A peer-reviewed version of this preprint was published in PeerJ on 5 June 2018.

View the peer-reviewed version (peerj.com/articles/4914), which is the preferred citable publication unless you specifically need to cite this preprint.

Ravee R, Mohd Salleh F', Goh H. 2018. Discovery of digestive enzymes in carnivorous plants with focus on proteases. PeerJ 6:e4914 https://doi.org/10.7717/peerj.4914

Discovery of digestive enzymes in carnivorous plants with focus on proteases

Rishiesvari Ravee [1], **Faris 'Imadi Mohd Salleh** [1], **Hoe-Han Goh** Corresp. [1]

[1] Institute of Systems Biology (INBIOSIS), Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia

Corresponding Author: Hoe-Han Goh
Email address: gohhh@ukm.edu.my

Background. Carnivorous plants have been fascinating researchers with their unique characters and bioinspired applications. These include medicinal trait of some carnivorous plants with potentials for pharmaceutical industry.

Methods. This review will cover recent progress based on current studies on digestive enzymes secreted by different genera of carnivorous plants: *Drosera* (sundews), *Dionaea* (Venus flytrap), *Nepenthes* (tropical pitcher plants), *Sarracenia* (North American pitcher plants), *Cephalotus* (Australian pitcher plants), *Genlisea* (corkscrew plants), and *Utricularia* (bladderworts).

Results. Since the discovery of secreted protease nepenthesin in *Nepenthes* pitcher, digestive enzymes from carnivorous plants have been the focus of many studies. Recent genomics approaches have accelerated digestive enzyme discovery. Furthermore, the advancement in recombinant technology and protein purification helped in the identification and characterisation of enzymes in carnivorous plants.

Discussion. These different aspects will be described and discussed in this review with focus on the role of secreted plant proteases and their potential industrial applications.

Discovery of digestive enzymes in carnivorous plants with focus on proteases

Rishiesvari Ravee, Faris 'Imadi Mohd Salleh, Hoe-Han Goh*

Institute of Systems Biology, Universiti Kebangsaan Malaysia, UKM Bangi 43600 Selangor, Malaysia.

*Corresponding Author: Hoe-Han Goh

Email address: gohhh@ukm.edu.my

Abstract

Background. Carnivorous plants have been fascinating researchers with their unique characters and bioinspired applications. These include medicinal trait of some carnivorous plants with potentials for pharmaceutical industry.

Methods. This review will cover recent progress based on current studies on digestive enzymes secreted by different genera of carnivorous plants: *Drosera* (sundews), *Dionaea* (Venus flytrap), *Nepenthes* (tropical pitcher plants), *Sarracenia* (North American pitcher plants), *Cephalotus* (Australian pitcher plants), *Genlisea* (corkscrew plants), and *Utricularia* (bladderworts).

Results. Since the discovery of secreted protease nepenthesin in *Nepenthes* pitcher, digestive enzymes from carnivorous plants have been the focus of many studies. Recent genomics approaches have accelerated digestive enzyme discovery. Furthermore, the advancement in recombinant technology and protein purification helped in the identification and characterisation of enzymes in carnivorous plants.

Discussion. These different aspects will be described and discussed in this review with focus on the role of secreted plant proteases and their potential industrial applications.

Introduction

Nitrogen is the most crucial mineral nutrient required by plants but its availability is largely limited in many terrestrial ecosystems (Behie & Bidochka, 2013). For adaptation to such unfavourable environment, carnivorous plants have developed the ability to attract, capture, and digest preys into simpler mineral compounds, which are then absorbed for plant growth and reproduction (Ellison, 2006). The first evidence on the ability of the plant to capture and digest insects was provided over 140 years ago (Darwin, 1875). Since then, more than 700 carnivorous species from 20 genera of 12 families (Givnish, 2015) have been identified with captivating morphological and physiological traits linked to carnivory (Król et al., 2011).

There are a few reviews on the evolution of carnivorous plants and their biotechnological applications (Król et al., 2011; Miguel, Hehn & Bourgaud, 2018). However, a systematic review with focus on digestive enzyme discovery and characterisation from all families of carnivorous plants is lacking. Furthermore, the pharmacological potentials of some of these carnivorous plants have also been largely overlooked. With the advent of omics technology which accelerated enzyme discovery in carnivorous plants for the past few years, there is a pressing need for a timely review on current progress of studies in this field. This review will be useful not only to researchers working on carnivorous plants, but also those with interest in commercially useful enzymes and natural products.

