starting point for further analysis.

Quality control was performed using both sequence alignment and inspection of the Rosetta structures; proteins that are missing one of the critical active residues (C158 or H292, papain numbering) were discarded, as were some lacking critical disulfide bonds or other structural features necessary for stability. After winnowing out sequences that are unlikely to produce active proteases, 44 potentially active proteases were chosen for further analysis. This methodology allows the development of hypotheses based on predicted 3D structure and activity, in contrast to focusing on the first discovered or most abundantly produced enzymes, enabling selection of the most promising targets for structural and biochemical characteri-

zation based on the priorities of technological utility rather than relative importance in the biological context.

4.2 Cysteine Protease Sequence Analysis

Multiple-sequence alignments for cysteine proteases from *D. capensis* and previously characterized plant cysteine proteases reveal diverse functionality. Annotated sequence alignments are shown for the DCAP cluster (Figure 4.1), the papain cluster, both catalytic domains (Figure 4.2) and (Figure 4.3), the vignain cluster, (Figure 4.4), the granulin domain cluster (Figure 4.5), the bromelain cluster (Figure 4.6) and the dionain cluster (Figure 4.7). The annotations highlight both specific amino acid properties and general sequence features. Followung the nomenclature of "Target Selection Pipeline" from Chapter 1, hydrophobic residues are shown in green, positively charged residues in blue, negatively charged residues in red, and cysteines in yellow. Conserved Cys residues involved in structure-stabilizing disulfide bonds are indicated with yellow asterisks, while other residues conserved across all the sequences considered are indicated with solid circles. Residues conserved within the cluster but not shared with papain are indicated with open circles. The residues of the catalytic dyad are indicated with colored arrows, yellow for Cys and purple for His. The position of the stabilizing Asn residue is indicated with a pink asterisk, although this residue is not conserved in all sequences. Strikethrough text indicates parts of the sequence that are expressed but removed during post-translational processing; for these proteins, this constitutes an N-terminal region comprised of the signal peptide and the pro-sequence. The presence and position of a signal sequence targeting the protein for secretion was predicted using SignalP, and is indicated in the figures by highlighting in light orange, with the predicted cut site indicated by underlining the residues on either side of the cleavage point. The position of the pro-sequences was predicted by sequence similarity to the reference sequences as well as comparison of the predicted structures to the crystal structure of the mature form of papain. The pro-sequences of many of the sequences studied here contain the ERFNIN motif ($EX_3RX_3FX_2NX_3I/VX_3N$) common to C1-family cysteine proteases. When present, this sequence is shown above the relevant residues in the alignments. The presence of localization tags, when present, is indicated by purple highlighting. Granulin domains, when present, are highlighted in blue.

Sequence consensus analysis for the sequences within each cluster defined in Figure 1.1, are mapped onto the structure of a representative member of the class in Figure 4.8. Percent conservation at each position is color coded (red = more conserved, white = intermediate values, blue = less conserved). These plots demonstrate that the degree of sequence conservation varies greatly among different clusters, i.e. the DCAP cluster has much less sequence conservation overall than the vignain cluster. In all the clusters, conserved residues are concentrated in the important secondary structure elements and near the active site cleft, whereas residues in loops and linkers away from the core of the protein are less likely to be conserved. All the predicted and equilibrated structures are available to download in the paper [30].

				•
DCAP_2263	WS PPISSAAIIA-VILAVVAA	A-TTTRASTFTE-ENPIRLVSD	-LREIESSVVE-AVGNSGRALS	EXX FARF-LHRYC-KRYETVEEMR
DCAP_7862 DCAP_3370				
DCAP_5561	FL-LLLSLTAA MVSLERERRA-HLWKKLREVR-LAIK¥I-VIPGMEPVI			LELVESYQLA-AEYSG-TELN
PAPAI CARPA	MAMI PSISKLLFVA ICL-FVYM	GL SFGDFSIVGYSQNDLTS	r erLiqi	FESW-MLKHN KIYKNIDEKI
DCAP_7834 DCAP_4959	INN SHISSLAFLL LI 	TSINVVASRYLSEF TSINVVASRYLSEF	S VSIREQ	FEEW-IVQYG RVYADNAEKE F EEW-IVQYG RVYADNAEKE
	XXAXXXQ XXXXXXXXXX XXIXXXN	E	E PQNCSAT	
DCAP_2263	LRFAVFKENL-RLIRSSNRNG MPYTLALNDF-ADMSWEEF	2K-HKLG	A AQNCSATLKG NHKMTDATLF	DMKDWREVGIVTPVK-N
DCAP_7862 DCAP_3370	LRFAVFKENL-RLIRSSNRNG-MPYTLALNDF-ADMSWEEF RRFAIFKANL-RRARRNQELD-PSAEHGVTKF-SDLTPREF			
DCAP 5561		SS-RNDFSLETHT-EGTTVSRLP		
PAPAI CARPA	YRFEIFKDNL-KYIDETNKKN NSYWLGLNVF-ADMSNDEF			EYVDWRQKGAVTPVK-N
DCAP_7834 DCAP_4959	KRFVIFQQNV-ARIEALRKLN-LCYCVGINKF-NDMTQEEF KRFVIFQQNV-ARIEALRKLN-LCYCVGINKF-NDMTQEEF			
				2 DOULDINDY
	••••• • • ••• •	• •	0	o *
DCAP_2263	QGHCGSCWTF STTGALEAAY SQAFGKSVSL SEQ-QLVD			
DCAP_7862 DCAP_3370	QGHCGSCWTF STTGALEAAY SQAFGKSVSL SEQ-QLVD QGSCGSCWSF STTGALEGSN YLATGKLVSL SEQ-QLVD			
DCAP_5561	NGVCGSCWAF SATGAIEGAN FIATGKLLNL SEQQQLID			
PAPA1 CARPA	QGSCGSCWAF SAVVTIEGII KIRTGNLNEY SEQ-ELLD			
DCAP_7834 DCAP_4959	GPSCGSCWAF AAIAAIEALH SAMYGDLRSL SEQ-QIID GPSCGSCWAF AAIAAIEALH SAMYGDLRSL SEQ-QIID			
_				
			1	
DCAP 2263	-GVRVVDSVN ITLGAEDELK HAVAFVRPVS VAF-QVVN			
DCAP_2203 DCAP_7862	-GVRVVESVN IILGAEDELK HAVAFVREVS VAF-QVVN			
DCAP_3370	-ATTVANFSV VSLDEDQIA- ANLVKNGPLA VGI-NAA-	M QTYIGGVSCP YICLRNI	DHGVLLVGYG SAGYAPIRFK	EKPYWIIKNS WGENWGENGY
DCAP_5561 PAPA1 CARPA	-SVKVVNFTT IPVDEKQIA- AHLVHHGPLA VGL-NAV- YAAK-TDGVR QVQPYNEGAL LYSIANQPVS VVLEAAGKI			
DCAP_7834	DPVTIVDGYE FVPSNSEIDL ARAVARRPVS VRV-QSGD			
DCAP_4959	DPVTIVDGYE FVPSNSEIDL ARAVARRPVS VRV-QSGD	AL QAYTGGIFKG -PCGKKI	NHAMTIVGYT	-PSYWIVKNS WGVWWGEDGY
	*			
DCAP_2263	FKMELGKNMCGKSIFQ TRSSTNP			
DCAP_7862 DCAP_3370	FKMELGKN MC			
DCAP_5561	YKICRGRNVCGVDSMV STVAALEISH TAATTQA- IAFAEGMVCAASTLW RKEMTK ARAENRNC	LKGYLHYWGN FGGKNYASS	TSLEYGOHOF SRTHS	
papaī_carpa	IRIKRGTGNS YGVCGLYTSSFYPVKN			
DCAP_7834 DCAP_4959	IRMRKGADGP AGLCGIAREPSYPFSY IQRNDS-S IRMRKGADGP AGLCGIAREPSYPFSY IQRNDS-S	<в NQQYYH В NOOYYH		
		Cys residue	signal sequence p	redicted minichain
	•	lis residue	propeptide	
	* C in disulfide bond * stabilizione	ng Asn residue	localization signal	
			isounzation signal	

Figure 4.1: The DCAP cluster contains sequences that are more closely related to other *D. capensis* sequences than to any of the references. Several have insertions not found in other sequences, potentially indicating specific functionalities. DCAP_2263 and DCAP_7862 contain the localization tag NPIR in their N-terminal pro-domain regions, indicating targeting to the vacuole.

PAPA1_CARPA			FG_DFSIVGYSQ= =====NDLTS_TERLIQLF	
DCAP_2570		MSYTIIAL-F-FFLLITHSYA-LDVSLPSS		DS-WLIKHGQT YNAINTIKVK
Droserain 3 DCAP 7627			GTWQPYN_LKA¥EKRF	KA-WOTTYGRE
DCAP 0302		MADILE LELLOC		DU MCMUUNVT UDTC
DCAP_5993				KA-WQNHYGKQYKDK
DCAP_5667 DCAP_5945		MATFIF-FLAIL		R-WLVKNRKN YNAT
DCAP_5945 ERVB TABDI				
CPGP1_ZINOF				
DCAP_6547 RD21A ARATH			SS DMSIISHNNK-V====NLRT DDEVMTLF AV-DMSIISYDEK-HGVSTTGGRS EAEVMSIY	
ORYA ORYSJ	MR			
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	EXXXRXXXF XXNXXXIXXX	N		•••••
PAPA1_CARPA			KY TGSIAGNYTTTE LSYEEVLN	
DCAP_2570 Droserain 3	FREIHRERVE ROMIENTORT	NKKVKS YWI GI NEEAD MTHDEEKS	SV-KYRDMAKDEE-LRDLYWEKRT-KKEERYLM RY-LG===LNAGL-PKS=====G-KASNEFTY	RD_MVDLPKSIDW_RKKGAVAPVK
	DEWILRFEIY QSNLQLIDFI	-NCQNYT_FTLTDKRIGR_HDNGRV	'QV-LNTSVSR-LMDAKPAN TR-LCLRDWD-RGDAVFEN-S-IGMEGMGE	LG NYTVLDSIDW REKGAVTLVK
DCAP_7627 DCAP_0302	QERQTRLSVF-LDNLAFVESH	-NSKEGTL-FSVSLNGFAD-LTVEEFRE	TR-LGLRDWD-RGDAVFEN-S-IGMEGMGE	RG-EGGVPGEVDW REEGAVTAVK
DCAP_5993	DEWVLRFGIY QTNLQFADFI	NSQ====NLS_FSLADNKFAD_LTNDEFKS	MY_LG===LLSDE_LPRVN==SED_KVNNVKLI	SR-DDSVPDSIDW RKKGAVTPVK
DCAP_5667 DCAP_5945	SEKETRFEIF-KDNLAFIEEH	NGN===EGRT YKVGLNKFAD_LTNDEYRF NGN===RNES_YKLGLNRFAD_LKNEEFKF	ITY_LG===TKLDP_KRVLL=RRQK_GGSKR¥AV MY_LG===KKGRG <mark>_ERRGNEAE</mark> R=_==YRFAGG	CD_EECLPENVDW ROKGAVAEVK
ERVB TABDI				LPSFVDW RSKGAVNSIK
CPGP1_ZINOF				DVLPDSIDW REKGAVVPVK
DCAP_6547 RD21A ARATH			NY-LG===VKPGV-KRPEKWADEK-RISERYAS K Y-LG===AKMEK-K=====GER-RTSLRYEA	
ORYA ORYSJ			NT-LGLRNKP-RRER-KVSDR¥LA	
-				
		al de la construcción de la construcción de la construcción de la construcción de la construcción de la constru		
PAPA1 CARPA	• * NOGSC	GSCWAFSAVV TIEGIIKIRT GNINEYSE	QE LLDCDR-RSY GCNGGYPWSA LQLVAQY-	SI HYRNTYPYEG VORYCRSREK
DCAP 2570	NQGHS	GSCWAFSAIG AVEGINQIVT GNFTSLSE	QE LVDCDA-NND GCDGGDLIYA FEFIVNN-	GI SFEEDYPYTG REGTCDQARK
Droserain 3	NQGSCESLST GANINIGSDA	GSCWAFSTVA AVEGINQIVT GNITTLSE	QE LIDCDTTYNS GCSGGLMDYA FAYIMSNG	GL RKEDDYPYIM EEGTCEDTKA
DCAP_7627 DCAP_0302	HQGKC	GSCWAFSTLA AVESINQIKT NKLISLSE	QQ LVDCDK-SNQ GCNGERMTRA LAYIIKNG QE LIDCDTSYNS GCGGGLMDYA YKFILDNG	GV TTQKNYPYTR KEGDCNAGKA
DCAP_0302 DCAP_5993	DQGSC	GACWSFSATG AVEGINKIST GSLVSLSE GSCWAFSAVA AVEGINOIRT KOLVSLSE	QQ LVDCDK-SDQ GCNGGLMEYA FTYIQENG	GI DTEADIPIQA ROTSCNENEL GL ATEKHYPYTG KDGKCSEK-I
DCAP 5667	DQGAC	GSCWAFSTIA TVEGINYVVT GNLTSLSE	QE LVDCDRAEDM GCNGGLMDNA FQFILQNG	GI DSEDDYPYTA EDGVCDQSRM
DCAP_5945			QE LVDCDTDYDS GCNGGLMDYA FEFIIKNG	
ERVB_TABDI CPGP1 ZINOF	NQKQC	GSCWAFSAVA AVESINKIRT GQLISLSE	QE LVDCDT-ASH GCNGGWMNNA FQYIITNG QQ LVDCST-RNH GCEGGWPYRA FQYIINNG	GI DTQQNYPYSA VQGSCKPYRL
DCAP 6547	DOGGC	GSCWAFSTIA AVEGINOIVI GELIILSE	QE LVDCDTSYNE GCNGGLMDYA FEFIINNG	GI DTDODYPYKG YDSTCDTYRK
RD21A_ARATH	DQGGC	GSCWAFSTIG AVEGINQIVT GDLITLSE	QE LVDCDTSYNE GCNGGLMDYA FEFIIKNG	GI DTDKDYPYKG VDGTCDQIRK
ORYA_ORYSJ	DQGGC	GSCWAFSAIA AVEGINQIVT GDLISLSE	QE LVDCDTSYNE GCNGGLMDYA FDFIINNG	GI DTEDDYPYKG KDERCDVNRK
			* ↓ •	
PAPA1 CARPA	GPYAAKTDGV ROVOPYNEGA	LLYSTANOPU SVULEAAGKD FOLYRGGI	FV GPCGNKVDHA VAAVGYGPNYILIK	NS WETEWEENEY INIKRETEN-
DCAP 2570	TARVVAIDGF ESVPRKDEKS	LQKAVASQPI SVSIDTGGRA VQLYESGI	FT GQCGTKLDHS VVLIGYGTEN GTDYWLLR	SS WGPRWGENGY LRLERNIKHA
Droserain 3	VAEMVSIDGY HDVPANEEQS	LLKALANQPI SIAIEASGRD FQFYNGGV	VFD GHCGTELDHG VAAVGYGSTM GIDYIIVK	NS WGPKWGEKGY IRMKRNTGK-
DCAP_7627 DCAP_0302			APCAANINHE AAAVGYGDES SKKYWIFK FT GPCSTALDHA VLIVGYGSEN GKEYWIVK	
DCAP 5993			FD GYCGTSLNHG VATVGYSNEN GTKYWIVK	
DCAP_5667	NKRVVSIDGY EDVPSYNERA	LKKAVAHQPV SVAIEASGRA LQLYESGV	FT GECGTALDHA VVAVGYGRDN GLDYWIVR	NS WGADWGEEGY IRIERNAAS-
DCAP_5667 DCAP_5945 ERVB_TABDI	NAKVVTIDGY EDVPANDEKS	LQKAVAHQPV SVAIEAGGRA FQLYESGV	FT GTCGTQLDHG VVAVGYGTED GLDYWIVR	NS WGSSWGENGY IRLERNINDT
ERVB_TABDI CPGP1 ZINOF	RVVSINGF QRVTRNNESA	LQSAVASQPV SVTVEAAGAP FQHYSSGI	FT GPCGTAQNHG VVIVGYGTQS GKNYWIVR FT GSCNISANHY RTVGGRETEN DKDYWTVK	NS WGQNWGNQGY IWMERNVAS-
DCAP 6547	NAKVVTIDDY EDVPYNDESA	LKKAVASOPV SVIEDAAGRD FOLYDSGI	FT GRCGTSLDHG VVAVGYGTEK GKDYWIVR	NS WGENWGESGI IRVERNIAE-
RD21A_ARATH	NAKVVTIDSY EDVPTYSEES	LKKAVAHQPI SIAIEAGGRA FQLYDSGI	FD GSCGTQLDHG VVAVGYGTEN GKDYWIVR	NS WGKSWGESGY LRMARNIAS-
ORYA_ORYSJ	NAKVVTIDSY EDVTPNSETS	LQKAVANQPV SVAIEAGGRA FQLYSSGI	FT GKCGTALDHG VAAVGYGTEN GKDYWIVR	NS WGKSWGESGY VRMERNIKA-
PAPA1 CARPA	SYGVCGLYTS SFYPVKN			
DCAP 2570	RTGKCGIAME AFYPLKTKE			
Droserain 3	PEGLCGINKM ASYPIKKK-			
DCAP_7627	TAGACGITKM ASYPVKDV-			
DCAP_0302	SKGICGINML ASYPTKASP TAGACGITKM ASFPVIDAS	XX signal cleavage site	🔶 active Cys residue	signal sequence
DCAP_5993 DCAP_5667	FTGRCGIAME PSYPVKRGQ	 conserved residue 	active His residue	
DCAP_5945	KTGKCGIAME ASYPLKAGA		*	propeptide
ERVB_TABDI	SAGLCGIAQL PSYPTKA	* C in disulfide bond	stabilizing Asn residue	
CPGP1_ZINOF DCAP 6547	SSGKCGIAIS PSYPIKEXX GNGKCGISME PSYPVKKGQ			
RD21A_ARATH	SSGKCGIAIE PSYPIKNGE			
orya_orysj	SSGKCGIAVE PSYPLKKGE			

Figure 4.2: Many of the reference sequences belong to the papain cluster despite the diversity of their sources. Several proteins in cluster also have C-terminal granulin domains, which are shown in Fig. S4.3.

