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Secreted major Venus flytrap chitinase enables digestion of Arthropod prey

Paulina Paszota [a], Maria Escalante-Perez [b], Line R. Thomsen [a,1], Michael W. Risør [a], Alicja Dembski [b], Laura Sanglas [a], Tania A. Nielsen [a], Henrik Karring [a,2], Ida B. Thøgersen [a], Rainer Hedrich [b], Jan J. Enghild [a], Ines Kreuzer [b,*], Kristian W. Sanggaard [a,**]

[a] Department of Molecular Biology and Genetics, Interdisciplinary Nanoscience Center (iNANO), Aarhus University, Gustav Wiedsvej 10C, 8000 Aarhus C, Denmark
[b] Institute for Molecular Plant Physiology and Biophysics, Biocenter, University Wuerzburg, D-97070 Wuerzburg, Germany

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ABSTRACT

Predation plays a major role in energy and nutrient flow in the biological food chain. Plant carnivory has attracted much interest since Darwin's time, but many fundamental properties of the carnivorous lifestyle are largely unexplored. In particular, the chain of events leading from prey perception to its digestive utilization remains to be elucidated. One of the first steps after the capture of animal prey, i.e. the enzymatic breakup of the insects' chitin-based shell, is reflected by considerable chitinase activity in the secreted digestive fluid in the carnivorous plant Venus flytrap. This study addresses the molecular nature, function, and regulation of the underlying enzyme, VF chitinase-I. Using mass spectrometry based de novo sequencing, VF chitinase-I was identified in the secreted fluid. As anticipated for one of the most prominent proteins in the flytrap's "green stomach" during prey digestion, transcription of VF chitinase-I is restricted to glands and enhanced by secretion-inducing stimuli. In their natural habitat, Venus flytrap is exposed to high temperatures. We expressed and purified recombinant VF chitinase-I and show that the enzyme exhibits the hallmark properties expected from an enzyme active in the hot and acidic digestive fluid of *Dionaea muscipula*. Structural modeling revealed a relative compact globular form of VF chitinase-I, which might contribute to its overall stability and resistance to proteolysis. These peculiar characteristics could well serve industrial purposes, especially because of the ability to hydrolyze both soluble and crystalline chitin substrates including the commercially important cleavage of α-chitin.

1. Introduction

The Venus flytrap (*Dionaea muscipula*) is a monotypic genus belonging to the Droseraceae within the order Caryophyllales. Exhibiting a carnivorous life style, Dionaea uses a snap trap to catch, digest, and "eat" animals. The snap trap performs one of the fastest movements in the plant kingdom and has fascinated scientists since Charles Darwin described these plants approximately 150 years ago [1–3]. The natural habitat of flooded grasslands and bogs around Wilmington, North Carolina is characterized by low-nutrient soils. Nutrients derived from their prey allow the Venus flytrap to cope with the limitations of its environment [3,4]. To digest the spiders and insects and assimilate the nutrients, the carnivorous plants first have to break down their chitin-rich shield (cuticle) before additional enzymes (e.g. proteases or lipases) can penetrate and degrade internal tissues. The external skeleton covers the entire surface of Venus flytrap's major prey [5]. Chitin, which is an insoluble linear polymer, similar to cellulose and composed of β-(1,4)-linked *N*-acetyl-glucosamine (GlcNAc), occurs in three different crystalline forms: α-, β-, and γ-chitin [6,7], and is mainly associated with Fungi and the Arthropoda phylum in the Animalia kingdom. The Arthropod cuticle is mainly composed of α-chitin where the chitin polymer chains exhibit an antiparallel orientation [6]. Next to cellulose, chitin is the most abundant polymer on earth, and these closely related polysaccharides are of large biological and economical importance [8]. The most abundant form in nature is α-type crystalline chitin. The commercial potential has not been fully realized because chitin is insoluble in common solvents [9]. Both in environmental and economic terms enzymes are a sustainable alternative to the use of harsh chemicals to solubilize and release polymer fragments. Thus, enzymatic release of chitin via chitinase cleavage activity would provide an attractive alternative for effective commercial utilization of chitin [10].