Survey Methodology

In this review, we provide perspectives on the latest research of different carnivorous plants, namely *Cephalotus, Drosera*, *Dionaea*, *Genlisea, Nepenthes*, *Sarracenia*, and *Utricularia*, on their digestive enzyme discovery and characterisation. In earlier studies, research interest on carnivorous plants was centred on axenic culture, ultrastructure of specialised trapping organs, foliar absorption of nutrients derived from preys, and the enzymatic studies of prey digestion (Adamec, 1997; Gorb et al., 2004; Farnsworth & Ellison, 2008). Thus, this review summarises the previous findings with focus on digestive enzymes discovered in carnivorous plants, especially proteases and their industrial applications. Literature survey was performed

exhaustively online using Google search engine and SCOPUS. Discussion will be mainly based on recent studies.

Different families of carnivorous plants

The emergence of carnivorous syndrome requires significant functional adaption in plant morphology and physiology. Carnivory trait has evolved independently in different orders of flowering plants, namely Caryophyllales, Ericales, Lamiales, Oxalidales, and Poales (Müller et al., 2004; Ellison & Gotelli, 2009; Król et al., 2011). This comprised of 12 different families of carnivorous plants with five distinct trapping mechanisms, including flypaper trap, snap trap, pitfall trap, suction trap, and eel trap (Table 1). The development of unique traps is one of the major indicators of carnivorous syndrome. These traps originate from the leaves specialised in trapping, digesting and absorbing nutrients from prey at the cost of reduced photosynthesis (Ellison & Gotelli, 2009). The modified leaves of carnivorous plants often form either an active or passive trap (Bauer et al., 2015). An active trap involves movement mechanics to aid prey capture, whereas a passive trap relies on its morphological structure to trap prey.

In Caryophylles, Droseraceae is one of the most species-rich families of carnivorous plants comprising over 160 species in *Drosera* genus of sundews with flypaper trap (Ellison & Gotelli, 2009). Earlier studies have reported the application of sundew plants as a remedy for pulmonary illnesses and coughs (Didry et al., 1998), in the form of tincture (Caniato, Filippini & Cappelletti, 1989). Compounds of pharmaceutical interest in *Drosera* include flavonoids, phenolic compounds, and anthocyanins. *Drosera* herbs have antispasmodic, diuretic, and expectorant properties (Banasiuk, Kawiak & Krölicka, 2012). Additionally, *in vitro* culture extracts of *Drosera* were reported with antibacterial and anticancer properties (Banasiuk, Kawiak & Krölicka, 2012). Interestingly, a crystal-like pigment from *D. peltata* can also be used as a dye in silk industry (Patel, 2014).

Venus flytrap (*Dionaea muscipula*) is another well-known member of Droseraceae due to its unique snap-trapping mechanism to capture small preys, primarily insects or spiders. Interestingly, the trapping signal of *Dionaea* is the fastest ever reported in the plant kingdom over 140 years ago (Darwin, 1875). The secretion of digestive fluid is highly induced by touch

stimulation of 'trigger hairs' on the trap sticky surface. Naphthoquinones were discovered from *in vitro* culture extract of Venus flytrap which is a traditional medicine for cough (Banasiuk, Kawiak & Krölicka, 2012). Plumbagin is another promising antitumor compound among the abundant beneficial secondary metabolites found in *D. muscipula* (Gaascht, Dicato & Diederich, 2013).

Cephalotaceae, Nepenthaceae, and Sarraceniaceae are three families of carnivorous plants which develop modified leaves shaped like a pitcher as a passive pitfall trap. A digestive zone is located at the lowest inner wall of the pitcher with abundant digestive glands responsible for the secretion of hydrolytic enzymes. In contrast, Bromeliaceae and Eriocaulaceae of Poales forms tube-like pitfall trap from overlapping erect leaves instead of a modified leaf organ. Most studies showed low production of enzymes in *Brocchinia*, *Catopsis*, and *Paepalanthus* in the absence of abundant specialised glands (Givnish et al., 1984; Adlassnig, Peroutka & Lendl, 2010). Some pitchers of *Nepenthes* and *Sarracenia* are so big that larger prey, such as frog and rodent are frequently found partially digested inside the pitcher (Adlassnig, Peroutka & Lendl, 2010). This phenomenon shows that preys of carnivorous plants are not restricted to only insects.

For *Sarracenia*, its pitcher acts as rainwater storage and at the same time secretes hydrolytic enzymes and other proteins for prey digestion. The secretions formed at the hood of pitcher lure insect prey, which eventually fall and drown in the pitcher fluid (Ellison & Gotelli, 2001). The prey is digested by the digestive enzymes, such as phosphatases, proteases, and nucleases in the pitcher fluid (Chang & Gallie, 1997). Interestingly, *Sarracenia* has been used as a traditional remedy for childbirth and as a diuretic agent (Patel, 2014). Moreover, tea made from its dried foliage can be used to treat fever and cold; whereas its roots can be consumed as a remedy for lung, liver, and smallpox diseases (Patel, 2014).