DCAP_0302 N DCAP_5667 E	STDMVTTVWD GGVARRNRP NPPPSP-RPGP EMNKPAWQFESE-EEQG NPPNPGPSPPTP-PPPD	AAAFIIV TKCDLFS / SSM	SVHMIILIAN SCGEGESCCC	XX GX LMDL ARSLLGFCLS	WRCCGLESAV		HDYPICDTQR	NQCLKV	
DCAP_5547 DCAP_6547 RD21A_ARATH ORYA_ORYSJ	NPPNPGPTPPSP-VKPP NPPNPGPSPPSP-IKPP NPPNPGPTPPSP-TPPP	TVCDSYY TQCDSYY	TCPESSTCCC TCPESNTCCC	AFEFGRYCFE LFEYGKYCFA	WGCCPLEGAT WGCCPLEAAT	CCDDHYSCCP CCDDNYSCCP	HTYPICNVEE HEYPVCDLDQ	GICQMSKGNP GTCLLSKNSP	LGVKMLKRTR FSVKALKRKP
DCAP_2570 DCAP_0302 DCAP_5667 DCAP_5945 DCAP_6547 RD21A_ARATH ORYA_ORYSJ	AKSYPKLLGG AGQIE ATPKWNLARR GFEGKGMSS: ATPFWSQGKN. AKPNLSPLFGNGKKS:	а А Г А			AYW	 conserve C in disulf conserved in granulir 	ide bond I Cys residue	g	rro-rich linker ranulin domain ocalization signa

Figure 4.3: The papain cluster granulin domains contain several examples homologous to the reference proteins RD21_ARATH and ORYA_ORYSJ. Papain itself lacks a C-terminal granulin domain, so it is not included in the alignment. DCAP_2570 and DCAP_5667 are truncated, and therefore do not contain both disulfide bonds stabilizing the granulin domains. DCAP_ 5945 contains an extra C-terminal extension not found in the reference sequences. The conserved sequence region characterizing animal granulin domains is shown above the corresponding sequences for comparison. The plant granulin sequences have two distinguishing features; an additional conserved Cys residue is present immediately after the first conserved CC pair in the animal sequence, and a 6-residue insertion containing another conserved C is present between the first and second CC pairs.

			• EVVVI	XXXFXXNXXX		
PAPA1 CARPA	MAMIPSISKL LEVAICLE- VYMGLSECDE S	SIVCYSONDL TSTERLIOLF			-IDETNKKNNS	YWLGLNVFAD
Aspain		EAYLHEHINL			-IHOHNOMNMP	YKLRLNEFAD
DCAP 6609	METTKI LLVALFLASV LCLAKSFDNA-E	EIFDFEEDDL SSDRTLWDLY	GRWRTHH-MV HRNHEEKRAL	R-FNVFKANVHH	-IHHHNKMGKP	YKLRLNKYGD
CYSEP_VIGMU	MAMKKL LWVVLSLSLV LCVAN	-SFDFHEKDL-ESEESLWDLY	ERWRSHH-TV SRSLCEKHK	R-FNVFKANVMH		YKLKLNKFAD
DCAP_7844	MDSKKF LLVTLSLVLV VGLSQ	-AFDFHEKDL-ESEESLWDLY	ERWRSHH-TV SRSIEDKHK	R-FNVFKENVFH		YKLKLNKFAD
DCAP_2555	<mark>KF LLVALSLALV VGLSQ</mark>	AFDFHEKDL ESEESLWDLY	ERWRSHH-TV SRSIEDKHK	R-FNVFKENVFH		YKLKLNKFAD
DCAP_6618		MALDL-ESEESLWNLY	ERWRNHH-TT SRSIEDKHK	R-FNVF <mark>EKNVLH</mark>		YKLRLNKFAD
DCAP_2122	MDSKKF ILVALSMALV IGLSQ	-GFDFHEKDL-ESEESLWNLY	ERWRSHH-TT SRSIEGMHK	R-FNVFKKNVLH	-VHEVNKMDKP	YKLKLNKFAD
PAPA1 CARPA	MSNDEFKEKY TGSIAGNYTTTELSYEE V	VLNDGDVNIP EYVDWRQKGA	VTPVKNQGSC G			SCWAFS
Aspain	MTNFEFQSFY -GSSIKHHRM YHPRSQTP F	FM¥EQADELP LSVNWRTKGE	VIPVKNQGKC GKFSCFRYP	QAAGFFLLCY	NNLVSWIMVC	VYVGSCWAFS
DCAP 6609	MTNDEFRSFFGSNIEHHRM-YHPRNQTS-F	FM¥AQADELP LSVDWRLRGA	VNPIKNQGKC G			SCWAFS
CYSEP VIGMU	MTNHEFRSTY AGSKVNHHKM FRGSQHGSGT F	FMYEKVGSVP ASVDWRKKGA	VTDVKDQGQC G			SCWAFS
DCAP 7844	LTNHEFRTLY ASSKIKHHLM LKGRAQGKCS F	FM¥ANVASLP PSI <mark>DWRTK</mark> GA	VTPVKNQGQC G			SCWAFS
DCAP_2555	LTNHEFRTLY ASSKIKHHRM LKGRAQGKCS F	FM¥ANVASVP PSI <mark>DWRTK</mark> GA	VTPVKNQGQC G			SCWAFS
DCAP_6618	LTSHEFRTQY ASSKIKHHRM LKGKARGSET F	FSYANVADVP PSVDWRDLGA	VTPVKNQGQC GAYSFK		YLF	NLGGSCWAFS
DCAP_2122	LTSHEFRTQY ASSKIKHHQM LKGKARGSET F	F SYANVAD VP PSVDWRDLGA	VTPVKNQGQC G			SCWAFS
		0000 0 0	•0000 •• • 0 *			
PAPA1 CARPA	AVVTIEGIIK IRTGNLNEYS EQELLDCDRR -			REKGPYAAKT	DGVROVOPYN	
Aspain	TVAAIEGINQ IRTNELVSLS EQELVDCDKS -					
DCAP 6609	AVGAIEGINQ IRTNQLVSLS EQELVDCDKD -					
CYSEP VIGMU	TIVAVEGINQ IKTNKLVSLS EQELVDCDKE E					
DCAP 7844	TVVSVEGINA IKTRKLVSLS EQELIDCDNG Q					
DCAP_2555	TVVSVEGINA IKTRKLVSLS EQELIDCDKG (
DCAP 6618	TVAAIEGINA IKTNNLVSLS EQELVDCDRA E					
DCAP 2122	TVAAIEGINA IKTNNLISLS EQELVDCDRA Q	-	-			
—						
			4	•		•
PAPA1 CARPA	QPVSVVLEAA GKDFQLYRG	OO # O GIEVGPCONK	VDHAVAAVGY GPNY		GEN-GYTRIK	RGTGNSYGVC
Aspain	QPVSVAVDAS GSSMQFYSS					
DCAP 6609	OPVSVSIDAG SSSMOFYSE					
CYSEP VIGMU	QPVSVAIDAG GSDFQFYSE	-	-			
DCAP 7844	QPVSVAIDAG GQDFQFYSE					
DCAP 2555	QPVSVAIDAG GQDFQFYSE					
DCAP 6618	QPVSVAIDAG GQDFQFYFEV TFHLRLLDYN K					
DCAP_2122	QPVSVAIDAG GQDFQFYFE					
PAPA1 CARPA	●000 0000 GLYTSSFYPV KN		XX signal cleavage site			_
Aspain	GIAMDASYPI KLSSNNTANC YAWTKT		_ • •	SI	gnal sequence	5
DCAP 6609	GIAMDASYPT KLSATNSTAS TPANI		 conserved residue 	pr	opeptide	
CYSEP VIGMU	GIAMMASYPI KNSSDNPT <mark>GS-LSSPKDEL</mark>		C in disulfide bond	Pi		
DCAP 7844	GIAMEASYPI KNSSVNPT PAESNDN E	DDDDWFSKKK-DEL		El	R retention sig	gnal
DCAP_2555	GIAMEASYPI KNSSVNPT PADSDDS E	DDDDWFSKRK-DEL	active Cys residue			
DCAP_6618	GIAMEASYPT INT <mark>DDDSNIP DAGTDGGDDS E</mark>	EDEDWFNKKD EL-	active His residue			
DCAP_2122	GIAMEASYPT INT <mark>DDDSNIP DAGTDGGDDS I</mark>	DDEDWFNKKD EL-	•			
		+	 stabilizing Asn residue 	:		

Figure 4.4: Many proteins in the vignain cluster, including vignain itself, are characterized by the localization tag KDEL at the C-terminus. This sequence element indicates that the protein is marked for retention in the endoplasmic reticulum.

	• EXXX RXXXEXXXX
DCAP_7656	MENHPVLAIM-CSQLRNQCHW-LPLLLLLLS TLACLSWALS SEYSITCYDP-KDLA-SDERISQLFQ-QWSQKHCKWYRHAKEAAK-RRENFKRNLK-
DCAP_5115	MCNQLRNQCHW-LPLLLLLLS TLACLSWALS-SEYSITCYDP-KDLA-SDERIAQLPQ QWSQKHCKVYKHAKEAAR-RRENFKWNLQ
DCAP_7518 DCAP_6097	
PAPA1 CARPA	MAMI-PEISKLIPVAI-CLPVAKGLEF CEPESIVCKSQ NDLT STBALIQLPE SWALKINKIYKNIDEKIY-REFIFYDALK
ORYA ORYSJ	M-RIS
RD21A_ARATH	<mark>MGFL-KPTMAILFLAMVAVSSA-UD</mark> MSIIS <mark>YDE</mark> -KHGVSTTGGR- <mark>SEAE</mark> VMSI¥ <mark>E</mark> -AWLVKHGKAQ-SQNSLV <mark>E</mark> KDR-RFEIFKDNLR
	•
	XIXXXN OO OO O O O O O O O O O O O O O O O
DCAP_7656	VIERNSKIN A-A-TOHTVCI, N-FADISNOE-FRENKISKVK- PVY NNH T-HIM OCHERS CDAPSSIDWR KKGVVTGVKD OCDCGSCWAF SSTGAIEGIN
DCAP_5115 DCAP 7518	VVIEKNSKEN-A-ATDHTVGL-NRFADLSNQE-FKENYLSKVK-KPAYKNNNET-RIMEQGRLRS-CDAPSSLDWR KKGVVTGVKD QGDCGSCWAF SSTGAIEGIN
DCAP 6097	YMIE NS NT S- TSITVGL N FADMSNEE FNN YLS W - P-Y NATAS VTGHRMRS-CDAPSSLDWR KKGVVTGVKN QCDCCSCWAF SSTGATGIN
PAPA1 CARPA	YIDETNIKNNSYWLGL NVFADNSNDE FEBYYTGSIA GNYTTTELSY EEVLNDCD VNIFEYVDWR QKGAVTFVKN QGSCGSCWAF SAVVTIEGII
ORYA ORYSJ	YIDEHNAAAD-AGVHSFRLGL-NRFADLTNEE-YRDTYLGLRN-FPRERKV8D-RYLAADN-EALPESVDWR TKGAVAEIKD QGGCGSCWAF SAIAAVEGIN
RD21A_ARATH	FVDEHNEKNLSYRLGL_TRFADLTNDE-YRSKYLGARM-EKKGERRTSL-RYEARVG-DELPESIDWR KKGAVAEVKD QGGCGSCWAF STIGAVEGIN
_	
	•••• •• • • • • • • • •
DCAP_7656	AITGDLVSL SEQELVSCOS T-NYGCEGGY MDYAFEWVIS NGGIDTESDY PYTGEDGTCD TTK
DCAP_5115	AITTGLUSI SEDELVSCOS T-NYGCEGGY MDYAFEWVIS NGGIDTESDY PYTGEDGTCD TSK
DCAP_7518 DCAP 6097	AISTGDLISL SEQELVDCDT T-NEGCDGGY MDYAFEWVVS NGGIDTESDY PYTGVDGTCD TSKVWLLASH IQRSQVVLLP PITSLHIYSC FQEEKKVVTI AISTGDLMSL SEQELVDCDT T-NEGCDGGY MDYAFEWVVS NGGIDTESDY PYTGVDGTCD TSK
PAPA1 CARPA	ATSIGNADA SQCLUDCDI I-REGLUGGI MILATEWVVS MODIFIEDU ISA
ORYA ORYSJ	QUVTGDLISL SEGELVDCDT SVNEGCNGGL MDYAFDFIIN NGGIDTEDDY PYKGKDERCD VNR
RD21A ARATH	QIVTGDLITL SEGELVDCDT SYNEGCNGGL MDYAFEFIIK NGGIDTDKDY PYKGVDGTCD QIR
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	• •• • • • • • • • • • • • • • • • • • •
DCAP_7656	DGYEDVDQ-S DSALLCATAT QPISVGIDGS AINFQLYTGG IYDGDCSSDP DDIDHAVLIV GYGSEGDEDY WIVKNSWGTS WGIQGYFYLR RNTDLEYGVC
DCAP_5115	NGYEDIDQ-S DSALLCGTVT QPISVGIDGS AIDFQLYTCG IYDGDCSSDP DDIDHAVLIV GYGSEGDEDY WIVKNSWGTS WGIQGYFYLK RNTDLEYGVC
DCAP_7518	DGYEDVDE-S DSALLCATVD QPISAGMDGS ALDFQLYTGG IYDGSCSSDP NDIDHAVLIV GYSSEDDVKY WUVKNSWGTS WGIDGYFYLE RDDTKEYGAC
DCAP_6097	DCEDVDE-S DGALLCATVD QPISAGMDGS AIDFQLYTGG IYDGSCSSDP NDIDHAVLIV GYSSEDDVKY WIVKNSWGTS WGIDGYFYLE RDDTKEYGAC
PAPA1_CARPA ORYA ORYSJ	DGVRQVQPYN EGALLYSIAN QPVSVVLEAA GKDFQLYRGG IFVGPCGNKVDHAVAAV GYGPNY ILIKNSWGTG WGENGYIRIK RGTGNSYGVC DSYEDVTPNS ETSLQKAVAN QPVSVAIEAG GRAFQLYSSG IFTGKCGT ALDHGVAAV GYGTENGKDY WIVRNSWGKS WGESGYVRME FNIKASSGKC
RD21A ARATH	DSTEDVINS EDIQUAVAN QVVVALAAG GARQUIDSG IFDGSCGTQLDHGVAV GYGTENGADU WIVNANGKS WEBSGIVANE ANIASSGKC DSYEDVINS EESIKKAVAH QUISIAIEAG GARQUIDSG IFDGSCGTQLDHGVAV GYGTENGKDY WIVNANGKS WEBSGYLANA ENIASSSGKC
	• • • xxcdxxxxc pdxxxc pdxxc ccexxx gxx ccexxxxcc xdxxkccexx
DCAP_7656	AVNAMASYPT KT PSSPSPYP SPAVPPPPPP TPPPPPPQSP PPPPPPSPSP TQCAII-SKI_TKSKPK
DCAP_5115	AVNAMASYPT KT <mark>SSSPSPYP SPAVPPPPPP TPPPPPPESP PPPPPSPSP TQCGDF-SYC-SSTETCCCIF-EVFDFCLIYG CCEYTDAVCC-TGTDYCCPND</mark>
DCAP_7518	AINAMASYPT KS <mark>SSTSSPPP SPSPGTPPPP PPPSPPPPP PSPPPSPSP SQCGDTSSYC-ASTETCCCIF-ELLDYCFVYG-CCEYTDAVCC-TGTEYCCPSD</mark>
DCAP_6097	AINAMASYPT KSSSTSSPPP SPSPGTPPPP PPPPSPP PSPPPSPSP SQCGDTSSYC-ASTETCCCIF-ELLDYCFVYG-CCEYTDAVCC-TGTEYCCPSD
PAPA1_CARPA	GLYTSSFYPV KN
ORYA_ORYSJ	GIAVEPSYPL KKGENPP
RD21A_ARATH	
DCAP 7656	
DCAP_5115	¥ PICD VDDCL-CLK
DCAP_7518	¥PICDTEECL-CLKSSCDYLCVE-AKKRKWANRK-LPWTKIEAND-EKTYEPLK-WKPNQLAAAQ
DCAP_6097	¥ PICDTEECL_CLKVLKSPEF-LSSCD¥LCVE-AKKRKWAKRK-LP WTKIEAND -E KTYEPLK-WKTNQLAAAQ
PAPA1_CARPA	
ORYA_ORYSJ	YPICNVQQCT CLMARDSPLAVK-ALKFPLARPN LSFLFCNCKK SAA
RD21A_ARATH	YPVCDLDQCT_CLL8KNSPFSVK_ALKRKPATPF_WSQGRK_NIA
	nal cleavage site 🛛 🖕 active Cys residue 🛛 🔻 conserved Cys residue 📃 signal sequence 📃 granulin domain
 COI 	nserved residue 🚽 active His residue in granulin domain propeptide Pro-rich linker
🐥 Ci	n disulfide bond * stabilizing Asn residue possible
	* stabilizing Ash residue

Figure 4.5: The granulin domain cluster contains proteins with C-terminal granulin domains. Although they are not closely related to any of the reference sequences, RD21_ARATH and ORYA_ORYSJ are shown in the alignment in order to compare sequence features among the granulin domains. As shown for the papain cluster granulin domains, the conserved sequence region characterizing animal granulin domains placed above the corresponding sequences. As in the papain case, there are two additional conserved Cs and a 6-residue insertion between the first and second CC pairs. In these sequence, a deletion of one residue relative to the animal sequence also occurs between the first and second conserved Cys residues in the granulin domain. DCAP_7656 is missing most of the granulin domain, and instead contains the localization tag SKL near the C-terminus, marking it for transport to the peroxisome.