Abbreviations: CM-Chitin-RBV, Carboxymethyl-Chitin-Remazol Brilliant Violet; DmACT, *Dionaea muscipula* actin transcript; DTT, dithiothreitol; GlcNAc, *N*-acetyl-glucosamine; MALDI, matrix-assisted laser desorption ionization; OPDA, 12-oxo-phytodienoic acid and 4-MU, 4-Methylumbelliferyl

* Corresponding author. Tel.: +49 931 318 6103; fax: +49 931 318 6157.
** Corresponding author. Tel.: +45 237 41497; fax: +45 871 54006.
E-mail addresses: ifuchs@botanik.uni-wuerzburg.de (I. Kreuzer), krs@mb.au.dk (K.W. Sanggaard).
[1] Present address: Enzyme Development, DuPont Industrial Bioscience, Edwin Rahrs Vej 38, 8220 Brabrand, Denmark.
[2] Present address: Institute of Chemical Engineering, Biotechnology and Environmental Technology, University of Southern Denmark, Niels Bohrs Allé 1, 5230 Odense M, Denmark.

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In contrast to chitin, chitinases are widespread across kingdoms and classes and are present in Bacteria, Plantae, and in several classes in the Animalia [11,12]. In plants, chitinases play a role in defense against pathogens, especially after fungal attack [13–15]. Consequently, plant chitinases are classified as pathogenesis-related proteins. The expression of these defense proteins is induced upon microbial infection of plants [13–15]. The chitinases primarily defend plants by degradation of chitin-containing fungal cell walls and thus inhibition of pathogen growth. Furthermore, some chitinases express lysozymal activity and target bacterial cell walls [12]. Chitinases hydrolyze the β-1,4 linkages in chitin-polymers, and they can be divided into two major categories: endochitinases that cleave internal bonds in chitin chains and generate low molecular mass multimers of GlcNAc and exochitinases targeting the end of the chitin chains. The exochitinases contain two subgroups namely the chitobiosidases, which release chitin disaccharides ($(GlcNAc)_2$) and acetylglucosaminidases, which generate GlcNAc monomers [11]. Based on the primary sequence five classes (I–V) of chitinases have been proposed and these classes are grouped into two families of glycosyl hydrolases, namely family 18 and 19, which are structurally unrelated [11]. Family 18 contains class III and IV, and family 19 contains the remaining three classes with chitinases of plant origin. The chitinases that express lysozymal activity belong to class III in family 18 [11].

Because carnivorous plants feed on chitin cuticle-shielded animals, the digestive fluid of species like Venus flytrap and the pitcher plant *Nepenthes* are likely to contain chitin-degrading enzymes [16–21]. Indeed, pitcher plant and Venus flytrap chitinases have recently been identified in proteomics studies of digestive fluid [22,23]. These chitinases are expected to i) degrade crystalline α-chitin, ii) be resistant towards proteolytic degradation, iii) have an acidic pH optimum, and iv) be functional at relatively high temperatures characterizing the plants' natural habitats.

Here, we further explore the molecular mechanism of prey degradation by Venus flytrap and characterize one of the most abundant chitinases present in the digestive fluid. This enzyme, which we named VF chitinase-I, was cloned, heterologously expressed, purified, and characterized for its enzymatic action. The VF chitinase-I gene is transcriptionally activated upon prey stimulation. Furthermore, the enzyme is upregulated specifically in the secreting glands supporting its major role for prey digestion. We show that VF chitinase-I degrades both soluble chitin and crystalline α-chitin and that it is relatively stable at acidic pH and high temperatures, which correlates well with the expected role in the Venus flytrap prey degradation. These enzymatic properties of VF chitinase-I might well suit industrial and biotechnological purposes.

2. Material and methods

2.1. Materials

Venus flytrap plants were purchased at the Lammehave nursery (Ringe, Denmark) and grown in a walk-in plant growth chamber at 26 °C and at 12:12 hour light:dark photoperiods. Alternatively, *D. muscipula* plants were purchased from CRESCO Carnivora and grown in plastic pots at 22 °C in a 16:8 hour light:dark photoperiod. All of the experiments were performed on healthy mature plants. The EasySelect™ *Pichia* Expression kit (including expression vectors and *Pichia* strains), *Pichia* EasyComp™ kit, and Zeocin were from Invitrogen. Long and Accurate PCR kit was from Clontech, NovaBlue Competent cells were obtained from Merck, and MWG delivered the primers. GeneJET™ kits (Gel Extraction and Plasmid Miniprep), FastDigest® restriction enzymes, and T4 ligase were obtained from Fermentas. HiTrap Chelating HP columns and an Ettan CAF™ MALDI sequencing kit were obtained from GE Healthcare. $ZipTip_{C18}$ pipette tips were from Millipore and C18 Stage Tips were from Proxeon Biosystems A/S. Carboxymethyl-Chitin-Remazol Brilliant Violet (CM-Chitin-RBV) was from Loewe biochemical and Moloney-Murine Leukemia Virus reverse transcriptase and MS-grade trypsin were from Promega. Chitinase Assay Kit (fluorometric), chitin from shrimp shells, *N*-acetylglucosamine, Glu-fibrinopeptide B, α-cyano-4-hydroxycinnamic acid, and all other chemicals were obtained from Sigma-Aldrich.