Nepenthes is a genus of tropical pitcher plants from the species-rich Nepenthaceae family with fascinatingly diverse pitcher structures adapted to different ecological niches and feeding habits. Despite the lack of a complete genome from this family, there are quite a few reports on transcriptome sequences. Recently, Mu'izzuddin et al., (2017) reported the first single molecule real time sequencing of full-length transcriptome sequences for *N. ampullaria*, *N. rafflesiana*, and *N. x hookeriana*. Ethnomedicinal properties of *Nepenthes* are well documented with boiled roots act as a remedy for stomach ache. The pitcher fluid can be consumed to cure urinary

diseases and used as eye drops to treat itchy eyes. Besides, the root and stem can serve as building materials for housing construction in place of rattan due to its elasticity and enduring property (Miguel, Hehn & Bourgaud, 2018). Asides that, *Nepenthes* pitchers have a distinct use in traditional cooking of glutinous rice snacks, which is practised by Bidayuh and Kadazan-Dusun people in Malaysia using *N. ampullaria* and *N. mirabilis* (Schwallier et al., 2015). Furthermore, Nepenthes also has a great potential as pest control agent in agriculture due to their ability to capture and kill insects, such as flies, ants, bees, and beetles; some even kill small animals like frog and rats (Miguel, Hehn & Bourgaud, 2018).

Genlisea and *Utricularia* are carnivorous plants under the family of Lentibulariaceae. These plants feed on microscopic preys and digest them in a closed trap under water. *Utricularia* spp. have reported usage for dressing wounds and as a remedy for urinary infections and cough (Patel, 2014). To date, *Genlisea aurea* (Leushkin et al., 2013) and *Utricularia gibba* (Lan et al., 2017) are among the four carnivorous plants with genome sequences publicly available, apart from *Drosera capensis* (Butts, Bierma & Martin, 2016) and *Cephalotus follicularis* (Fukushima et al., 2017). The availability of genome sequences has contributed greatly to enzyme discovery and improving our understanding of carnivory mechanisms and evolution in different carnivorous plant families.

Digestive Enzyme Discovery, Identification and Characterisation

Digestion of prey in carnivorous plants relies on enzymes which could be associated with morphologically diverse trapping mechanisms. There are a few studies which reported that the secretion of the digestive enzymes is strongly induced by prey capture. However, there are also certain digestive enzymes which are readily secreted in the absence of prey. This indicates plant regulation of enzyme secretion because the production and secretion of enzymes incur energetic costs.

To date, numerous studies had reported the discovery of distinct digestive enzymes in carnivorous plants (Table 2). Similar enzymes with various enzymatic properties were shared among different carnivorous families. With the genome sequencing of *Cephalotus follicularis*, various digestive enzymes were discovered, namely esterases, proteases, nucleases,

phosphatases, glucanases, and peroxidases (Takahashi et al., 2009; Fukushima et al., 2017). Similar classes of enzymes were also detected in other carnivorous families, such as Droseraceae, Lentibulariacea, Sarraceniaceae, and Nepenthaceae. This suggests significant role of common hydrolytic enzymes, especially phosphatases, proteases, and chitinases, in prey digestion of various carnivorous plants regardless of different families or trapping mechanisms. Recently, Yilamujiang et al., (2017) reported the presence of a novel digestive enzyme urease in *N. hemsleyana* which has developed a symbiosis relationship with bat.

However, investigation related to the identification of proteins found in the pitcher fluid is highly challenged by unusual amino acid composition and limited carnivorous plant genome or protein sequence database (Lee et al., 2016). Early study by Amagase (1972) utilised zymography technique to determine the protease activity found in fluid of *Nepenthes* spp. and *D. peltata.* The fluids were purified and characterised for acid protease and demonstrated similar protease activity from two distinct families. Later, Hatano & Hamada, (2008) conducted proteomic analysis on the digestive fluid of *N. alata* in which secreted chitinase, glucanase, and xylosidase were identified through in-gel trypsin digestion, *de novo* peptide assembly, and homology search using public databases. Recently, a transcriptomic approach was taken for *N. ampullaria* and *N.* x *ventrata* (Wan Zakaria et al., 2016a; Wan Zakaria et al., 2016b), which can serve as reference sequences for identifying more digestive enzymes. A proteomics informed by transcriptomics approach was taken by Schulze et al., (2012) to determine the proteins highly expressed in the digestive fluid of Venus flytrap. They discovered a coordinated prey digestion mechanism facilitated by various enzymes, such as chitinases, lipases, phosphatases, peroxidases, glucanases, and peptidases. Fluorescent resonance energy transfer (FRET) based technique can be utilised as an efficient and rapid detection of proteolytic activities in the pitcher fluid of various *Nepenthes* species (Buch et al., 2015). Rey et al., (2016) applied a similar approach to assess proteolytic efficiency of the protein secreted in the pitcher fluid of *Nepenthes* species.