	0	E XXXEXXEXX NXXIXXN
PAPA1 CARPA	MAMI-PSISKLLFVA-ICLEVYMGLS-FGDFSIVGYS_QNDLTSTERL-I	
brom1_anaco	<mark>MASKVQLV_F-LFLFLCAM_WASPS</mark> A_A <mark>SRDEPNDP</mark> M_M	
SAG39_ORYSJ		ARHERWMAQ YGRV¥RDDAE-KARRFEVFKA-NVAFIESFNA-CN-HNFWLGV
DCAP_3872 Droserain 2	MASI-KCCAQLIFLA-V-LFFSVY FASQTSS- ARLINPELSM S(
DCAP 0995	MALI-NQHFHILCFA-V-LLFVL-VR-FAPCVS <u>ST-RLLCSNDLTM-K(</u> MAFI-NKHRQYIFLA-L-LLAI-VL-FSCRCSSSRLPNSELSM-RI	DEHEOWMED VCENVENCVE KEEEEVKIEKK NVEETEESNKLIDEELCU-DA
DCAP 8396	MAFT NQPSLCILLV-F=LFL=LGLL_AAQ===-GSA_SRLLSSESLM_E	
DCAP_7614 DCAP_8215	G SRPLGRFMLLA S-LFLGLQLL-AIQG SRPLNSNSSM-VI	
DCAP_8215		ERHEQWMAE-HGRVYADDIE-KEARFKIFRE-NVEKIEAYNL-MD-LGFTLGV
DCAP_2190 DCAP_0551		KRHEEWMIQ HGRTYKDHAE-KDARFQIFQK-NVELIEAHNK-MG-KSYTLSV KKHEDWMVQ YGRTYKDNAE-KAMRFEIFKK-TVEFIEAHNR-LN-KGYTLSV
DCAP_0551 DCAP_4240		KKHEDWMVQ IGRTYKDNAE AAMRFEIFKK-TVEFIEAHNR-LN-KGYTLSV KKHEDWMVQ YGRTYKDNAE KAMRFKIFKK-TVQFIEAHNR-LN-KGYTLSV
-		
PAPA1 CARPA	NVFADMSNDE FKEK¥TGSIA CN	
BROM1 ANACO	NQFTDMTKSE_FVAQYTGVSL_PLNIE	OSIDWRDYC AVNEVKNONE CCSCWAFSAV VIIEGIIKIK IGNINEISEQ
SAG39 ORYSJ	NGFADLTNDE FRWMKTNKGFIPST TRVPTGFR¥E NVNIDAL PA	
DCAP 3872		
Droserain 2	NAFADLTNAE-FLASRTCYKR-QTVCLS-SKKSTTFR¥E-NNSVL P/ NAFADLTNEE-FRASHTCYKR-QRSLDSS-RS-KPRSTSFR¥E-NLTCI P	RSIDWRRKG AVTPIKDQGL CGSCWAFSAV AAIEGLTKIK TGKLISLSEQ
DCAP_0995	NAFADLTNEE FRASHTGFKS QKARELAKSS KSKSSSFKYE NFTVV A	
DCAP_8396 DCAP_7614	NAFTDLTSEE FFASHTGYKA PEDLDS SFESTPFMYE NITAV PC	
DCAP_7614 DCAP_8215	NSFTDLSDEE FQATYTGLKG IENLES MYNSASFKYE NITSA PA NAFADLTPEE FRGSYTGYKK SS-SAS SLKSTTFRYE NLTDV PA	ASVDWVARG AVTPINHQGQ CGSCWIFATS ATTEGLHRIT TGRLVSLSEQ
DCAP 2190	NQFADLTDEE_FEKM¥AS¥KG_PESLRH_DSKHTSFK¥E_NYASV PA	ASINWVSMG AVTPVKNOGT CGSCWAFAAI ATVESLTWLY TRKSYDLSEO
DCAP_0551	NAFADLTDEE-FRKTYTGYKR-HESSNP-SLKSTSFR¥K-NFTDV PS	SSVNWVAAG AVTPIKNQGT CGSCWAFASV ATIESLCEIT TGNLISLSEQ
DCAP_4240	NAFADLTDEE-FRKTYTCYKR-HESSNS-SLKSTSFR¥K-NFTDV PS	SSVNWVAAG AVTPIKNQGT CGSCWAFASV ATIESLCEIT TGNLISLSEQ
PAPA1 CARPA BROM1_ANACO SAG39_ORYSJ DCAP_3872 Drosērain 2 DCAP_0995 DCAP_8396 DCAP_7614 DCAP_8215 DCAP_2190 DCAP_2551 DCAP_4240	ELLDCDRR SYGCNGGYPW SALQLVAQY- GIHYRNTYPY EGV-QRYCRS RI EVUDCAVSYGCRGGWUN KAYDFIISNN GVITEENYPY LAX-QGTCNA N ELVDCDVHGE DQGCEGGLMD DAFKFIIKNG GLITESNYPY IAX-DDKCKS V ELVDCDTN DGGCAGGLMD TAFAFVNTNG GLARESTYPY TGT-GDTCNR RI QLVDCDVDYG DEGCEGGLMD NAFTYINNG GLITENYPY TGT-GDTCNR RI QLUDCDTGGR DLGCRGGFSE SAYQFVIRNG GLITENYPY VGY-QQFCRR G- QILDCDIHGH QLGCKGGFYT EAYLFVKING GLITEINYPY VGR-QQTCKK N- EVVDCDVHS DLGCNGGTPD GAFSYMIRNG GLITEINYPY VGR-QQTCKK N- EVVDCDVHS DLGCNGGFAD GAFSYMIRNG GLITESAYPY VGRQUFCVG G- EILDCDVNGE DHGCNGGFAD GAYQFVINNG GLITEDNYPY VGYQENYCNG N- EILDCDVNGE DHGCNGGFAD GAYQFVINNG GLITEDNYPY VGYQENYCNG N-	S-FPNSAYI TGYSYVRRDD ERSMMYAVSN QPIAALIDAS -ENFQYYNGG S-NSVASI KGYEDVPANN EAALMKAVAN QPVSVAVDGG DMTFGYYKGG KASKIAAKI ISYEDVPNNN EAALKAAVAH QPVSVAVDGG DNFGYSSG LKASRAASI KGYKDVPSNN EAALLQAVAN QPVSVAVDGG DNNFGYSSG EEAQHAATI SGYEDVPSNN EQALUQAILAH QPVSVAVDGG DNNFGYSSG UPAAAKI GGYQVPSNN ECALUQAILAH QPVAVVIDAQ SYQLKHYQGG VPVAAKI GGYQVPNNN EKALQNAVAN QPVAVAVDSN STTFKNYKSG KASQVAAKI STYEDVPTNN EGAMQQAVSM QPISVALEAS GFFFGYYMGG DQVAVTI SGYENVPNND ESSMQQAVAN QPIAVVLDA
PAPA1 CARPA BROM1_ANACO SAG39_ORYSJ DCAP_3872 DCAP_0995 DCAP_8396 DCAP_7614 DCAP_7614 DCAP_8215 DCAP_8215 DCAP_0551 DCAP_0551 DCAP_4240	* CONTRACTOR OF CONTRACT OF CO	SSSSGVCGI AMAPLFPTLQ SGANAEVIKM VSET SDKRGMCGL AMEPSYPTA SNKEGTCGI AMPSYPIA SSKKGLCGI AMPSFPIA SSKKGLCGI AMMPSFPIA KDRKGRCGV AMFPAPYTN SDPRGLCGV AMMPSYPVA RDPRGLCGV AMYPAYPTN
	🛛 🕹 xx signal cleavage site	signal sequence
		propeptide
	 C in disulfide bond stabilizing Asn resi 	idue

Figure 4.6: The bromelain cluster is characterized by strong sequence identity with pineapple fruit bromelain.

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PAPA1_CARPA			<mark>MAMIP</mark>					-IQLFESWMLK-		
Dionain 1			MALTNMSS							
DCAP_0624 DCAP_3192			MASRN	QYCLEVIVA=	- ALCVL	CTEVENASSS	-RVLS-SEPSM-	AERHEQWMAK	YCRVYRDNAE VCRVYRDNAE	VETREFEIERK
DCAP_7132			MASRN	OYCLEVIVA-	-ALCVL	CTEVENASSS	RVLS-SEPSM	AERHEOWMAK	YCRVYRDNAE	KEIRFEIFKK
DCAP 6781			MASRN	QYCLFVIVA-	-ALCVL	CTEVSIASS	RVLS-SDPSM	AERHEQWMAK	YGRVYRDNAE	KEIRFEIFKK
DCAP_3193			<mark>M</mark>		-ALFVA	GIF <u>SS</u> NVCSA	RPLT-SEPSM	AERHEQWMAQ	YGRVYKDAAE	-KEMRFNLFKK
DCAP_7131			<mark>M</mark>				RPLT-SEPSM			
DCAP_7714			<mark>MD</mark>	MASTNMILV-	-VLFVL	GVFASSVSSS	RLLN-TQPSM	RERHEQUMSE	YGRVYKSDVE	KEARFQVFKD
DCAP_0107 DCAP 1658	MYRHKRRKNL				-VLFIL					
DCAP 4793							RLLS-SEPSM			
Dionain 3									YGRVYEDNAE	KERRFNIFKK
DCAP_5360			<mark>MS</mark>		-TIIAV					
DCAP_4952										
Droserain 1				тазузтык-		GPPUSHANA92	-Kris-seism	TERHEQWING	- IGRV IRNAVE-	-NYKKEBIEKK
	NXXXIXXXN	• • •		000			• • •		****	•
PAPA1_CARPA	NLKYIDETNK	-KNNSYWLGLN	VFADMSNDEF	-KEKYTGSIAG			PEYVDWRQKG	AVTPVKNQGS	CGSCWAFSAV	
Dionain l			AFSDLTNEEF							
DCAP_0624			EFSDMNNEEF							
DCAP_3192 DCAP_7132	NARYMEAKNK	INPORTICIN	EFSDMTNEEF	-RATHNGYKRH	ARHASSVEPT	SFRYENLTDV	PASIDWRSQG	AVTPIKNQGQ	CGCCWAFAAI	AATEGITEIN
DCAP_6781	NVEYMEAMNR	-INRGETLGIN	EFSDMTNEEF	BATHNCYKRH	ARHASSVKPT	SEKYENLTDV	PASIDWRSOG	AVTPIKNOGO	CGCCWAFAAT	AATEGITEIK
DCAP_3193			AFSDMSNREF							
DCAP_7131			AFSDMSNKEF							
DCAP_7714			AFTDMTSEEF							
DCAP_0107			AFTDMTSEEF							
DCAP_1658 DCAP_4793			AFTDMTNEEF AFTDMTNMEF							
Dionain 3	NVERIESMNK	-LNRPFTLGVN	-AFTDLTLEEF	-RASHNGYKO-	-RPVGSLKAT	SFKYENFTDV	PDSINWVTNG	AVTPVKDOGO	CGCCWAFSAV	ASTEGIHSIN
DCAP_5360	NVERIEAMNK	-LNRSFTLGVN	-AFTDLTTQEL	-RSSRNGYKK-	QSTMVSRT	SFKYENFTDV	PASVDWVANG	AVTPVKDQGQ	CGCCWAFSAV	ASTEGINEIR
DCAP_4952	NIEYIENMNR	-LNRSFSLGLN	AFTDLTLEEF AFTDLTTQEL AFTDLTNEEF	-RASHNGYRR-	PASNLRST	-FFKYENFTDV	PSSVDWVANG	AVTPVKNQGQ	CGCCWAFSAV	AATEGITEIS
Droserain 1	NIEYIENMNR	-LNRSFSLGLN	AFTOLTNEEF	-RASHNGYRR-	PASNLRST	-FFKYENFTDV	PSSVDWVANG	AVTPVKNQGQ	CGCCWAFSAV	AATEGITEIS
	• •	*	*****	000	• ••	o *			• •	•• • ••
PAPA1_CARPA	TGNLNEYSEQ		SYGCNGGYPW	SALQLVA-QY	GIHYRNTYPY	EGVQRYC				ANQPVSVVLE
Dionain 1			DKGCNGGTPD							
DCAP_0624			DQGCNGGYAD DQGCNGGYAD							
DCAP_3192 DCAP_7132			DQGCNGGIAD							
DCAP_6781			DQGCNGGYAD							
DCAP 3193	SGTLTSLSEQ	EIVDCDTNGN	DQGCNGGTPD	GAFTYIINNG	GITTESNYPY	QGTQGTC	NSNSF-PAAA	TLSSFQDVPT	NSEASLQQAV	ANQPVSVAID
DCAP_7131 DCAP_7714 DCAP_0107 DCAP_1658 DCAP_4793			DQGCNGGTPD							
DCAP_7714			DMGCGGGTMD DQGCNGGTAD							
DCAP_0107			DQGCNGGTAD							
DCAP 4793	TGTLTSLSEQ	EIVDCDTNGN	DQGCNGGTAD	GAFSFIMSNG	GITTESNYPF	VGSQGTC	NTNAG-PVAA	TLSSYQDVPS	NSESALLNAV	SNQPVSVAID
Dionain 3	TKKLVSLSEQ	QVLDCDTNGQ	DQGCNGGMPQ	GAFEYMISNG	GLTTEDAYPY	TGSQGWWC	NLW-FDAIAA	KISNYENVPS	D-EGSLQKAV	ANQPCSVAID
DCAP_5360			DQGCNGGMPD							
DCAP_4952			DQGCNGGTPD DQGCNGGTPD							
Droserain 1	IGNEISESEQ	QIVDCDIDGN	DQGCNGGIPD	GATSIIINAG	GLIIEDNIFI	NGNQGS===C	NGN=GGQPAA	1LSSIQDVPS	S-EDSTOOMAA	ANQPVSVALD
	•• •	• *	∳ ∞∞ ●	••	@000		•	* *0 • •		
PAPA1_CARPA			KVD HAVAAVG	YGPN	YILIKNSWGT	GWGENGYIRI	KRGTGNSYGV			
Dionain 1 DCAP_0624			NLDHAVTVVG NLDHAVTAVG							
DCAP_3192			NLDHVVTAVG							
DCAP_7132			NLDHAVTAVG							
DCAP_6781			NLDHAVTAVG							
DCAP_3193			NLDHAVTAVG							
DCAP_7131 DCAP_7714			NLDHAVTVVG NLDHAVTIIG							
DCAP_7714 DCAP_0107			NLDHAVTIVG							
DCAP 1658			NLDHAVTVVG							
DCAP_4793			NLDHAVTVVG							
Dionain 3	ASCDDFMQYS	GGVFSESCGD	NLDHAVTAGG	YGTTD-DGTD	YWLVKNSWGT	SWGENGYIRM	QRNVGG-DGM	CGIATDASYP	TI-	
DCAP_5360			DLDHAVTVVG							
DCAP_4952 Droserain 1			DLDHAVTVVG DLDHAVTVVG							
Proserdin 1	MOGODIQUIS	Gevr:GSCGT	DIDINA I V VG	1919 0-0 910	THEADNOL	SWGENGIIKM	Zveree-cer	<u>uainnQAD</u> 1P	1N-	
		XX signal of	cleavage site	🔶 a	ctive Cys resi	due	signal	sequence		
		 conservation 	ved residue	🖌 a	ctive His resid	due	proper	tide		
		🖌 C in dis	sulfide bond				higher			
				★ SI	abilizing Asn	residue				

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Figure 4.7: The dionain cluster contains many cysteine proteases that appear to be specific to Caryophylalles carnivorous plants; this cluster contains the dionains from D. muscipula as well as several proteins from D. capensis, but none of the reference sequences from other sources.

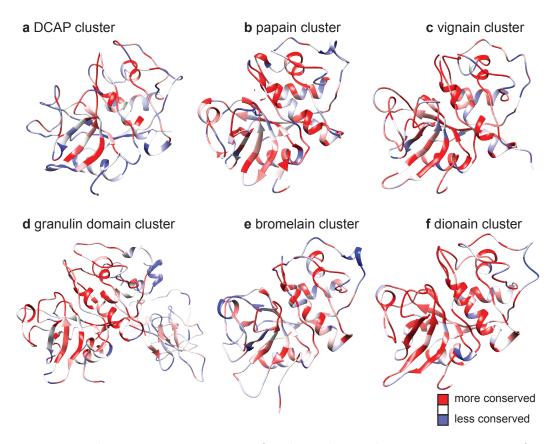


Figure 4.8: The percent conservation of each residue in the consensus sequence for each cluster is shown mapped onto a representative member of the cluster. The color scale ranges from red (more conserved) to blue (less conserved). a. DCAP cluster (DCAP_2263) b. papain cluster (papain) c. vignain cluster ((DCAP_2122) d. granulin domain cluster (DCAP_5115) e. bromelain cluster (droserain 2) and f. dionain cluster (DCAP_0624).

4.3 Results and Discussion

I discuss some important and relevant results from the paper [30] in this section- author contributions can be found in the paper. Molecular modeling, protein network analysis and all the predicted and equilibrated structures are available to download from our paper [30].

4.3.1 *D. capensis* Cysteine Proteases Cluster Into Distinct Families Based on Resemblance to Known Homologs

All *D. capensis* sequences previously annotated as coding for MEROPS C1 cysteine proteases using the MAKER-P (v2.31.8) pipeline [49] and a BLAST search against SwissProt (downloaded 8/30/15) and InterProScan [50] were clustered by sequence similarity. Several previously-characterized cysteine proteases that have been identified from other plants are also included as reference sequences. Clustering of the *D. capensis* cysteine protease sequences reveals a broad range of cysteine protease types, some of which are homologous to known plant proteases (Figure 4.9). Three of the six clusters contain only proteins from *D. capensis* or the related Venus flytrap *Dionaea muscipula*, while many of the reference sequences cluster together despite coming from a variety of different plant species from diverse orders including both monocots and eudicots. The general types of plant protease features found correlate well with previous surveys of cysteine proteases in *Arabidopsis thaliana* [119], *Populus sp.* [104], and more recently, soybeans [120] and a broader group of plant proteases from a variety of species [7].

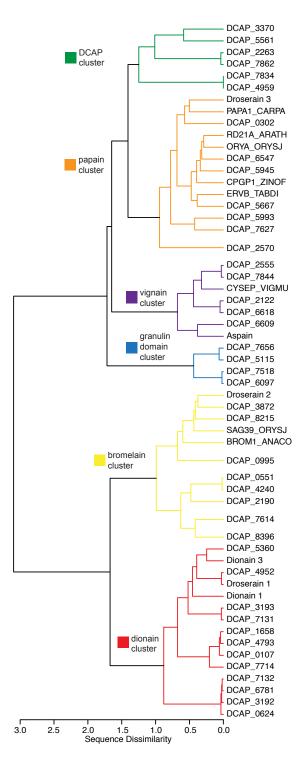


Figure 4.9: Clustering of cysteine protease sequences identified from the *D. capensis* genome. Many are homologous to known plant cysteine proteases, including dionain 1 and dionain 3 from the Venus flytrap, *Dionaea muscipula*. Dissimilarity between clusters is defined by the *e*-distance metric of [4] (with $\alpha = 1$), which is a weighted function of within-cluster similarities and between-cluster differences with respect to a user-specified reference metric. The underlying input metric employed here is the raw sequence dissmilarity (1 - (%identity)/100).