2.2. Collection of the digestive fluid from the Venus flytrap and SDS-PAGE

For the protein analyses, magnet-based mechanostimulation of the leaf was used to initiate the secretion of the digestive fluid, as previously described [23]. The plants were allowed to secrete the fluid for 48 h. Afterwards, the material was collected, centrifuged, and the proteins in the supernatant denatured and reduced in SDS sample buffer containing 30 mM dithiothreitol (DTT). Then the proteins were resolved in 5–15% acrylamide gradient gels [24] and finally visualized using silver-staining.

2.3. In-gel digestion and mass spectrometry-based de novo sequencing

The major bands from the gel were excised and in-gel digested with trypsin as described before [25]. The resulting peptides were purified, eluted directly onto matrix-assisted laser desorption ionization (MALDI)-target plates using an α-cyano-4-hydroxycinnamic acid matrix, and finally analyzed using a Q-TOF Ultima mass spectrometer (Waters/Micromass) operated in positive-ion and reflector MALDI-mode. To facilitate the fragmentation and interpretation of the generated MS/MS spectra chemical assisted fragmentation methods (4-sulfophenyl isothiocyanate (SPITC) and Ettan CAF™ MALDI sequencing kit) were employed [26]. Prior to the analyses the instrument was calibrated using polyethylene glycol mixture (from *m/z* 50 to *m/z* 3000), and in addition each MS spectrum was externally calibrated using Glu-fibrinopeptide B (*m/z* 1570.68). Masslynx MS software (Waters) and Biolynx software (Waters) were used for data processing and for de novo sequencing, respectively. The obtained peptide sequences were subsequently analyzed using MS-BLAST (http://genetics.bwh.harvard.edu/msblast/) [27].

2.4. Cloning of full-length cDNA encoding VF chitinase-I

The digestion process of Venus Flytrap was initiated by feeding the plants with yellow mealworm beetles (*Tenebrio molitor*). After 40–88 h the trap leaves were collected, rinsed in water to remove all the remains from the digested beetles, snap-frozen in liquid nitrogen, and stored at −80 °C. Total RNA was isolated using a Cetyltrimethylammonium Bromide (CTAB) protocol. Afterwards mRNA was purified from the pellet generated after LiCl-precipitation and the mRNA used for cDNA synthesis, using an oligo(dT)-linker-primer introducing the sequence: 5′-GCGGCCGCAGTGAGTGACAGCCAAGGTCCA GAGATCG-3′ after the poly-A tail. After cDNA amplification, with Long and Accurate PCR, outer and inner primers (5′-AGCAGCTTCAAT AAADGCRTTRTA-3′ and 5′-AAAAGCGTTATATGTATARAANCC-3′, respectively), deduced from the obtained chitinase peptide sequence (GFYTYNAFL(I)EAAR), were used in nested 5′-RACE PCR. The obtained fragment was purified, cloned, and sequenced. From the sequence a new primer, 5′-GCAAAGTCACAATTAGAAAATAC-3′, binding upstream from the start codon was constructed and used together with a primer (5′-CGATCTCTGGACCTTGGCTGTCACTCACTGCGGCCGC-3′) that anneals to the adapter-sequence introduced with the oligo(dT)-linker-primer at the cDNA synthesis step. Using this method the full-length cDNA sequence was obtained. The generated fragment was cloned in vector pBlueScript II sk^+ and sequenced.

2.5. Bioinformatics and structure prediction

The sequence was analyzed using the GPMAW 9.1 software from Lighthouse data (http://www.gpmaw.com), Pfam from Wellcome Trust Sanger Institute (http://pfam.sanger.ac.uk), BLAST from National