On the other hand, purification of digestive enzymes from carnivorous fluid is extremely challenging due to low amount of secreted fluid and enzyme. Furthermore, pitcher fluids are often diluted with rainwater and even contaminated by decomposing prey. Nevertheless, there are studies which manage to purify and characterise digestive enzymes from carnivorous plants

(Table 3). Based on the reported purification and characterisation studies, proteases are the most abundant enzymes characterised from the digestive fluid of carnivorous plant. The very first purification of protease from pitcher fluid of *Nepenthes* species was performed by Steckelberg, Lüttge & Weigl (1967) using Ecteola column chromatography and its optimum activity was detected at pH 2.2 with stability at 50^{o}C. To date, the common purification strategies applied by various studies are column chromatography, affinity chromatography, ultrafiltration, and dialysis. Although many digestive enzymes have been identified from carnivorous plants, only few studies have purified and characterised the enzymes. Therefore, further studies on the purification and characterisation of various digestive enzymes are needed.

Most of the characterised enzymes can catalyse various substrates and activities of the same category of enzymes from different carnivorous plants are similar in terms of optimum pH, temperature, and substrate specificity (Table 3). For instance, most of the characterised proteases from different families function optimally at acidic condition. Interestingly, there are a few proteases reported to function optimally at high temperature ranging from 40-60^{o}C. Additionally, the secreted enzymes demonstrate higher stability against various chemicals and denaturing agents than similar enzymes from other sources. This is because prey digestion often occurs over long period under varied conditions, thus digestive enzymes are important to be active and stable (Butts, Bierma & Martin, 2016). Subtle variations in enzymatic characteristics of digestive enzymes from different carnivorous plants remain to be explored. Furthermore, nomenclature of enzymes reported from different carnivorous plants need to be standardised for comparative studies.

There are only a few reports on the structural characterisation of the digestive enzymes secreted by carnivorous plants. To date, proteases and chitinases are the most characterised in structural and enzymatic properties (Ishisaki et al., 2012a; Fukushima et al., 2017; Jopcik et al., 2017; Unhelkar et al., 2017). Athauda et al. (2004) was the first to report a complete model of purified Nepenthesin from *N. distillatoria*. Interestingly, nepenthesin contains extra three disulphide bonds in the N-terminal compared to only three disulphide bonds in porcine pepsin A (Figure 1). Comparison of predicted protease structures of Nepenthesin I and Nepenthesin II from *N. alata* show similarities in the location of catalytic Asp residues. Nepenthesin is distinct from pepsin with a nepenthesin-type aspartic protease (NAP)-specific insert with four conserved

cysteine residues believed to confer higher protein stability. Further structural analysis on proteases from carnivorous plants can refer to a recent study by Butts, Bierma & Martin (2016).

On the other hand, feeding with insect or chitin induces the secretion of enzymes in digestive fluid. Clancy & Coffey, (1977) have reported the maximal secretion of digestive enzymes, specifically phosphatases and proteases in *Venus flytrap* and *Drosera* within 3 to 4 days after feeding. Apart from that, mechanical irritation also stimulates the increase in the activity of phosphatases and phosphodiesterases in *Drosera* (Mcnally et al., 1988). Moreover, the quantity of enzymes secreted often associates with the size of prey (Darwin, 1875; An, Fukusaki & Kobayashi, 2002). These reports suggest a signal transduction mechanism which stimulates the expression of digestive enzymes, allowing plants to respond accordingly toward prey for optimal cost-benefit ratio (Chang & Gallie, 1997).