4.3.2 Residues Conserved in *D. capensis* Cysteine Proteases Include Active Sites and Important Sequence Features

A defining feature of C1A cysteine proteases is the Cys-His catalytic dyad, which is often accompanied by an Asn residue that stabilizes the protonated catalytic His [121, 122]. The mechanism of these enzymes requires using the thiolate group on the deprotonated cysteine as a nucleophile to attack a carbonyl carbon in the backbone of the substrate. Preliminary sequence alignments comparing putative cysteine proteases from D. capensis were used to discard sequences lacking the conserved Cys and His residues of the catalytic dyad due to either substitution or truncation. Other conserved features were observed in many of the sequences, but were not treated as necessarily essential for activity. Reference sequences used include zingipain 1 from Zingiber officianale (UniProt P82473), pineapple fruit bromelain (Ananas comosus, UniProt O23791), RD21 from Arabidopsis thaliana (UniProt P43297), oryzain alpha chain (UniProt P25776) and SAG39 (UniProt Q7XWK5) from Oryza sativa subsp. japonica, ervatamin b from *Tabernaemontana divaricata* (UniProt P60994), and dionains 1 and 3 from the related *Dionaea muscipula* (UniProt A0A0E3GLN3, and A0A0E3M338, respectively). Several of the reference sequences, e.g. zingipain-1 [123], were characterized by mass spectrometric analysis of the mature enzyme; these sequences therefore lack the signal peptide and pro-sequence found in the initially transcribed sequence. Sequence alignments for the individual clusters (seen above) are annotated to highlight individual amino acid properties, residues conserved within the cluster and/or shared with papain, as well as functional sequence features, as described above. In addition to the cluster-specific reference sequences, all clusters include papain (*Carica papaya*, UniProt P00784) in order to have a common reference for all the C1A proteases discussed in this work.

Most of the clusters are named after a reference sequence or a distinguishing feature of its members. The DCAP cluster is highly diverse, yet it contains only sequences from D.

capensis. The papain cluster contains many of the reference sequences, as well as several D. capensis proteases, some of which have granulin domains (Figures 2 and 3), a feature that is peculiar to plant cysteine proteases. The vignain cluster (Figure 4) contains vignain from Vigna mungo (UniProt P12412) as well as D. capensis homologs. Many of the proteins in the vignain cluster have C-terminal KDEL tags, indicating retention in the ER lumen, suggesting that they are involved in germination and/or senescence. In the granulin domain cluster (Figure 5), every sequence but one contains a granulin domain connected to the catalytic domain by a proline-rich linker of about 40 residues; the one exception is truncated after the proline-rich region. Several sequences in the papain cluster also contain granulin domains, however the Pro-rich linkers in those sequences contain only about 16 residues and the sequence identity between the two types of granulin domains themselves is not high. The bromelain cluster (Figure 6) contains homologs of both defensive and senescence-related enzymes. Every sequence in the dionain cluster (Figure S7) contains an extra Cys residue immediately prior to the active site Cys. This CCWAF structural motif has been previously observed in the Arabidobsis protein SAG12 and homologs [7]; however, the function of the double Cys in unknown. It may have cataytic relevance, perhaps providing a second nucleophilic thiolate or operating as a redox switch.

Like many other proteases, the papain-family enzymes are expressed with an N-terminal prosequence blocking the active site. This sequence is cleaved during enzyme maturation, often upon the protein's entering a low-pH environment. This pro-sequence was found in most of the C1A proteases from *D. capensis* (highlighted with pink boxes in figures 1-7 in the above subsection). Plant C1A protease pro-sequences are often bioactive in their own right, acting as inhibitors of exogenous cysteine proteases. This enables them to deter herbivory by insects [124], nematodes [125], and spider mites [126], protecting the plants from damage. This can be technologically exploited by producing transgenic crop varieties with protective cysteine proteases they would otherwise lack [127]. This approach has proven useful in protecting crops from Bt-resistant pests [128]. Despite some variation in the lengths of the C-terminal and N-terminal regions, all the cysteine proteases investigated here show substantial similarity in the pro-sequences; in particular, the ERFNIN motif ($EX_3RX_3FX_2NX_3IX_3N$) often found in the pro-sequence of C1A proteases [129] is conserved in many sequences spanning all the clusters. Interestingly, the alternative sequence $EX_3RX_3FX_2NX_3AX_3Q$, which is characteristic of the RD19 family of plant cysteine proteases, is found in only one of the *D*. *capensis* proteases, DCAP_3370 in the DCAP cluster. For all previously uncharacterized sequences, SignalP 4.1 [26] was used to predict the location of the signal sequences, if any, while the pro-sequence annotations were then used as the basis for further structure prediction and functional analysis.

In addition to the common sequence features in the N-terminal pro-region, other variations are observed, such as the presence of C-terminal granulin domains in some sequences and extra insertions that may be responsible for specific activities in others. Examples of organelle-specific targeting sequences are observed; several sequences have a C-terminal KDEL sequence targeting them for retention in the endoplasmic reticulum, while others have targeting sequences indicating their destination in the cells, including signals indicating transport to the vacuole (NPIR, but not FAEAI or LVAE) or the peroxisome (SSM at the C-terminus). The level of sequence conservation among the members of each cluster varies dramatically, as can be seen in Figure 8, where sequence conservation is mapped onto the structure of a representative member of each cluster. The sequences in the DCAP cluster are less closely related to each other than the members of any of the other clusters, and some are homologous to reference sequences used by Richau et al. [7].

Another interesting result was of DCAP_2263 and DCAP_7862, which belong to the Richau

aleurain (cathepsin H) cluster. In humans, cathepsin H is an aminopeptidase that processes neuropeptides in the brain [130], as well as acting as a lysosomal protein in other tissues. Its barley (*Hordeum vulgare*) homolog, aleurain, has both aminopeptidase and endopeptidase activity [131], suggesting that DCAP_2263 and DCAP_7862 may have both types of activity as well. This hypothesis is supported by the presence of the Cathepsin H minichain sequence in its plant orthologs, as discussed in the section devoted to these proteins. DCAP_3370 is related to the Richau RD19 (cathepsin F) cluster, and is the only protease in this set that contains the characteristic pro-sequence motif (EX₃RX₃FX₂NX₃AX₃Q), of the RD19 (cathepsin F) family. Human cathepsin F is distinguished by its unusually long pro-domain, which is approximately 100 residues longer than that of other cysteine proteases and adopts a cystatin fold [132]. In contrast, the pro-sequence of DCAP_3370 is about 140 residues, typical for a plant cysteine protease. The last enzyme in the DCAP cluster, DCAP_5561 is not closely related to anything in either reference set. A BLAST search yields numerous matches to uncharacterized predicted cysteine proteases from a variety of plant genomes, however, the specific function of this enzyme remains enigmatic.

4.3.3 Some Cysteine Proteases Are Targeted to Specific Locations

Several of the cysteine proteases identified from D. capensis contain known targeting signals that mark the protein for delivery to specific cellular locations. The most common such signal is the N-terminal signal peptide targeting the protein for secretion. As expected, the majority of proteins in this set contain such a secretion signal. In plants, the secretory pathway delivers proteins to the vacuole, the vacuolar membrane, the cell wall, and the the plasma membrane. In D. capensis, digestive enzymes are also expected to be secreted into the mucilage. In addition to the N-terminal signal sequences, tri- or tetrapeptides indicating that the protein is destined for a particular subcellular compartment are also found in many cases. Figure 4.10 shows the structures predicted by Rosetta for three full-length cysteine proteases containing targeting signals, DCAP_2263, DCAP_5667, and DCAP_2122. Ribbon diagrams are shown for all three enzymes; a surface is also shown for DCAP_2122 in order to assist with visualization of the relationship of the pro-sequence, N-terminal signal peptide, and Cterminal localization sequence to the rest of the protein. The positioning of the pro-sequences (pink) and signal peptides (light orange) is highly variable, although in each example the pro-sequence blocks the active site and the signal sequences and other localization tags (light purple) are in highly exposed positions as expected based on their function.

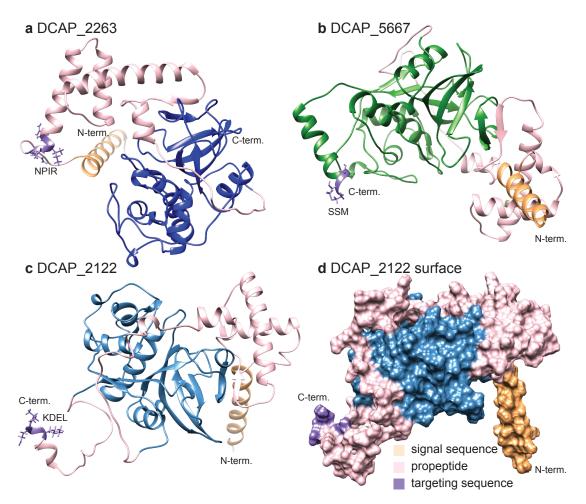


Figure 4.10: Predicted structures for three full-length cysteine proteases. The secretion signals are highlighted in light orange, the pro-sequences in pink, and the localization tags in light purple. a. DCAP_2263 contains the target sequence NPIR, indicating localization to the vacuole. b. DCAP_5667 ends in the tripeptide SSM at the extreme C-terminus, indicating transport to the peroxisome c. and d. DCAP_2122 ribbon diagram and surface model, respectively. DCAP_2122 ends in the ER-retention signal KDEL, indicating that it is retained in the ER lumen.

In plants, the subsequence NPIR in the N-terminal region of a protein indicates targeting to the vacuole, a large acidic compartment that is specific to plant cells and serves the same function as the lysozome in animal cells. These compartments, which often occupy most of the volume of the cell, contain a variety of hydrolases, including both aspartic and cysteine proteases, which normally act to recycle damaged or unneeded cellular components. Upon infection by viruses or fungal pathogens, the vacuole can also fuse with the plasma membrane to release defensive proteases into the extracellular space. Two putative vacuolar proteases, (DCAP_2263 and DCAP_7862) are found in the DCAP cluster. The NPIR tag is located in an exposed position between the secretion signal and the beginning of the N-terminal pro-sequence, as shown for DCAP_7862 in Figure 4.11. These proteases display sequence homology to mammalian cathepsin H, a lysozomal protein that is important in development and also implicated in cancer proliferation [133, 134].

In human cathepsin H, aminopeptidase activity is modulated by the minichain sequence (EPQNCSAT). DCAP_2263 and DCAP_7862 (and aleurain, but no others in this set) contain the sequence AAQNCSAT, which may have a similar function. The hypothesis that this plant-specific minichain serves a similar role in modulating the substrate specificity is supported by comparing the predicted structures with the crystal structure of porcine cathepsin H (PDBID: 8PCH) [135]. Figure 4.11 shows the predicted structures of mature DCAP_2263 (blue) and DCAP_7862 (green) overlaid with the crystal structure of porcine cathepsin H (gray). The predicted structures of the plant proteins coincide with the porcine protein in the major secondary structure elements, albeit with substantial variation in loops and linkers. The minichain sequence (EPQNCSAT in the porcine protein and AAQNC-SAT in the *D. capensis* proteins) occupies a similar position in all three structures, allowing substrate approach to the active site cleft from one side (Figure 4.11a), but not the other (Figure 4.11b). Biochemical characterization of human cathepsin H has shown that deletion of the minichain abolishes aminopeptidase activity [136], making this protein a standard endopeptidase. Based on sequence homology and examination of the predicted structures, we hypothesize that this sequence plays a similar role in modulating the substrate specificity and activity patterns of DCAP_2263 and DCAP_7862.

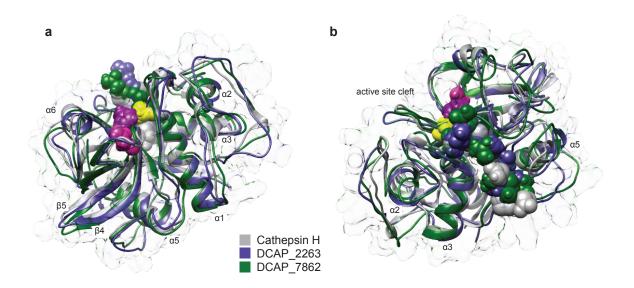


Figure 4.11: Predicted structures for two vacuolar cysteine proteases (DCAP_2263, blue and DCAP_7862, green) with sequence homology to cathepsin H (PDBID: 8PCH gray). The active site residues and the minichain are shown as space-filling models. a. One side of the active site cleft is open and accessible to substrate. b. The other side of the active site cleft is blocked by the minichain. In cathepsin H, this partial occlusion of the active site confers aminopeptidase specificity.

Other proteases are targeted to the peroxisomes, organelles that bud from the ER membrane and primarily break down long-chain fatty acids, but are also involved in the synthesis of functional small molecules, such as isoprenoids, polyamines, and benzoic acid [137]. Some proteases in the peroxisome are involved in the maturation of other enzymes imported to this organelle, as well as disposal of oxidized proteins that build up in this challenging redox environment [138]. Others are active during different developmental stages, such as differentiation of seed glyoxysomes to mature leaf peroxisomes [139]. The most common type of targeting signal for transport to the peroxisome is one of several C-terminal tripeptides. The canonical example is SKL, but others have been discovered in a variety of plant proteins [140]. DCAP_5667, which is in the papain cluster (Figure 3), has the tripeptide SSM at its extreme C-terminal end, indicating targeting to the peroxisome. DCAP_7656, which is in the granulin domain cluster (Figure 5), contains the SKL sequence not at the C-terminal end, but at a highly exposed position near the C-terminus, suggesting possible peroxisome targeting for this protein also. DCAP_7656 contains the proline-rich linker common to this cluster, but its granulin domain is truncated. Another possibility is that the short sequence region following the SKL tripeptide may be cleaved under some circumstances, acting as a switch that determines whether this enzyme is sent to the peroxisome or elsewhere. Peroxisome-targeted proteases represent attractive targets for biotechnological studies, because they are optimized to remain stable and maintain their activity under harshly oxidizing conditions.

Proteins with the sequence KDEL at the C-terminus are retained in the lumen of the endoplasmic reticulum, enabling them to be stored in specialized vesicles as zymogens and released to mediate programmed cell death in response to a stressor or during a particular developmental phase. KDEL-tailed proteases such as vignain from *Vigna mungo* and CysEP from *Ricinus communis* play an important role during germination, when proteins stored stored in endosperm tissue are degraded for use as the cotelydons develop. A C-terminal propeptide including the KDEL tag is removed along with the N-terminal pro-sequence during maturation, to yield the soluble, active enzyme [141]. The crystal structure and biochemical characterization of a homologous KDEL-tailed protein from the castor bean indicates that this enzyme has a strong preference for large, neutral amino acids in the substrate peptides, and has an unusually large and possibly flexible substrate-binding pocket that can accommodate a variety of sidechains, including proline [142].

4.3.4 Several Discovered Proteases Possess Novel Granulin Domains

Cysteine proteases with a C-terminal granulin domain are specific to plants, where they are involved in response to dessication or infection by pathogenic fungi [143]. This type of domain is found in two of the *D. capensis* protease clusters, the papain cluster Figure 3 and the granulin domain cluster Figure 5. The reference sequences RD21A (RD21A_ARATH) from arabidopsis and oryzain (ORYA_ORYSJ) from rice both contain granulin domains, as do three proteins in the papain cluster (Figure 3) and three in the granulin domain cluster (Figure 5). An additional two sequences in the papain cluster and one in the granulin domain cluster contain truncated versions that do not contain all four cysteine residues necessary to form the two disulfide bonds stabilizing the granulin domains. The granulin domain is separated from the catalytic domain by a proline-rich linker region. In RD21A, which is found in both the vacuole and the ER bodies [144], the granulin domain is removed from the mature enzyme. Maturation within the vacuole is relatively slow and involves accumulation of an intermediate where the N-terminal pro-sequence is removed and the C-terminal granulin domain remains attached [145]. This intermediate species forms aggregates that slowly release active enzyme following cleavage of the granulin domain, which is performed by RD21 itself [146]. This suggests that aggregation mediated by the granulin domain provides a mechanism for regulating protease activity during leaf senescence.

The granulin domain is attached to the catalytic domain by a proline-rich linker of variable length, as illustrated in Fig. 4.12. Granulins in animals act as growth factors, and contain distinct sequence and structural features: the characteristic sequence motif consists of four pairs of cysteine residues, with single conserved cysteines on both sides, and the resulting fold consists of β hairpins held together by disulfide bonds [147]. In plants, the granulin domain has two additional cysteines and an insertion of 6 residues between the first two Cys pairs, slightly modifying the structure (Fig 4.13a). Clustering of the granulin domains themselves, separately from the catalytic domains, yields three clusters (Fig 4.13b), two of which contain proteins from the *D. capensis* papain cluster and one of which is made up entirely of proteins from the *D. capensis* granulin domain cluster. The cluster analysis of Richau, et al [7] identified two subfamilies of granulin domain-containing cysteine proteases; comparison with those results places DCAP_0302 in their XBCP3 cluster, while DCAP_5945 and DCAP_6547 are in their RD21A cluster. Notably, the *D. capensis* granulin domain cluster represents a new subfamily of plant cysteine proteases that is not closely related to either of the previously described subfamilies.

The key sequence region of the canonical animal granulin motif is shown above the sequence alignment for comparison (Fig 4.13c). The plant granulin sequences have two distinguishing features; an additional conserved Cys residue is present immediately after the first conserved CC pair in the animal sequence, and a 6-residue insertion containing another conserved C is present between the first and second CC pairs. In the granulin domain cluster, there is also a one-residue deletion between the first two conserved Cys residues. The first conserved glycine in the animal sequence is not conserved in the plant granulin domains, and in fact all of the examples shown here contain a bulky residue (F, Y, or L) at that position.