The origin of enzymes found in digestive fluid has been controversial on whether all are plant secreted or derived from microbial community found in the digestive fluid. A study reported high expression of hydrolytic enzymes in the digestive zone of pitcher trap (An, Fukusaki & Kobayashi, 2002). Meanwhile, a study on *Sarracenia* pitcher showed there is a symbiotic interaction between microbial community in the pitcher fluid and the plant in prey digestion (Koopman et al., 2010). This study suggests that some carnivorous plants could be co-opting microbes for initial prey digestion and secrete digestive enzymes for later stage of digestion. From a different perspective, prey digestion through plant enzymes could be enhanced through symbiotic relationship with microbes or fungi to decompose prey into simpler form of nutrients. This mutualistic interaction with microbial community in the digestive fluid will boost digestion and nutrient absorption. However, there must be a balance point or even selection of microbial community (Takeuchi et al., 2015) to prevent competitive loss of nutrients as indicated by various defence-related proteins (Lee at al., 2016; Rottloff et al., 2016) and antimicrobial naphthoquinones (Buch et al., 2012) found in the pitcher fluid.

Secreted Proteases in Different Families of Carnivorous Plants

Carnivorous plants attain substantial amount of nitrogen from prey through specialised trapping organs which accumulate acidic fluid containing protease. Early reports of digestive enzymes

involved in carnivorous plants were initiated by Sir Joseph Hooker's studies of protease activity in the pitcher fluid of *Nepenthes* plants (Renner & Specht, 2013). Independent evolution of carnivorous plants might have resulted in convergent evolution of diverse digestive enzymes serving similar functions (Fukushima et al., 2017).

Aspartic proteases (APs), such as nepenthesin, are one of the most abundant and well characterised enzymes found in the digestive fluid (An, Fukusaki & Kobayashi, 2002; Rottloff et al., 2016). AP have been purified and characterised from sterile pitcher fluid of several *Nepenthes* species (Jentsch, 1972; Tokes, Woon & Chambers, 1974). In a study conducted by Nakayama & Amagase (1968), a protease from pooled pitcher fluids of *N. mixta* and *N. maxima* was only partially purified and characterised due to insufficient amount. Amagase (1972) investigated aspartic proteases found in *N. ampullaria, N. mixta, N. rafflesiana, N. maxima*, and *N. dyeriana* compared to leaf extract from *Drosera peltata*. Lately, acid protease from *Nepenthes* and *Drosera* genus are partially purified and characterised (Takahashi, Tanji & Shibata, 2007; Tokes, Woon & Chambers, 1974). Surprisingly, both the purified proteases from *Nepenthes* and *Drosera* share common characteristics. An, Fukusakhi & Kobayashi, (2002) cloned homologous AP genes and examined their expression in *N. alata*. The protease secreted in the pitcher fluid is pepsin-like and active at acidic condition (Rudenskaya et al., 1995). Although they have been categorised as APs, none of the native enzymes was purified to homogeneity, mainly due to difficulty in obtaining sufficient amount of pitcher fluid. Later, Athauda et al., (2004) for the first time purified and characterised two APs, namely Nep1 and Nep2, from pitcher fluid *N. distillatoria.* They also characterised the amino acid sequences of the enzymes by cloning the cDNAs from pitcher tissue of *N. gracilis.* Recently, five nepenthesins were reported to be secreted in *Nepenthes* pitcher fluid (Lee et al., 2016). However, little is known about why there are various AP genes expressed in *Nepenthes* pitcher fluid and their differential regulations if any. It is key to a better understanding of the regulation of nitrogen-acquisition mechanism in *Nepenthes* plants.

Apart from aspartic proteases, there is also presence of cysteine proteases in carnivorous plants. Lately, it also has been found that cysteine protease is the primary protease found in digestive fluid of *Dionaea* (Venus flytrap). Prey proteins found in the digestive fluid of *Dionaea* are degraded by cysteine endopeptidases in association with serine carboxypeptidases (Risør et

al., 2016). This is highly distinct to the digestive fluids found in *Nepenthes* and *Drosera* with aspartic proteases (Athauda et al., 2004). However, there is also presence of both aspartic and cysteine proteases in *N. ventricosa* as reported by (Stephenson & Hogan, 2006). Takahashi, Tanji & Shibata (2007) conducted comparative enzymatic characterisation of acid proteases from crude digestive fluid of various carnivorous plants namely *Nepenthes*, *Chepalotus*, *Drosera*, and *Dionaea*, with distinct trapping mechanisms. The study demonstrated significant variations between them, which might be due to the presence of different classes of proteases in different families. This reflects the phylogenetic diversity of these carnivorous plants.

There are attempts on the recombinant expression of the enzymes from carnivorous plants (Morohoshi et al., 2011; Ishisaki et al., 2012b; Kadek et al., 2014b). Kadek et al., (2014b) reported an efficient way to obtain high amount of Nepenthesin I (Nep1) from *N. gracilis* through heterologous expression in *Escherichia coli.* The characteristics of the recombinant protein obtained are similar to the native enzyme isolated from the pitcher fluid. More recently, Nep1 from *N. gracilis* was successfully purified and crystallised (Fejfarová et al., 2016).

On the other hand, the evolution of different trapping mechanisms for carnivorous plants to survive in harsh environments with limited nutrients may result in enzymes with novel properties. For instance, a novel class of prolyl endopeptidase called neprosin 1 and neprosin 2 (Npr1 & Npr2) was recently discovered in *Nepenthes* species to be distinct from commonly known proline-cleaving enzymes, which consists of two novel neprosin domains (Lee et al., 2016). Schrader et al., (2017) characterised neprosin to be a proline-cleaving enzyme through recombinant approach and demonstrated that it has the potential to be utilised for whole proteomic profiling and histone mapping. This is because neprosin is a low molecular weight prolyl endopeptidase and extremely active at low concentration and pH. Combined actions of a neprosin and nepenthesin from *Nepenthes* pitcher fluid showed potential of effective gluten detoxification, which broaden the prospects for enzyme supplementation approach to circumvent celiac disease (Rey et al., 2016)

Although the proteolytic activity in the digestive fluid is of great interest, low yields of secreted enzymes make it very challenging for native enzyme purification. Furthermore, prey digestion is likely to be concerted activities of various proteases and other enzymes in the

digestive fluid, hence it is interesting to compare the enzyme assays between crude digestive fluid extracts and individual purified proteases.

Applications of proteases from carnivorous plants

The metabolic activity of most living organisms including plants, animals, fungi, bacteria, and viruses requires proteolytic enzymes. Proteases are one of the largest groups of hydrolytic enzymes that cleave the peptide bonds in the polypeptide chains. The two major groups of proteases are endopeptidases that cleave non-terminal peptide bonds, and exopeptidases that can be classified to carboxypeptidases or aminopeptidases based on their ability to cleave the C or N terminal peptide bonds respectively. The four major classes of proteases are aspartic proteases, serine proteases, cysteine proteases, and metalloproteases.

Proteases are the dominant class of industrial enzymes with diverse applications, such as leather products, detergents, meat tenderisers, food products, as well as pharmaceutical and waste processing industry (Rao et al., 1998; Lakshmi & Hemalatha, 2016). Almost 60% of the total worldwide production of the enzymes are dominated by proteases (Usharani & Muthuraj, 2010). Microbes and animals are currently the major source of proteases with only a few commercialised plant proteases. Interest has been growing in plant proteases, which have significant commercial values due to high stability in extreme conditions (Canay, Erguven & Yulug, 1991; Houde, Kademi & Leblanc, 2004; Karnchanatat et al., 2011). Examples of proteases from plant sources are listed in Table 4.

Broad substrate specificity, high activity in wide range of pH, temperature, and high stability in the presence of organic compounds are the major factors that attributed for special attention towards proteolytic enzymes from plant sources. Furthermore, ethical/religious reasons and/or regulatory limitations, which restrict the applications of non-plant proteases (animal and recombinant sources) in certain countries pose a need for new plant proteases. In plants, aspartic proteases are widely distributed in the seed, flower, leaf, as well as in the digestive fluid of carnivorous plants. Several plant aspartic proteases, such as oryzasin from rice and phytepsin from barley have been purified and well characterised. Proteases found in the digestive fluid of carnivorous plants are the only extracellular proteinase of plant origin. Most plant proteases are

known to be intracellular vacuolar enzymes. Kadek et al. (2014a) and Yang et al. (2015) successfully immobilised nepenthesin-1 and nepenthesin-2 respectively as a molecular tool for digestion in hydrogen/deuterium exchange mass spectrometry (HXMS) to track exchange patterns in protein structure, especially useful for biopharmaceutical industry. Nep1 is shown to exhibit wide substrate cleavage specificity and high stability towards denaturing reagents compared with pepsin for digesting protein into small peptides with overlapping fragments to provide necessary coverage of protein sequences.

Therefore, carnivorous plants signify a unique source of proteases for various biotechnological applications. The proteases discovered in the trap secretions could be distinct and provide wide range of functional temperature, stability and pH activity profiles. Furthermore, differential substrate specificity among the proteases could provide specialised applications, such as that of demonstrated for a new mass spectrometry technique. The common plant proteases, such as bromelain and papain, denote only small population of plant proteases which are yet to be discovered. On the other hand, inhibiting protease activity in digestive fluid will be critical when using carnivorous plants as hosts for expressing functional plant-made proteins.