4.4 Conclusion

In summary, 44 cysteine proteases were identified directly from the genomic DNA of *D. capensis*, and sorted into clusters based on sequence homology to known plant cysteine proteases in our paper [30]. Molecular modeling and network analysis indicate that these proteases have distinct structural properties suggesting potential diversity in functional characteristics (e.g., thermal stability, substrate affinity). One particularly attractive potential

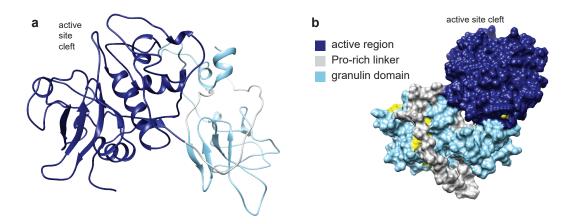


Figure 4.12: a. Ribbon diagram for the predicted structure for a representative member of the granulin domain cluster (DCAP_5115), showing the catalytic domain (dark blue), the proline-rich linker (gray) and the granulin domain (light blue). b. Surface representation of the same structure rotated to show how the proline-rich linker interacts with the granulin domain.

application for these proteases is in mass spectrometry-based proteomics. Identification and characterization of new proteases from diverse sources, including carnivorous plants, adds to the repertoire of cleavage patterns that can be used in proteomics research. The diverse properties make this class of proteins an attractive target for further characterization studies, with rich potential for biotechnology applications. Please look at the paper for detailed results, supplementary data, downloadable structures and author contributions.

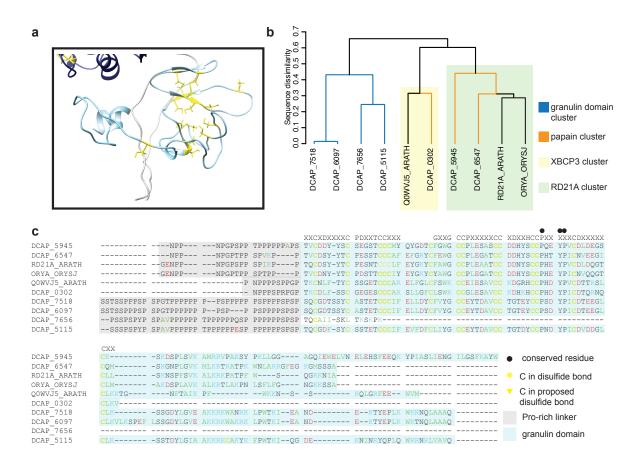


Figure 4.13: a. Ribbon diagram of the DCAP_5115 granulin domain, with cysteine residues highlighted in yellow. b. Cluster analysis of granulin domains from *D. capensis* cysteine proteases and reference sequences. Solid colors denote membership in the clusters of Fig 4.9, while the transparent boxes correspond to the clusters previously identified by Richau et al. [7]. Notably, the *D. capensis* granulin domain cluster appears to represent a new type of plant cysteine protease granulin domain. c. Sequence alignment of all the granulin domains found in the *D. capensis* cysteine proteases with reference sequences.

Chapter 5

Leveraging molecular modeling, experimental chemistry and bioinformatics to study amyloid fibril kinetics

5.1 Introduction

Amyloid fibrils are locally ordered protein aggregates that self-assemble under a variety of physiological and *in vitro* conditions. Their formation is of fundamental interest as a physical chemistry problem and plays a central role in Alzheimer's disease, Type II diabetes, and other human diseases. In fact, more than 40 different human diseases, many of which are both fatal and incurable, are associated with the formation of amyloid fibrils [8, 9, 148, 149]. These locally ordered protein aggregates are characterized by a cross- β structure, in which β -strands composed of adjacent monomers stack in a repeating linear pattern, analogous to

a crystal that grows along a single dimension rather than in all three [150]. This characteristic structure is conserved across amyloid fibrils, independent of the secondary structure of the protein monomer prior to fibrillization [151]. Despite the universality of this overall arrangement, the patterns of connectivity among monomers that form the repeating subunits of amyloid fibrils vary widely and are incompletely characterized. Indeed, the same fibrillizing monomer has been found to form different periodic structures in different experiments, ruling out simple sequence-based explanations: for example, the PDB structures 5KK3 [9] and 2BEG [152] exhibit markedly different periodic patterns along their respective growth axes, yet both fibrils are comprised of the same $A\beta_{1-42}$ peptide. High-resolution structures have shown a diverse set of fibril subunit geometries displaying subtle but distinctive differences, e.g., linear vs. annular structures and parallel vs. antiparallel β -sheet arrangements [8, 153, 154, 155]. These structural differences have demonstrated clinical relevance: for instance, they have been shown to directly correlate with toxicity and disease progression for strains of both β -amyloid [156] and α -synuclein [157] fibrils. The key feature differentiating these subunit geometries from each other is the periodic pattern of non-covalent bonding between monomers. This study refers to such motifs of non-covalently bonded connectivity among the protein monomers making up each fibril type as *fibril topology*, the characterization and modeling of which are the focus of the present work.

As an initial illustration of how fibril topology can be extracted from high-resolution structures and specified using graph theoretic formalisms, Figure 5.1 compares the patterns of non-covalent connectivity for human Iowa mutant β -amyloid fibrils, associated with an earlyonset hereditary form of Alzheimer's disease (PDBID:2LNQ [8]), with that of a fibril formed by wild-type A β_{1-42} (PDBID:5KK3 [9]). As detailed below and in our paper [14], the study represents the topology of each fibril by a network in which each node represents a single protein monomer, with ties indicating monomers that are non-covalently bound to one another. Application of this coarse-grained representation to solved amyloid structures demonstrates that it is sufficient to distinguish a wide range of fibril topologies while also

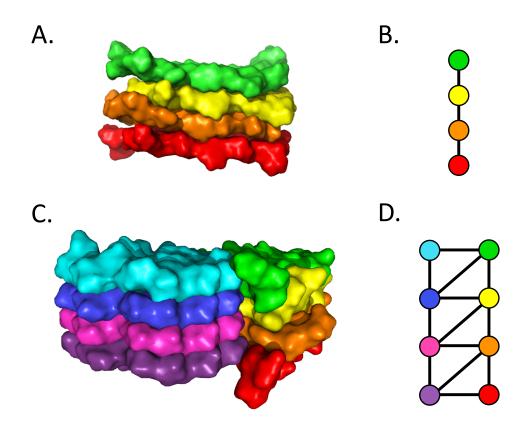


Figure 5.1: Two examples of the mapping of 3-dimensional fibril structures into their equivalent graph representations, where the color coding indicates different protein monomers. Each node in panels B and D corresponds to a protein monomer, with ties between nodes whose monomers are non-covalently bound. Panels A. and B. show the molecular structure and graph representations, respectively, of a fibril segment formed from β -amyloid D23N (PDBID:2LNQ [8]). Using the typology developed in this paper, this fibril structure is classified as a 1-ribbon. Panels C. and D. show the molecular structure and its corresponding graph representation for a segment of wild-type $A\beta_{1-42}$ (PDBID:5KK3 [9]). In our typology, this structure is classified as a 1,2 2-ribbon.

being compact enough to serve as the basis for scalable models of fibrillization kinetics that are able to simulate the fibrillization of hundreds or thousands of protein monomers. The study's examination of experimentally solved amyloid fibril structures reveals several distinct and previously unrecognized topological classes of fibrils; to describe them, this study introduces a systematic nomenclature for fibril structure akin to the naming conventions for organic polymers or protein secondary structure. This system, demonstrated in Figure 5.5, describes all presently known fibril topologies, and is extensible to others not yet found.

A second motivation driving the present work is the need for models of fibril formation that

are able to represent the diversity of observed structures and to capture fibrillization kinetics on experimentally relevant time scales (many hours) and system sizes (hundreds to thousands of peptide monomers). Because both the number of degrees of freedom and the timescales of these events extend far beyond the reach of current atomistic methods, this study developes a mathematical formalism for a *network* Hamiltonian describing the physics of interactions between fibrillizing protein monomers in graph theoretic, or *connectivity* terms rather than in atomistic detail; our approach exploits a formal connection between the statistical mechanics of the fibrillization process and a framework for network modeling (Exponential family Random Graph Models, or ERGMs) originally developed for studying social networks. This study also describes simulation methodology that generates representative fibril topologies under this Hamiltonian, recapitulating the topologies of all currently known amyloid fibril structures reported in the Protein Data Bank (PDB [158]).

In the remainder of this paper [14], we: 1) introduce a general topological formalism for protein aggregates (fibrillar or otherwise), including a quantitative mapping from atomicresolution structures to their topological representations; 2) introduce a typology and nomenclature for amyloid fibrils that encompasses all fibril structures found in the Protein Data Bank; 3) introduce a specific family of Exponential family Random Graph Models (ERGMs) that can reproduce all currently known amyloid fibril topologies (as well as some not yet experimentally observed); 4) employ Markov-chain Monte Carlo (MCMC) simulations to examine the equilibrium behavior of our models; and 5) employ a dynamic extension of ERGMs based on local energy differences to probe the kinetics of fibril formation.

Our work has resulted in two papers [14] and [159]. I analyzed every amyloid fibril structure from the PDB, was involved in topological classification and wrote the manuscript with my coauthors. Dr. Gianmarc Grazioli and Dr. Yue Yu developed and implemented the quantitative topological classification method and simulation studies with Prof. Carter Butts and Prof. Rachel Martin. A portion of the papers [14, 159] is reproduced in this chapter to understand the importance of the results found in this study. For more details, in-depth analysis and author contributions, please refer the papers [14, 159].

5.2 Network-based Classification and Modeling of Amyloid Fibrils

5.2.1 Methods

Defining Protein Aggregation States with Graphs

To capture the process of fibril formation, we require a simple representation that can accommodate protein monomers, small oligomers, larger unstructured aggregates, and the repeating units of fibrils themselves. Graph theory provides a natural language for this purpose, and enables use of the ERGM formalism that has been extensively developed for modeling graphs in social network and neuroscience applications to simulate realizations of the aggregation process. [160] A graph is composed of a set of nodes or vertices, together with a set of *ties* or *edges* representing pairwise interactions between nodes. Here, the nodes represent individual protein monomers, with two nodes being joined by an edge if their corresponding monomers are non-covalently bonded. We refer to such structures as aggregation graphs. Because our focus is on fibril topology, we do not distinguish among types of intermolecular interactions (e.g. hydrogen bonds, salt bridges, non-polar interactions, etc.); in practice, protein monomers are held together by a combination of different interactions, whose combined effects are of primary interest. As Figure 5.1 illustrates, a topological representation is rich enough to distinguish among different fibril forms. Additional forms are shown in the examples throughout this work. In the case of fibril structure A. from Figure 5.1, we note that the structure displays a motif in which each protein monomer is bonded exclusively to its two immediate neighbors along the fibril growth axis; the minimal repeating subunit is a single protein monomer. In contrast to this, the latter structure is characterized by two chains of linear growth, whereby the minimal repeating subunit is a pair of monomers (one from each chain, and of equal fibril growth axis index) that are bonded to: 1) each other; 2) both of their neighbors along the same chain; and 3) the monomer on the opposite chain with a fibril growth axis index offset by one and opposite in sign. Although our representation is by intention coarse-grained, it has some advantages over (and can be used in conjunction with) more fine-grained approaches.

Directly modeling aggregation states via topology dramatically reduces the degrees of freedom that must be explicitly represented, allowing substantial gains in computational efficiency versus geometric representations. Modeling aggregation states in this fashion, however, requires a different approach from e.g. atomistic methods. The above-mentioned ERGM framework provides a parsimonious means of parameterizing and simulating draws from probability distributions on graphs, in this case representing patterns of connectivity between protein monomers. Here, we exploit a property of this framework that allows us to relate the ERGM specification to a Boltzmann distribution over aggregation states (Eq. 5.1).

$$\Pr(G = g|\theta, t) = \frac{\exp\left(\theta^T t(g)\right)}{\sum_{g' \in \mathcal{G}} \exp\left(\theta^T t(g')\right) h(g')} h(g), \tag{5.1}$$

This correspondence also provides a basis for defining Hamiltonian functions that describe the energy of formation for all graph theoretic features sufficient for recapitulating a particular aggregation state (Eq. 5.2).

$$\mathcal{H}(g) = (\phi_e + k_B T) t_e(g) + \phi_{2s} t_{2s}(g) + \phi_{NSP1} t_{NSP1}(g) + \phi_{NSP2} t_{NSP2}(g) + \phi_{ESP0} t_{ESP0}(g) + \phi_{ESP1} t_{ESP1}(g) + \phi_{C5} t_{C5}(g) + \phi_{C6} t_{C6}(g) + \phi_{C7} t_{C7}(g).$$
(5.2)

Finally, we employ the graph Hamiltonian to define a family of kinetic models whose equilibrium distributions correspond to a target ERGM distribution, and that we show to be able to qualitatively recapitulate behaviors seen in experimental studies. Further details regarding the mapping of aggregation states to graphs and the statistics describing their formation to ERGMs can be found in the paper [14]. Although the ERGM portion was performed by Giannmarc, Yue and Prof. Butts, I had to mention it here to understand the importance of the results in the next section. For more details, please look at the paper [14].

Identifying Amyloid Fibril Structures from the Protein Data Bank

To examine the diversity of fibril topologies observed to date, I performed an exhaustive search of the PDB for all amyloid fibril structures. Although the PDB is not a random sample from nature, it is a reasonable census of the known fibril structures so far discovered by the scientific community and their classification into topological classes is useful for showing what fibril forms have been found. The fibril structures were downloaded from the Protein Data Bank (PDB; http://www.rcsb.org/pdb/) [158] after running multiple advanced searches on the website. The search criteria included finding fibrils of sufficient size that the repeating subunit could be clearly identified. Search terms used included "Fibril," "A-beta," "Protein Fibril," and "Lysozyme." Any fibril attached to a ligand, metal or macro-scaffold was discarded. To be retained for analysis, a putative fibril structure was required to display 1) the cross-beta sheet structure definitive of amyloid fibrils (distances between adjacent β -strands of 4.7 +/- .4 Å with 10 +/- 3 Å between β -sheets) as seen in Figure 5.3(a) [161], and 2) a periodic pattern of connectivity between monomers along a single fibril growth axis as seen in Figure 5.3(b,c). Structures not satisfying these conditions were discarded. Then, the structures were inspected individually in VMD [162] and Chimera [163]. Using PDB ID 4RIK [164] for representation in Figure 5.3 (b) and (c), each structure was color coded and mapped on the basis of topology using simple ball and stick model. The structure was also checked for its correctness, any structure that was repeating as a crystal, or did not conform to the definition of an amyloid fibril, was discarded. Figure 5.4 highlights the criteria of acceptance. Figure 5.4 panel (1a) shows the accepted amyloid fibril structure, PDB ID 4RIK, panel (1b) shows the supercell constructed in Pymol [12] and panel (1c) shows the amyloid fibrils distances between adjacent β -strands of 4.8 Å with 9.4 Å between β -sheets [11]. Figure 5.4 panel (2a) shows the rejected fibril structure, PDB ID 4RXFO, panel (2b) shows the supercell constructed in Pymol [12] and panel (2c) shows the fibrils distances between adjacent β -strands of 4.4 Å with 7.8 Å between β -sheets [13]. In case of 4XFO, the cross-layer contacts look really weak and 4XFO did not pass energy calculations performed by Dr. Gianmarc Grazioli, details can be found in the paper [14]. Figure 5.4 panel (3a) shows the rejected fibril structure, PDB ID 3FOD, panel (3b) shows the supercell constructed in Pymol [12] and panel (3c) shows the fibrils distances between adjacent β -strands of 5.3 Å with 10-11.4 Å between β -sheets [15], which does not conform to the amyloid fibril criteria of the amyloid fibrils distances between adjacent β -strands of 4.8 Å with 9.4 Å between β -sheets [11]. 3FOD did not pass energy calculations performed by Dr. Gianmarc Grazioli, details can be found in the paper [14].

This individual inspection not only help collect the accurate data samples to study but also formed the basis of GUI and a traditional interface developed by Dr. Gianmarc Grazioli and Dr. Yue Yu that automated the process of identifying and defining amyloid fibrils found in the PDB and can be found in our paper [14]. In addition to the summary of our classification results shown in Figure 5.2, a detailed list of structures satisfying our criteria is given in Table

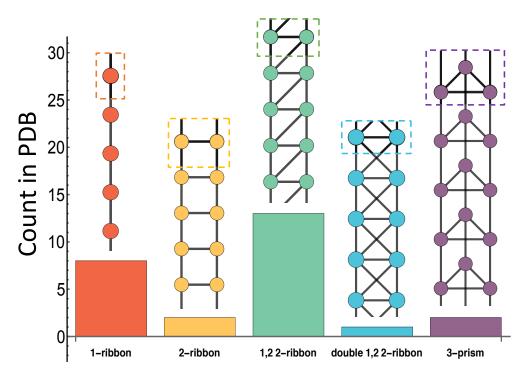


Figure 5.2: The five unique amyloid fibril topologies found in the PDB, sorted by relative complexity. Bar heights indicate the number of structures in the PDB with the indicated topology.

5.1.

Supplementary Video

We have provided a narrated video that features an animated visualization of one of our kinetic simulations of fibrillization, as well as a brief introduction to this body of work. The file name for the video is FibrilTopologyMovie.mp4. Additionally, a high resolution version of the same video can be viewed via the following link: Fibril Topology Movie.