Conclusions

The search for new industrially viable plant enzymes is a continuous effort in which carnivorous plants serve as great resources for exploration. There are numerous studies on the properties of digestive fluid of carnivorous plants towards a better understanding of carnivory mechanism and evolution. Further extensive biochemical and morphological studies on carnivorous plants will still be needed to help in further understanding the regulation of hydrolytic enzyme secretion. In addition, successful purification and characterisation of the secreted enzymes will encourage their exploitation for industrial applications. Future research efforts in studying regulatory mechanisms of digestive enzymes or metabolites responsible for attracting prey will not only be useful to fill in current gap of knowledge, but also advancing novel utilisation of carnivorous plants for producing plant-made proteins. Comparative genomics approach will help in elucidating the evolutionary history of these fascinating plants. With the advent of omics

346 technologies, a holistic understanding on the molecular mechanisms of carnivory in various
347 carnivorous plants will be achievable along with more exciting discoveries.

References

Adamec L. 1997. Mineral nutrition of carnivorous plants: A review. *The Botanical Review* 63:273–299. DOI: 10.1007/BF02857953.

Adlassnig W., Peroutka M., Lendl T. 2010. Traps of carnivorous pitcher plants as a habitat: composition of the fluid, biodiversity and mutualistic activities. *Annals of Botany* 107:181–194. DOI: 10.1093/aob/mcq238.

Amagase S. 1972. Digestive enzymes in Insectivorous plants: Acid proteases in the genus *Nepenthes* and *Drosera peltata*. *Journal of Biochemistry* 72:73–81.

Amri E., Mamboya F. 2012. Papain, a Plant Enzyme of Biological Importance : A Review. *American Journal of Biochemistry and Biotechnology* 8:99–104. DOI: 10.3844/ajbbsp.2012.99.104.

An C-I., Fukusaki E., Kobayashi A. 2002. Aspartic proteinases are expressed in pitchers of the carnivorous plant *Nepenthes alata* Blanco. *Planta* 214:661–667. DOI: 10.1007/s004250100665.

Athauda SBP., Matsumoto K., Sanath R., Rajapakshe S., Kuribayashi M., Kojima M., Kubomura N., Inoue H., Shibata C., Takahashi K. 2004. Enzymic and structural characterization of nepenthesin, a unique member of a novel subfamily of aspartic proteinases. *Journal of Biochemistry* 381:295–306.

Banasiuk R., Kawiak A., Krölicka A. 2012. *In vitro* cultures of carnivorous plants from the *Drosera* and *Dionaea* genus for the production of biologically active secondary metabolites. *Journal of Biotechnology, Computational Biology and Bionanotechnology* 93:87–96. DOI: 10.5114/bta.2012.46572.

Bauer U., Paulin M., Robert D., Sutton GP. 2015. Mechanism for rapid passive-dynamic prey capture in a pitcher plant. *Proceedings of the National Academy of Sciences* 112:13384–13389. DOI: 10.1073/pnas.1510060112.

Behie SW., Bidochka MJ. 2013. Insects as a nitrogen source for plants. *Insects* 4:413–424. DOI: 10.3390/insects4030413.

Buch F., Kaman WE., Bikker FJ., Yilamujiang A., Mithöfer A. 2015. Nepenthesin Protease Activity Indicates Digestive Fluid Dynamics in Carnivorous *Nepenthes* Plants. *PLoS ONE* 10:1–15. DOI: 10.1371/journal.pone.0118853.

Buch F., Rott M., Rottloff S., Paetz C., Hilke I., Raessler M., Mithöfer A. 2012. Secreted pitfall-trap fluid of carnivorous *Nepenthes* plants is unsuitable for microbial growth. *Annals of Botany* 111:375–383. DOI: 10.1093/aob/mcs287.

Buddrick O., Cornell HJ., Small DM. 2015. Reduction of toxic gliadin content of wholegrain bread by the enzyme caricain. *Food Chemistry* 170:343–347. DOI: 10.1016/j.foodchem.2014.08.030.

Butts CT., Bierma JC., Martin RW. 2016. Novel proteases from the genome of the carnivorous plant *Drosera capensis*: Structural prediction and comparative analysis. *Proteins* 84:1517–1533. DOI: 10.1002/prot.25095.

Canay S., Erguven S., Yulug N. 1991. The function of enzyme in removing candida accumulated on denture plaque. *Journal of Islamic Academy of Sciences* 4:87–89.

Caniato R., Filippini R., Cappelletti EM. 1989. Naphthoquinone contents of cultivated *Drosera* species. *International Journal of Crude Drug Research* 27:129–136.

Chanalia P., Gandhi D., Jodha D., Singh J. 2011. Applications of microbial proteases in pharmaceutical industry: an overview. *Reviews in Medical Microbiology* 22:96–101. DOI: 10.1097/MRM.0b013e3283494749.