5.2.2 Fibril Nomenclature Rules for Chords

By default, it is assumed that each monomer within a repeating unit is adjacent to the corresponding monomer in the next unit along the fibril axis. Where additional adjacencies are present, these are indicated via the specification of cross-cutting ties that we refer to as

PDB ID	Fibril Type	Description	Reference
2BEG	1-ribbon	Αβ 1-42	[152]
2E8D	1-ribbon	β 2-microglobulin	[165]
2LMQ	1-ribbon	Αβ 1-40	[166]
2LMP	1-ribbon	Αβ 1-40	[166]
2LNQ	1-ribbon	A β D23N 1-40	[8]
2MXU	1-ribbon	Αβ 1-40	[167]
2NNT	1-ribbon	TTCERG1 Y22F CA150	[168]
50QV	1-ribbon	$A\beta$ 1-42	[169]
2LMN	2-ribbon	$A\beta$ 1-40	[166]
2LMO	2-ribbon	$A\beta$ 1-40	[166]
2M5N	1,2 2-ribbon	TTR 105-115	[11]
2M5K	1,2 2-ribbon	TTR 105-115	[11]
2M5M	1,2 2-ribbon	TTR 105-115	[11]
20MQ	1,2 2-ribbon	Insulin 12-17	[170]
20MM	1,2 2-ribbon	Yeast Prion sup35 7-13	[170]
3FTK	1,2 2-ribbon	IAPP 64-70	[171]
3FVA	1,2 2-ribbon	Elk Prion	[171]
3ZPK	1,2 2-ribbon	TTR 105-115	[11]
4R0U	1,2 2-ribbon	α -synuclein 72-78	[172]
4RIL	1,2 2-ribbon	α -synuclein 68-78	[164]
4ZNN	1,2 2-ribbon	α -synuclein 47-56	[164]
5K2H	1,2 2-ribbon	Yeast Prion sup35 7-13	[170]
5KK3	1,2 2-ribbon	A β 1-42	[9]
2MVX	double 1,2 2-ribbon	Αβ 1-40	[173]
2MPZ	3-prism	A β D23N 1-40	[174]
2M4J	3-prism	A β 1-40	[156]

Table 5.1: The following table is a comprehensive list of all fibrillar structures found in the PDB at the time this analysis was done, as well as their topological classification.

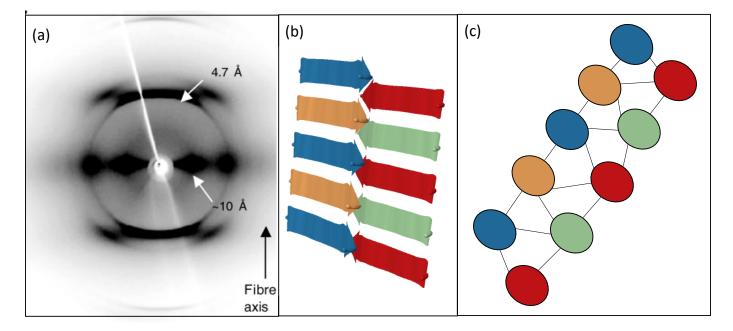


Figure 5.3: Panel (a) represents the characteristics of amyloid fibril appearance. X-ray fiber diffraction pattern from aligned IAPP amyloid fibrils, showing the positions of the 4.7 Å meridional and 9.8 Å equatorial reflections in a cross- pattern. The figure is taken from the paper [10]. Using PDB ID 4RIK [11], (c) is the color coded topology representation of (b).

chords. A chord is indicated by a pair of numbers, indicating the repeating units joined by the chord in question. The first number is always 1, referring to the focal unit; succeeding units are numbered as 2, 3, etc. Thus, a chord from the focal unit to the next unit is a 1,2 chord. A chord from the focal unit to the unit after next is a 1,3 chord, and so on. By default, a chord is assumed to connect each element in the focal unit not having an incoming chord of the same type from a previous unit to the next vertex along the ribbon or prism in the unit to which it connects. Where specified explicitly, these are called *trans* chords. When a chord connects to the corresponding element in a subsequent unit, it is known as a *cis* chord. (Fig. 5.5 gives examples of both trans and cis chords.) Hence a 2-ribbon in which the first vertex in the ribbon in a focal unit is adjacent to the second ribbon vertex in the next unit is called a trans-1,2 2-ribbon, or simply a 1,2 2-ribbon (a cis-1,2 chord would be a reference to an inherent edge between subunits, thus all all 1,2 chords must be trans, ergo trans is a redundant qualifier for a 1,2 chord). Where chords connect both previous and subsequent elements in the ribbon or prism structure, the chords are said to be *doubled*.

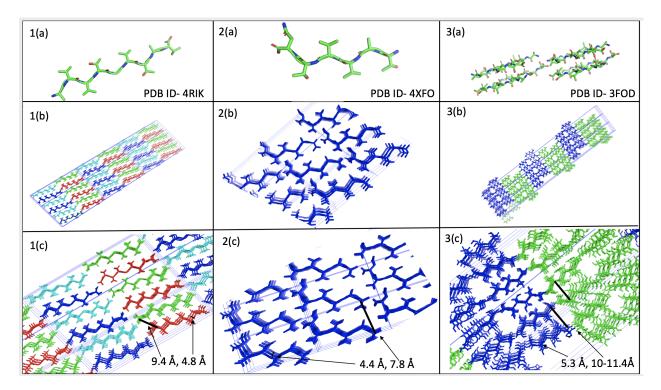


Figure 5.4: Panel (1a) shows the accepted amyloid fibril structure, PDB ID 4RIK, panel (1b) shows the supercell constructed in Pymol [12] and panel (1c) shows the amyloid fibrils distances between adjacent β -strands of 4.8 Å with 9.4 Å between β -sheets [11]. Panel (2a) shows the rejected fibril structure, PDB ID 4RXFO, panel (2b) shows the supercell constructed in Pymol [12] and panel (2c) shows the fibrils distances between adjacent β -strands of 4.4 Å with 7.8 Å between β -sheets [13]. In case of 4XFO, the cross-layer contacts look really weak and 4XFO did not pass energy calculations performed by Dr. Gianmarc Grazioli, details can be found in the paper [14]. Panel (3a) shows the rejected fibril structure, PDB ID 3FOD, panel (3b) shows the supercell constructed in Pymol [12] and panel (3c) shows the fibrils distances between adjacent β -strands of 5.3 Å with 10-11.4 Å between β -sheets [15], which does not conform to the amyloid fibril criteria of the amyloid fibrils distances between adjacent β -strands of 4.8 Å with 9.4 Å between β -sheets [11]. 3FOD did not pass energy calculations performed by Dr. Gianmarc Grazioli, details can be found in the paper [14].

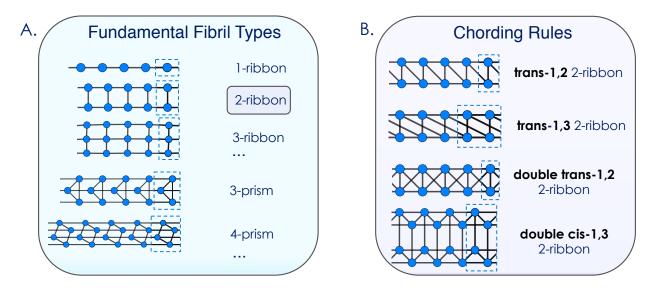


Figure 5.5: The topology of all amyloid structures can be described using a simple network framework. Shown in A are the fundamental fibril forms: the n-ribbon and the n-prism. These fundamental forms are the basis for describing any fibril by either adding (chording) or deleting (nulling) edges between nodes in a repeating pattern. In B, we demonstrate various chording operations to the 2-ribbon. Chords are indexed by the subunits they connect, e.g. consecutive chorded subunits are labeled 1,2, while subunits two positions apart are labeled 1,3. Cis- and trans- indicate whether chords are between subunits occupying equivalent or different embedded 1-ribbon "backbones," respectively.

A double trans-1,2 2-ribbon hence has chords from both vertices within each repeating unit to the opposite vertex in the next repeating unit. When multiple chords types are present, they are listed sequentially from longest to shortest; one may thus have e.g. a cis-1,3 double trans-1,2 2-ribbon, or any other combination that forms a valid aggregation graph.

Extracting Topology from Atomistic Protein Structures

After identifying an amyloid fibril structure, we then extract its underlying topology (i.e., the pattern of bound interactions among monomers). For structures derived from x-ray crystallography, this requires distinguishing between crystal contacts and the much stronger interactions that define the fibril structure itself; in the case of NMR and EM structures, the corresponding problem is distinguishing ephemeral and dynamically unstable contacts from structural ones. In both cases, we employ an energy scoring protocol to remove spurious contacts.

Although we use the same scoring scheme for all structures, the definition of crystal structures in terms of a repeating asymmetric unit requires special processing. This is performed as follows. To ensure that we are working with a collection of fibril segments, we first generate supercells consisting of multiple repeats of the reported crystal unit cell [12]. We then calculate the score for the total interaction energy between each spatially adjacent monomer in the supercell (using the approach described below), using a -10 kcal/mol cutoff to filter out spurious contacts. If this yields a periodic fibril structure along a single axis (and not, e.g. a three-dimensional, sheet-like, or non-periodic structure), the resulting ties are taken to define the fibril topology. If this does not yield such a structure, then we lower the energy cutoff until either such a structure is observed, or until the structure decomposes into independent monomers; in the latter case, the structure is considered not to meet our criteria of being composed of a repeating one-dimensional pattern of bound monomers, and is rejected. Although the focus of the current work is fibril units held together by strong interactions, we note that this methodology can be extended to more complex treatments in which valued graphs are used to represent strong and weak interactions. In this case, multiple scoring thresholds would be used to classify edges into their respective classes.

Since the NMR and EM structures employed here were not obtained from crystallized protein, we do not need to build supercells prior to scoring inter-monomeric interactions. Instead, we simply score contacts among spatially adjacent monomers within the structure, as before beginning with a -10 kcal/mol cutoff and lowering the energy threshold until a periodic structure is obtained. For the NMR and EM structures examined here, it was not necessary to lower the default cutoff in order to obtain periodic structures. Most of this work was done by Gianmarc and Yue, and further details can be found in the paper [14].

Computational Experiments

Other experiments performed in this paper, mainly by Gianmarc and Yue include the following and further details can be found in [14]- Binding Energy Scoring Protocol for Edge Determination, Kinetic Extension of the Fibrillization ERGM, Statistical Mechanics of the Aggregation Graph, Aggregation Model Reference Measure, Aggregation Model Behavior at Extreme Temperatures, Extended Stability Testing of Fibrils, Definition of Fibril Epochs and Sample Code for 2-Ribbon Simulation.

5.2.3 Results and Discussion

A Systematic Nomenclature for Fibril Topology

In the study of individual protein structures, the paradigm of secondary structure has provided researchers with a tool for concisely describing structural details of proteins based on commonly observed hydrogen bonding patterns. Here we present an analogous, systematic, and standardized nomenclature system for fibril topologies that encompasses all of the diverse amyloid fibril forms currently represented in the PDB, and which can straightforwardly be extended to describe forms yet to be discovered (Figure 5.5). The goal of the present study is to develop a formalism for describing amyloid fibrils purely in network terms, using connectivity among monomers. Although it differs in the details, the general approach is part of the rich tradition in computational chemistry of adopting coarse-grained models that capture the most salient features of the system of interest, while discarding other details that are judged to add complexity but not essential information [175, 176]. Such coarse-grained representations of protein systems often necessitate use of a specialized force field, whereby the coarse-grained mapping is performed in the most literal sense, i.e. atomistic degrees of freedom deemed to be unnecessary for capturing the phenomena of interest are fused together to create the "coarse grains" that compose less computationally expensive models with fewer degrees of freedom.[177, 178] We address this objective below.

Amyloid fibrils are characterized by interesting structural features at multiple scales, from the atomistic details of the molecular structures all the way to the distribution of plaques in the brain. [179] The former have been the subject of considerable experimental investigation, leading to details about the shapes of individual monomers within both parallel and antiparallel assemblies. Types of fibrils include S- [9] (or tilde) [180] and double-horseshoe [181] shapes, along with more complicated supramolecular assemblies. Our particular coarsegrained model focuses on fibril topology at the level of interacting monomers, thus opening the door to similar investigations at a higher level of structure, including potential relationships with disease etiology (to which other features are hypothesized to be related [182]) and connections between atomistic and topological features. Because all fibrils, by definition, have a unique axis along which fibril growth occurs, we first posit that all fibrils are chains of repeating subunits that are non-covalently bonded end to end. Each unique fibril topology is identified by this one-dimensional repeating pattern of linked subgraphs, as summarized in Figure 5.5. Our convention for visually indicating the axis of fibril growth in the graphs is to depict "stubs" (i.e., endpoints of cross-unit edges) at both ends of the fibril segments shown. In all examples, the repeating pattern of nodes, edges, and stubs that defines the repeating unit of the fibril form is enclosed with dotted lines.

The subunit topologies observed to date can be divided into two categories: toroidal (i.e., cyclic) and linear. If the subunit is linear, we call it an *n*-ribbon, and if it is toroidal we call it an *n*-prism (see Figure 5.5). For example, if a subunit comprises 3 monomers i, j, and k, and the edges they share within the subunit can be represented as $\{i \leftrightarrow j, j \leftrightarrow k\}$, the fibril type is a 3-ribbon. On the other hand, if the set of edges between monomers i, j, and k in a single subunit are $\{i \leftrightarrow j, j \leftrightarrow k, k \leftrightarrow i\}$, the fibril type is a 3-prism. The simplest case, where the repeating subunit is a single monomer, is called a 1-ribbon.

We can further extend the naming convention to describe ties between monomers from different unit cells. We introduce a convention for describing these additional ties of the form i, j, where i and j give the indices of the subunits between which the ties occur. Additionally, if the ties between unit cells connect nodes along the same embedded ("backbone") 1-ribbon, we add the term *cis*, while the term *trans* indicates that the tie is between nodes on different ribbon segments. For example, if we have a 2-ribbon, where an additional tie connects each unit cell to its immediate neighbor (a 1,2 tie), and the tie is between nodes that belong to the opposite constituent 1-ribbons that make up the 2-ribbon (trans), the 2-ribbon becomes a trans-1,2 2-ribbon (see Figure 5.5).

Characterizing Fibril Topologies Observed in Nature

In order to demonstrate that our system of nomenclature is general enough to be useful, we have categorized the complete set of amyloid fibrils in the Protein Data Bank by topology (Fig. 5.2). The amyloid fibril type classification follows a two-step protocol (see Methods). First, we confirm that the PDB entry possesses the characteristic cross-beta sheet structure definitive of amyloid fibrils (i.e. distances of 4.7 +/- .4 Å between strands within β -sheets and 10 +/- 3 Å across β -sheets [161]). We carry out this step using the distance measurement tool in PyMOL [12]. Second, we apply an energy scoring criterion to identify monomer pairs whose interactions are sufficiently strong to constitute an edge in the aggregation graph. The interaction energy score between two monomers is calculated in a style similar to free energy difference scoring commonly practiced in molecular docking simulations. A conservative minimum energetic threshold of -10 kcal/mol is employed to ensure that the inter-monomeric interactions included in the graph representation of the fibril are at least an order of magnitude more enthalpically favorable than a typical hydrogen bond between water molecules. For the 28 amyloid fibril structures solved to date that meet our inclusion criteria, we find 5 unique topological classes (Figure 5.2). We have identified simulation parameters for our mathematical model of fibril formation that are capable of recapitulating all 5 of these known amyloid fibril topologies, as described in the following sections.

Computational Experiments and Simulation Results

Prof. Carter T. Butts, Dr. Gianmarc Grazioli and Dr. Yue Yu were successful in recapitulating fibril structure in equilibrium by adapting exponential family random graph (ERGM) techniques originally developed for the study of social networks. Details on how we employed the ERGMs using a Hamiltonian function and Markov Chain Monte Carlo (MCMC) can be found in the paper [14] [183, 160]. We were also successful in constructing the fibrilization kinetics simulations [14]. Our followup study to the above paper [14] deals with the mechanisms of amyloid fibril formation, a problem of considerable interest in terms of both basic science and medical applications [159]. Our distinctive approach is to employ a graph-theoretic formalism to represent the underlying topology of fibril structure, giving us a parsimonious way of describing fibrils that is nevertheless flexible enough to accommodate a wide range of aggregation states [159]. Further details can be found in the papers [14, 159]

5.2.4 Experimental Validation of the Predicted Statistical Model: *in vitro* Amyloid Fibril Kinetics

Network statistical characterization of the pathways to fibrillization predicted by network Hamiltonian models [14, 159] offer a uniquely capable approach toward identifying potential intermediates in the process of amyloid fibril formation for experimental validation or invalidation. Most papers used two-step nucleation induced mechanism to study amyloid fibril kinetics [184, 185]. To understand the disease and treatment better, we need to understand the actual process, including formation of monomers, to better understand pathology, process and disease treatment and management As I want to study the process of amyloid fibril kinetics, I propose to use unseeded, no nucleation method to understand the kinetics better.

Materials and Methods

Hen egg white lysozyme (HEWL), Enzyme Commission (EC) Number 3.2.1.17, CAS molecular weight 14.4 kDa, was obtained from Millipore Sigma. γ -crystallin was expressed in the lab [186].

Sample Preparation- 1 mg/ml, 2 mg/ml or 2.5 mg/ml of HEWL or γ S-crystallin in different buffers were made by vortexing in a test tube. Tested buffers included phosphate buffer (pH 2.15, 6.8, 12) and carbonate buffer (3.2, 9.2) as amyloid fibrils tend to form generally at extreme pH, temperatures and agitation [184, 185, 187, 188]. Aliquots of 120 μ L of sample were taken in an eppendorf tube, placed in the Digital Heating Shaking Drybath (Thermofisher) with or without shaking at temperatures between 70-76 degree celcius.

Thioflavin Binding Experiments- Thioflavin binding-dependent fluorescence was routinely assessed on kinetic time points as described in literature [184, 185, 187, 188]. ThT was sourced from Sigma Aldrich. 10 μ L aliquot of a 2.5 mM stock of ThT was added to 90 μ L to the aliquot of the amyloid fibril sample in fluorescence 96-well plate. The fluorometer was set to an excitation wavelength of 450 nm and an emission wavelength of 489 nm. Calculations were performed using excel and blank and negative controls were used.

Results

Although incomplete, the results seen below are promising and the project will continue on the experimental path. Some results are presented in Figure 5.6, 5.7, 5.8, 5.9 and 5.10 General trend shows a minimum concentration 2 mg/ml at pH 3.7 with agitation (150 RPM) at 75 degree Celsius shows proprising results. In Figure 5.6, we see ThT assay results of

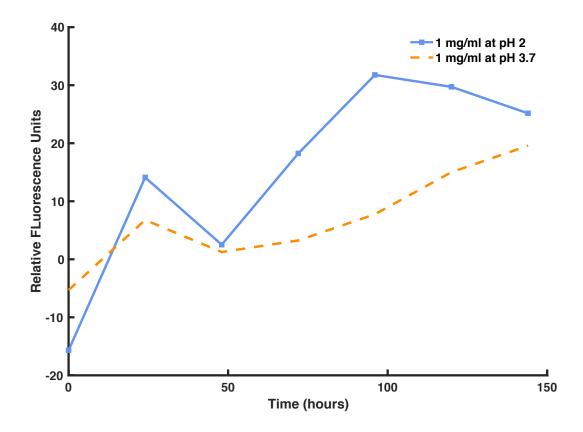


Figure 5.6: ThT assay results of 1 mg/ml HEWL using phosphate buffer at pH 2 and pH 3.7 with agitation (150 RPM) at 75 degree celcius.

1 mg/ml HEWL using phosphate buffer at pH 2 and pH 3.7 with agitation (150 RPM) at 75 degree Celsius. Some amyloid fibril growth is observed however my hypothesis is the fibrils crashes out of the solution. In figure 5.7, ThT assay results of 1 mg/ml and 2 mg/ml γ S-crystallin using phosphate buffer at pH 1 and pH 2 with agitation (150 RPM) at 75 degree Celsius. In Figure 5.8, ThT assay results of 1 mg/ml and 2 mg/ml γ S-crystallin using phosphate buffer at pH 2 and pH 3.7 with agitation (150 RPM) at 75 degree Celsius. In Figure 5.9, ThT assay results of 0.5 mg/ml and 1 mg/ml HEWL using carbonate buffer at pH 2 and pH 3.7 with agitation (150 RPM) at 75 degree 5.10, ThT assay results of 1 mg/ml γ S-crystallin using phosphate buffer at pH 3.7 with agitation (150 RPM) at 75 degree 5.10, ThT assay results of 1 mg/ml γ S-crystallin using phosphate buffer at pH 3.7 with agitation (150 RPM) and without agitation at 75 degree Celsius.

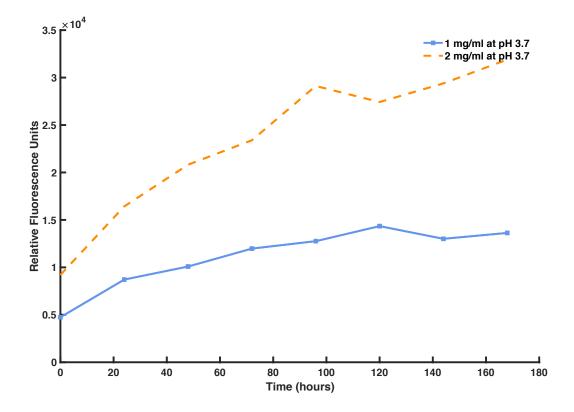


Figure 5.7: ThT as say results of 1 mg/ml and 2 mg/ml γS -crystallin using phosphate buffer at pH 1 and pH 2 with a gitation (150 RPM) at 75 degree celcius.

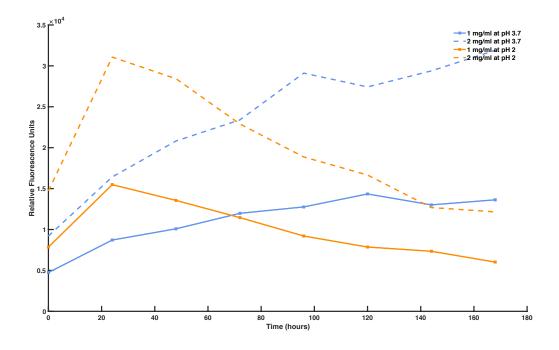


Figure 5.8: ThT as say results of 1 mg/ml and 2 mg/ml γS -crystallin using phosphate buffer at pH 2 and pH 3.7 with a gitation (150 RPM) at 75 degree celcius.

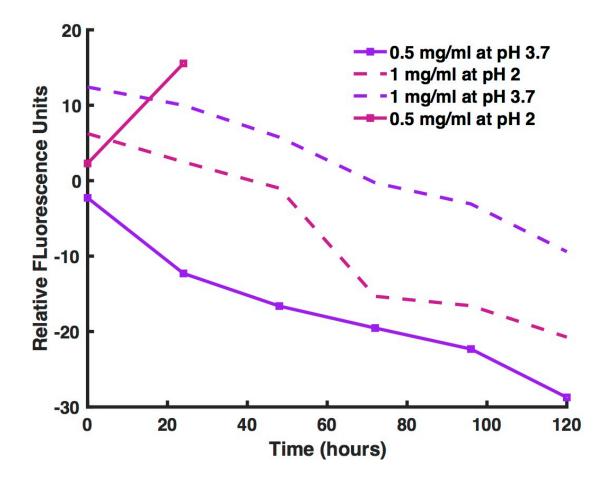


Figure 5.9: ThT assay results of 0.5 mg/ml and 1 mg/ml HEWL using carbonate buffer at pH 2 and pH 3.7 with agitation (150 RPM) at 75 degree celcius.

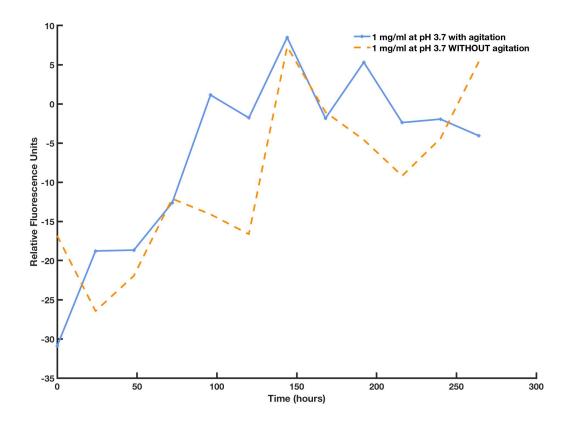


Figure 5.10: ThT as say results of 1 mg/ml $\gamma \rm S$ -crystallin using phosphate buffer at pH 3.7 with a gitation (150 RPM) and without agitation at 75 degree celcius.

Chapter 6

Biophysical characterization and solution-state NMR assignments of J2 crystallin: Novel eye lens protein from the box jellyfish

In this chapter, I describe the preparation, biophysical characterization, and solution-state NMR structure determination of J2-crystallin, a previously uncharacterized eye lens protein from *Tripedalia cystophora* (box jellyfish).

6.1 Background

The crystallins of the eye lens are extremely stable, soluble proteins that are responsible for the transparency of this highly specialized tissue. The high refractivity of the eye lens results from two major contributions; the high protein concentration (up to 1000 mg/mL in some fish species) [189, 190] and the high refractive indices of the crystallin proteins [191, 192]. Many crystallins are derived from either metabolic enzymes or physiological stress proteins and appear to have undergone selection for increased refractive index after gene duplication [193]. Mammalian lenses mostly consist of two strongly conserved classes of crystallins, the chaperone α -crystallins, and the structural $\beta\gamma$ -crystallins [194, 195, 196, 193, 197, 198].

Taxon-specific crystallins with diverse structural properties are found in other species, including the ϵ -crystallin in avians and reptiles and the S-crystallins in cephalopods [194, 196]. Unlike other non-cephalopod invertebrates, in which typical visual systems consist of simple ocelli and/or compound eyes made up of an array of ommatidia [199, 200], the box jellyfish, *Tripedalia cystophora*, has camera-type eyes similar to those of vertebrates and cephalopods [199]. Although the lenses are capable of forming sharp images, the eyes are used for navigation rather than detection of detailed objects [201], as the distance between the lens and the retina is not optimized for maximum visual acuity. *T. cystophora* has a total of 24 eyes split among four rhopalia (specialized structures that sense light), around the bell of the medusa, each containing two camera-type eyes as well as an array of simpler pigment cup eyes [202, 200]. The camera-type eye lenses are composed of the J1-, J2and J3-crystallin proteins [202]. J1- and J3-crystallins are homologous to known enzymes, (ADP-ribohydroglycosylase and saposins, respectively) [202], whereas J2-crystallin (J2) has no known homologs [201, 203]; a BLAST search of the Protein Data Bank (PDB) found no proteins above 37% similarity [204].

To my knowledge, J2 has not previously been expressed and characterized. Here I focus on the box jellyfish eye lens protein J2-crystallin. This 157-amino acid protein has a molecular weight of 18.2 kDa [194] and a theoretical isoelectric point (pI) of 9.25 [205]. As expected for an eye lens protein, the thermal stability of J2 crystallin is high, with an unfolding temperature of 75.2 C. The project was initiated by Dr. Domarin Khago [192] in the lab. She pioneered the expression and optimization of J2 crystallin [192] and was also involved in biophysical characterization and taking initial HSQC and NMR data. Please refer to her thesis [192] for more details on expression and optimization. I will be focusing on the biophysical studies and 3D NMR data that I collected for J2 crystallin [206, 207].

As seen in Figure 6.1, Rosetta and iTasser servers were used to predict J2 crystallin protein models.

Rosetta, of Robetta server [206, 207], is an automated protein structure prediction software developed by the Baker laboratory for *ab initio* and comparative modeling. On submission of the fasta sequence, in this case J2 crystallin, Rosetta first searches for structural homologs using BLAST, PSI-BLAST, and 3D-Jury, by breaking down the target sequence in small portions of individual domains, or independently folding units of proteins. It then matches the sequence to structural families in the Pfam database. Domains with structural homologs then follow a "template-based model" (i.e., homology modeling) protocol [206, 207]. The lowest energy model as determined by Rosetta energy function is then selected as the final predicted structure [206, 207].

iTASSER server [208, 209] is an on-line software that implements the iTASSER based algorithms for protein structure and function predictions from amino acid (fasta) sequences. When user submits an amino acid sequence, the server first tries to retrieve template proteins of similar folds (or super-secondary structures) from the PDB library by LOMETS, a locally installed meta-threading approach developed Zhang Lab [208, 209]. Next, the continuous fragments excised from the PDB templates are reassembled into full-length models using Monte Carlo simulations with the threading unaligned regions built by *ab initio* modeling [208, 209]. In the following step, the fragment assembly simulation is performed again starting from the SPICKER cluster centroids, where the spatial restrains collected from the LOMETS templates and the PDB structures are used to fasten the simulations [208, 209]. The final structure is predicted from the consensus of top structural matches between model and templates as evaluated by TM-score, and the sequence identity in the structurally aligned regions [208, 209].

As seen in Firgure 6.1, there is variability and high dissimilarity in the models predicted by iTasser [208, 209] and Rosetta [206, 207]. As these both tools use homology modelling of the known protein structures, I hypothesize J2 crystallin has a unique protein fold (structure).

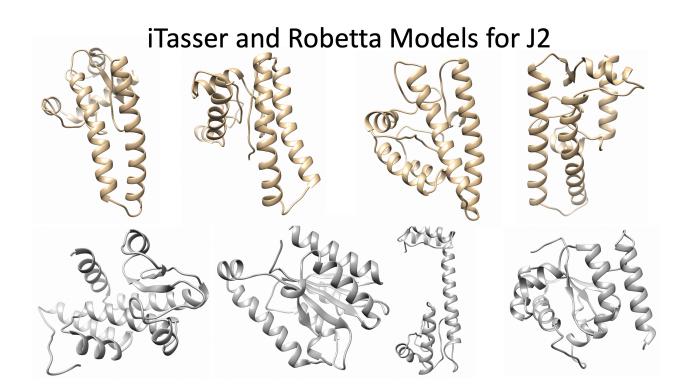


Figure 6.1: Rosetta (gold) and iTasser (silver) servers were used to predict J2 crystallin protein model. Even within the server, there is variability and high dissimilarity of the predicted models. As these both tools use homology modelling of the known protein structures, I hypothesize J2 crystallin has a unique protein fold (structure).

6.2 Materials and Methods

6.2.1 Expression and purification of ¹⁵N-labeled and ¹³C-labeled J2-crystallin

For the starter culture of J2 crystallin, 50 mL of LB media was inoculated with a single colony of Rosetta (DE3) Escherichia coli cells containing a pET28(+)a vector with J2-crystallin gene inserts was grown at 37 °C for 16 hours with shaking at 225 RPM [192]. Optical density measurement (OD), defined as a logarithmic intensity ratio of the light falling upon the material, to the light transmitted through the material, measures the concentration of cells in the solution [210]. Until an OD of 0.60 was reached, the cultures were grown at 37 °C with shaking at 225 RPM. The cells were then collected in 500 mL batches by centrifugation at 3000 RPM for 30 minutes and each 500 mL batch was resuspended in 1 L ¹⁵N-labeled ¹³C-labeled minimal media cultures [192]. The 1 L minimal media cultures were grown for an additional 2 hours at 37 °C at 225 RPM. Protein overexpression was induced using IPTG (Gold Biotechnology) at a final concentration of 0.10 mM at 25 °C for 30 hours [192]. Cells were collected via centrifugation at 6000 RPM, and pellet was resuspended in 40 mL of 50 mM sodium phosphate buffer with 300 mM sodium chloride, 10 mM imidazole, and 0.05%sodium azide at pH 7.4 [192]. Cells were lysed by sonication in 30 second intervals for a total of 10 minutes, followed by centrifugation at 15000 RPM for 90 minutes. The supernatant was filtered with through a 0.22 μ m filter (Millipore) before being loaded onto a Bio-Rad Duo-Inject FPLC system (Hercules, CA) [192]. The His-tagged crystallin was purified and cut by a His-tagged TEV protease, using a Ni-IDA (Bio-Rad) and a second application to a Ni-IDA column was done to remove TEV protease and His-tag [192]. The purified labeled crystallin was dialyzed into 10 mM sodium phosphate buffer with 0.05% sodium azide at pH 6.0 [192]. The final 1.8 mM concentrated J2-crystallin sample was prepared in 10 % D₂O and 2 mM TMSP [192].

6.2.2 NMR experiments

A Varian ^{Unity}INOVA spectrometer (Agilent Technologies, Santa Clara, CA) operating at 800 MHz coupled with a ¹H⁻¹³C⁻¹⁵N 5 mm tri-axis PFG triple-resonance probe, using an 18.8 Tesla superconducting electromagnet (Oxford Instruments) was used to perform most of the 2D and 3D experiments (HNCA and HN(CO)CA were performed on a Bruker Spectrometer operating at 800 MHz at University of California, San Diego NMR Facility). ¹H chemical shifts were referenced to TMSP, and ¹⁵N and ¹³C shifts were referenced indirectly to TMSP. NMR data were processed using NMRPipe [211] and analyzed using CcpNMR Analysis [212]. Center operating frequencies and (unless otherwise stated) center frequency offsets were as follows:

Center	1 H: 799.7988955 MHz	15 N: 81.04252684 MHz
Offset	¹ H: 4.81 ppm	¹⁵ N: 118.70 ppm

6.3 Results and Discussion

6.3.1 Biophysical characterization of J2-crystallin reveals a stable protein

Circular dichroism (CD) spectroscopy and aggregation propensity were used for biophysical characterization of J2-crystallin. As expected of a structural protein, J2 crystallin is a stable protein, aggregating at 73.5 degree Celcius and unfolding at 76.5 degree Celcius. A part of biophytical characterization was performed by Dr. Domarin Khago and the results can be found in her thesis [192].

Aggregation under thermal stress measured as a function of temperature using Dynamic Light Scattering (DLS)

Dynamic Light Scattering is robust and sensitive technique that can be used to characterize protein aggregates in solution, because of its ability to resolve molecular or particle sizes ranging from sub-nanometer to several microns [213]. DLS measurements to understand the aggregation propensity of J2 crystallin were obtained on Zetasizer Nano ZS (Malvern Instruments, Malvern, U.K.). The sample was prepared at the concentration of 1.0 mg/ml in 10 mM phosphate buffer at pH 6.9. At each temperature, the sample was allowed to equilibrate for 2 min before measurements were obtained, after which scattering measureazments were performed in triplicate, resulting in a heating rate of 0.5 C/min. The average apparent particle size is plotted as a function of temperature. J2 aggregates at 73.5° Celsius as seen in Figure 6.2.

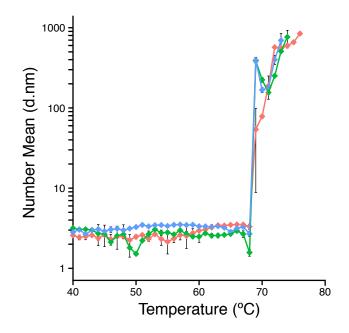


Figure 6.2: DLS measurements of thermally induced aggregation of J2 crystallin over a range of 40-80 degree celcius. Salmon, green and blue colors represent the data taken in triplicate. The average apparent particle size is plotted as a function of temperature. J2 aggregates at 73.5 degree Celsius.

Circular Dichroism (CD) to study the thermal unfolding curves of J2 crystallin

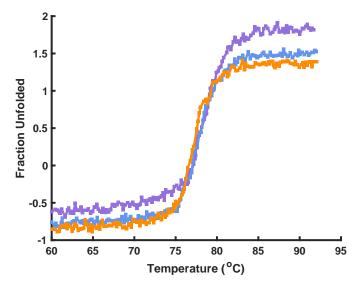


Figure 6.3: Thermal unfolding curve of J2 crystallin measured by monitoring the circular dichroism signal at 218 nm with the Tm value for J2 is 76.5° celcius.

Thermal denaturation provides complementary information regarding protein stability and aggregation propensity. Thermal unfolding curves of J2 are measured by monitoring the circular dichroism signal at 218 nm on a J-810 spectropolarimeter (JASCO, Easton, MD) equipped with a thermal controller. For unfolding measurments, the samples were heated at a rate of 2° C/ minute. The midpoint of the unfolding transition (Tm) is itself a useful measure of protein stability. Tm value for J2 is 76.5° Celcius exhibiting characteristics of a stable protein as seen in the Figure 6.3.

For the sake of comparison, Figure 6.4, shows the Tm comparison of J2 crystallin with other crystallins studied in the lab. Figure from the paper [16].

6.3.2 Backbone assignment of J2 crystallin using Nuclear Magnetic Resonance

Solution state NMR was used to collect the data for J2 crystallin structure determination.

protein variant	<i>T</i> _m (°C)
γS-WT	72.0 ± 0.1
γS-G18V	63.6 ± 0.1
γS-G106V	58.9 ± 0.1
γS-G18V/R20A	67.1 ± 0.1
γS-G18V/R20M	63.1 ± 0.1
γS-G106V/M108R	62.7 ± 0.1

Table 1. Thermal Unfolding Temperatures for yS-Crystallin Variants

Figure 6.4: Tm comparison of J2 crystallin with other crystallins studied in the lab. Figure from the paper [16].

¹⁵N HSQC

To assess the suitability of J2-crystallin for structural studies, a ¹H–¹⁵N heteronuclear singlequantum correlation experiment (HSQC) was collected using samples of ¹⁵N-labelled J2crystallin. The first of many solution-state NMR experiments in a structure determination effort, this experiment determines how well-folded the protein is by the distribution of cross peaks [192]. The cross peaks are representative of the amide N-H pairs of the protein backbone and sidechains. Clean and separated cross peaks allow for distinction among the 157 amino acid residues that should be seen in the spectrum. An HSQC also can indicate the signal-to-noise ratio at the particular pH and concentration of the sample [192]. J2-crystallin HSQC, as seen in Figure 6.5, shows well-separated cross peaks in both spectral dimensions, indicating that the protein is folded and monomeric.

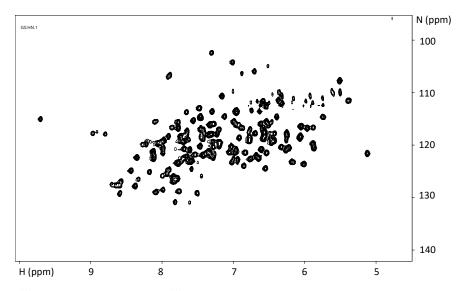


Figure 6.5: ${}^{1}\text{H}{-}^{15}\text{N}$ HSQC spectrum of ${}^{15}\text{N}$ -labelled J2-crystallin acquired at 25 °C, indicating that the protein is folded and monomeric. The crystallin sample was prepared in 10 % D₂O and 2 mM TMSP at a final concentration of 1.8 mM.

¹⁵N-Temperature dependent HSQC

 ${}^{1}\text{H}-{}^{15}\text{N}$ HSQC spectrum of ${}^{15}\text{N}$ -labelled J2-crystallin acquired at 20, 25, 30, 35 and 40 degree Celcius. indicating that the protein is folded, monomeric and stable. The crystallin sample was prepared in 10 % D₂O and 2 mM TMSP at a final concentration of 1.8 mM. Temperature dependent HSQC does not reveal a major shift in the peaks showing that J2 is a stable protein as seen in Figure 6.6.

15 N- 13 C HNCA

HNCA experiment is useful for backbone assignment when used in conjunction with the HN(CO)CA [214]. This is a 3D experiment because the chemical shifts are evolved for ¹HN, ¹⁵NH and ¹³C α [214]. The magnetisation is passed in the following order- ¹H - ¹⁵N - N-C α J-coupling - ¹³C α - ¹⁵N - ¹H hydrogen, where it is detected [214]. Since the amide nitrogen is coupled both to the C α of its own residue and that of the preceding residue, both these transfers occur and peaks for both C α s are visible in the spectrum [214]. HNCA spectrum

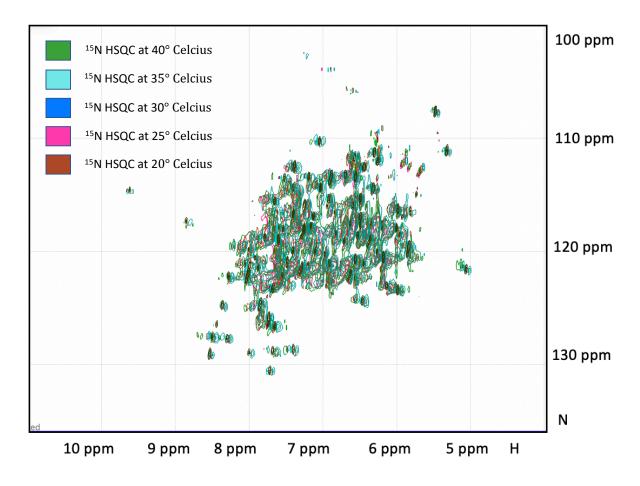


Figure 6.6: Temperature dependent HSQC does not reveal a major shift in the peaks showing that J2 is a stable protein.

for J2 crystallin is seen in Figure 6.7.

¹⁵N-¹³C HN(CO)CA

HN(CO)CA experiment is useful for backbone assignment when used in conjunction with the HNCA [214]. The magnetisation is passed as follows- ¹H to ¹⁵N- to ¹³CO, then transferred to ¹³C where the chemical shift is evolved [214]. The magnetisation is then transferred back via ¹³CO to ¹⁵N and ¹H for detection. The chemical shift is only evolved for the ¹HN, the ¹⁵N and the ¹³C, but not for the ¹³CO α [214]. HNCA spectrum for J2 crystallin is seen in Figure 6.8.

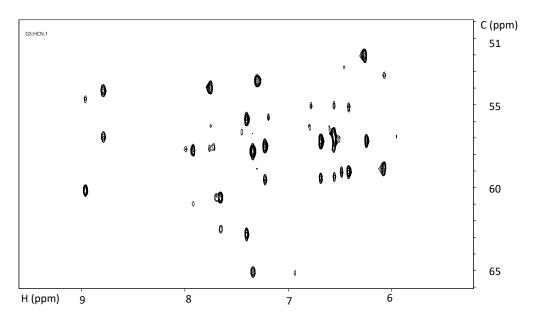


Figure 6.7: Slice of ${}^{1}\text{H}{-}{}^{15}\text{N}$ HNCA spectrum of ${}^{15}\text{N}$ ${}^{13}\text{C}$ labelled J2-crystallin acquired at 25 °C. The J2 crystallin sample was prepared in 10 % D₂O and 2 mM TMSP at a final concentration of 1.8 mM.

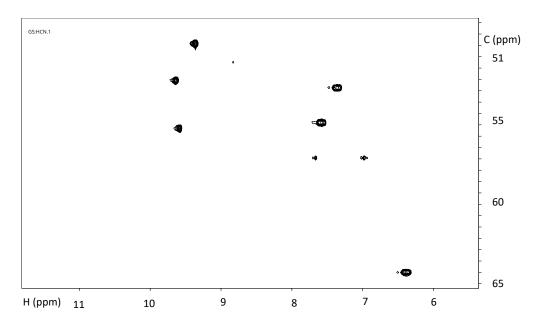


Figure 6.8: Slice of ${}^{1}H{}^{-15}N$ HNCOCA spectrum of ${}^{15}N$ ${}^{13}C$ labelled J2-crystallin acquired at 25 °C. The crystallin sample was prepared in 10 % D₂O and 2 mM TMSP at a final concentration of 1.8 mM.

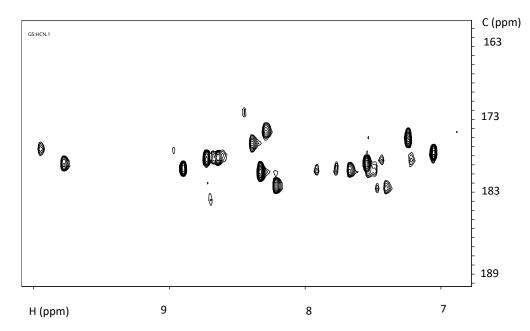


Figure 6.9: Slice of ${}^{1}\text{H}{-}{}^{15}\text{N}$ HNCO spectrum of ${}^{15}\text{N}$ ${}^{13}\text{C}$ ${}^{1}\text{H}$ labelled J2-crystallin acquired at 25 °C. The crystallin sample was prepared in 10 % D₂O and 2 mM TMSP at a final concentration of 1.8 mM.

¹⁵N-¹³C HNCO

This is the most sensitive triple-resonance experiment as in addition to the backbone CO-N-HN correlations, asparginine and glutamine are also visible [214]. Magnetisation is passed in the following order ¹H to ¹⁵N to the carbonyl ¹³C through ¹⁵NH–¹³C J-coupling to ¹⁵N to ¹H where it is detected [214]. HNCO is used in conjunction with the HN(CA)CO [214]. HNCO spectrum for J2 crystallin is seen in Figure 6.9.

¹⁵N-¹³C HNCACO

Magnetisation is transferred in the following order ¹H to ¹⁵N and then via the N-C J-coupling to the ¹³C α to ¹³CO via the ¹³C α -¹³CO J-coupling [214] and then detected on H. HNCACO spectrum for J2 crystallin is seen in Figure 6.10 which can be used in conjunction with HNCO.

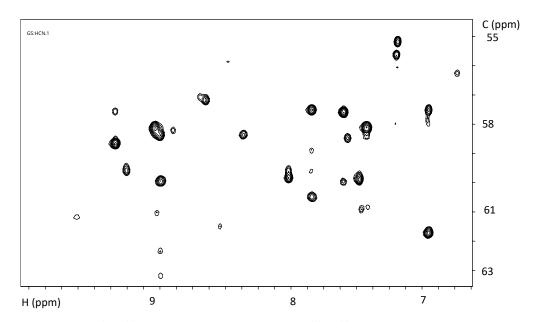


Figure 6.10: Slice of ${}^{1}\text{H}{-}{}^{15}\text{N}$ HNCACO spectrum of ${}^{15}\text{N}$ ${}^{13}\text{C}$ labelled J2-crystallin acquired at 25 °C. The crystallin sample was prepared in 10 % D₂O and 2 mM TMSP at a final concentration of 1.8 mM.

¹⁵N-¹³C CBCACONH, ¹⁵N-¹³C CBCANH and ¹⁵N-¹³C HNCACB

¹⁵N-¹³C CBCACONH, ¹⁵N-¹³C CBCANH and ¹⁵N-¹³C HNCACB, as seen in Figures 6.11, 6.12 and 6.13, are a standard set of experiments needed for backbone assignment especially for large proteins which tend to have a big signal to noise ratio [214].

HCCH-COSY

HCCH-COSY is used for side-chain assignment. Magnetisation transfer is from the side-chain hydrogen nuclei to their attached ¹³C nuclei which is then exchanged between neighbouring ¹³C nuclei via the J-coupling and finally transferred back to the side-chain hydrogen atoms for detection [214]. Figure 6.14 shows the HCCH-COSY spectrum of J2 crystallin.

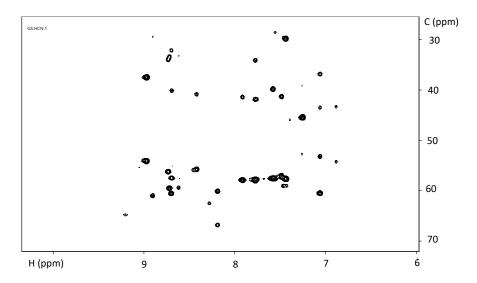


Figure 6.11: Slice of ${}^{1}H{-}^{15}N$ ${}^{15}C$ CBCACONH spectrum of ${}^{15}N$ -labelled J2-crystallin acquired at 25 °C. The crystallin sample was prepared in 10 % D₂O and 2 mM TMSP at a final concentration of 1.8 mM.

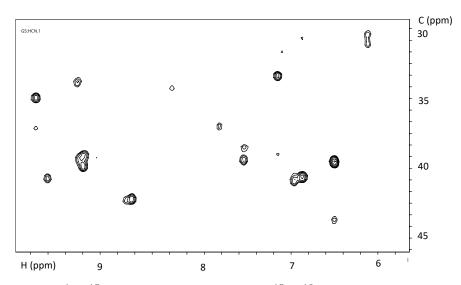


Figure 6.12: Slice of ${}^{1}\text{H}{-}{}^{15}\text{N}$ CBCANH spectrum of ${}^{15}\text{N}$ ${}^{13}\text{C}$ labelled J2-crystallin acquired at 25 °C. The crystallin sample was prepared in 10 % D₂O and 2 mM TMSP at a final concentration of 1.8 mM.

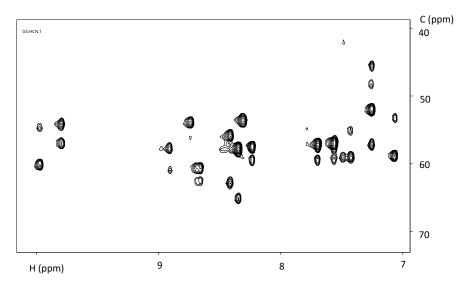


Figure 6.13: Slice of ${}^{1}\text{H}{-}{}^{15}\text{N}$ HNCACB spectrum of ${}^{15}\text{N}$ ${}^{13}\text{C}$ labelled J2-crystallin acquired at 25 °C. The crystallin sample was prepared in 10 % D₂O and 2 mM TMSP at a final concentration of 1.8 mM.

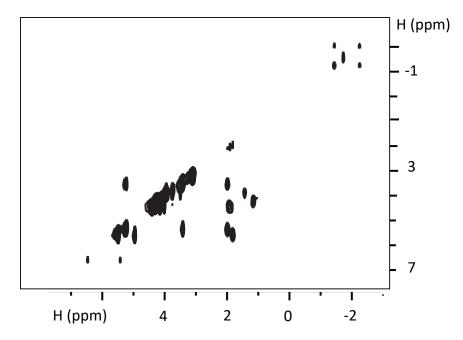


Figure 6.14: Slice of HCCH-COSY spectrum of 15 N 13 C labelled J2-crystallin acquired at 25 °C. The crystallin sample was prepared in 10 % D₂O and 2 mM TMSP at a final concentration of 1.8 mM.

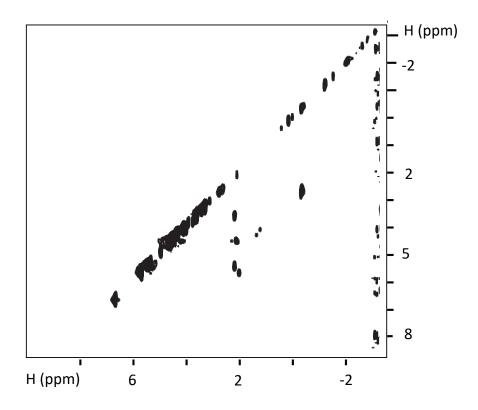


Figure 6.15: Slice of HCCH- TOCSY spectrum of 15 N 13 C labelled J2-crystallin acquired at 25 °C. The crystallin sample was prepared in 10 % D₂O and 2 mM TMSP at a final concentration of 1.8 mM.

HCCH-TOCSY

HCCH-TOCSY is used for side-chain assignment. FIgure 6.15 shows the HCCH- TOCSY spectrum of J2 crystallin. Magnetisation transfer begins from the side-chain hydrogen nuclei to their attached ¹³C nuclei, followed by isotropic ¹³C mixing and back to the side-chain hydrogen atoms where it is detected [214].

¹³C HSQC Aliphatic and ¹³C HSQC Aromatic

¹³C HSQC Aliphatic and ¹³C HSQC Aromatic experiments (Seen in Figure 6.14, provide correlations between carbons and its attached protons and helps circumvent the issue of splitting of signal due to homonuclear ¹³C—¹³C J couplings making the spectral resolution

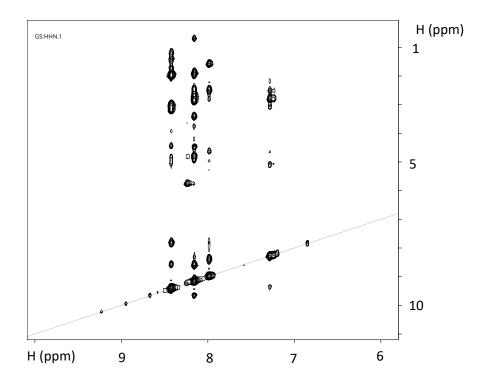


Figure 6.16: Slice of ${}^{1}H{}^{-15}N$ NOESY spectrum of ${}^{15}N$ ${}^{13}C$ labelled J2-crystallin acquired at 25 °C. The crystallin sample was prepared in 10 % D₂O and 2 mM TMSP at a final concentration of 1.8 mM.

better.

NOESY

This spectrum can be used to obtain restraints for structure calculations and can also help in assigning the backbone [214]. J2 crystallin NOESY is seen in Figure 6.16.

6.3.3 J2 Crystallin triple resonance backbone assignment

Sequential protein backbone assignments for J2 crystallin using triple-resonance experiments is seen in figure 6.17. Most spectra used for triple resonance backbone assignment have a ¹H, ¹⁵N and ¹³C dimension each. Assigning the backbone is step one on the path to structure from NMR spectra. In Figure 6.17, Alanine 54 and 55 and Serine 56 residue are assigned using the CBCA(CO)NH and HNCACB 3D experiments.

6.4 Conclusion

As this is a structural protein, the structure is of paramount importance in determining its function. J2-crystallin is a novel eye lens protein that has been expressed recombinantly and purified in high yield [192]. In agreement with the secondary structure prediction and CD spectroscopy, J2-crystallin has primarily α -helical character and thermal studies have concluded a melting temperature of 75.2 °C [192]. A ¹H–¹⁵N HSQC shows the protein is well-folded, allowing for further NMR experiments to be performed. Next steps include spin assignments and residual dipolar coupling.

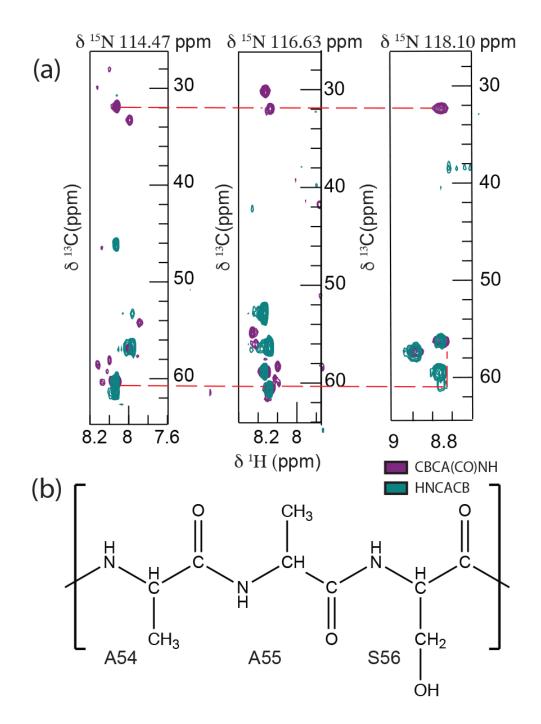


Figure 6.17: Sequential protein backbone assignments for J2 crystallin using triple-resonance experiments where the Alanine 54 and 55 and Serine 56 residue are assigned using the CBCA(CO)NH and HNCACB 3D experiments.

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