394 Chang SC., Gallie DR. 1997. RNase activity decreases following a heat shock in wheat leaves
395 and correlates with its posttranslational modification. *Plant physiology* 113:1253–1263.
396 DOI: 10.1104/pp.113.4.1253.
397 Choudhury D., Roy S., Chakrabarti C., Biswas S., Dattagupta JK. 2009. Production and recovery
398 of recombinant propapain with high yield. *Phytochemistry* 70:465–472. DOI:
399 10.1016/j.phytochem.2009.02.001.
400 Clancy FG., Coffey MD. 1977. Acid phosphatase and protease release by the insectivorous plant
401 *Drosera rotundifolia*. *Canadian Journal of Botany* 55:480–488.
402 Darwin C. 1875. *Insectivorous plants*. New York: D. Appleton and Company.
403 Didry N., Dubreuil L., Trotin F., Pinkas M. 1998. Antimicrobial activity of aerial parts of
404 *Drosera peltata* Smith on oral bacteria. *Journal of Ethnopharmacology* 60:91–96. DOI:
405 10.1016/S0378-8741(97)00129-3.
406 Eilenberg H., Pnini-cohen S., Schuster S., Movtchan A., Zilberstein A., Aviv R. 2006. Isolation
407 and characterization of chitinase genes from pitchers of the carnivorous plant *Nepenthes*
408 *khasiana*. *Journal of Experimental Botany* 57:2775–2784. DOI: 10.1093/jxb/erl048.
409 Ellison AM. 2006. Nutrient limitation and stoichiometry of carnivorous plants. *Plant Biology*
410 8:740–747. DOI: 10.1055/s-2006-923956.
411 Ellison AM., Gotelli NJ. 2001. Evolutionary ecology of carnivorous plants. *Trends in Ecology*
412 *and Evolution* 16:623–629. DOI: 10.1016/S0169-5347(01)02269-8.
413 Ellison AM., Gotelli NJ. 2009. Energetics and the evolution of carnivorous plants - Darwin's
414 "most wonderful plants in the world." *Journal of Experimental Botany* 60:19–42. DOI:
415 10.1093/jxb/ern179.
416 Farnsworth EJ., Ellison AM. 2008. Prey availability directly affects physiology, growth, nutrient
417 allocation and scaling relationships among leaf traits in 10 carnivorous plant species.
418 *Journal of Ecology* 96:213–221. DOI: 10.1111/j.1365-2745.2007.01313.x.
419 Feijoo-Siota L., Villa TG. 2011. Native and Biotechnologically Engineered Plant Proteases with
420 Industrial Applications. *Food and Bioprocess Technology* 4:1066–1088. DOI:
421 10.1007/s11947-010-0431-4.
422 Fejfarová K., Kádek A., Mrázek H., Hausner J., Tretyachenko V., Koval' T., Man P., Hašek J.,
423 Dohnálek J. 2016. Crystallization of nepenthesin I using a low-pH crystallization screen.
424 *Acta Crystallographica Section:F Structural Biology Communications* 72:24–28. DOI:
425 10.1107/S2053230X15022323.
426 Frazao C., Bento I., Costa J., Soares CM., Veríssimo P., Faro C., Pires E., Cooper J., Carrondo
427 MA. 1999. Crystal structure of cardosin A, a glycosylated and Arg-Gly-Asp-containing
428 aspartic proteinase from the flowers of *Cynara cardunculus* L. *The Journal of Biological*
429 *Chemistry*. 274:27694–27701. DOI: 10.1074/jbc.274.39.27694.
430 Fukushima K., Fang X., Alvarez-Ponce D., Cai H., Carretero-Paulet L., Chen C., Chang TH.,
431 Farr KM., Fujita T., Hiwatashi Y., Hoshi Y., Imai T., Kasahara M., Librado P., Mao L.,
432 Mori H., Nishiyama T., Nozawa M., Pálfalvi G., Pollard ST., Rozas J., Sánchez-Gracia A.,
433 Sankoff D., Shibata TF., Shigenobu S., Sumikawa N., Uzawa T., Xie M., Zheng C., Pollock
434 DD., Albert VA., Li S., Hasebe M. 2017. Genome of the pitcher plant Cephalotus reveals
435 genetic changes associated with carnivory. *Nature Ecology and Evolution* 1:59. DOI:
436 10.1038/s41559-016-0059.
437 Gaascht F., Dicato M., Diederich M. 2013. Venus Flytrap (*Dionaea muscipula* Solander ex Ellis)
438 Contains Powerful Compounds that Prevent and Cure Cancer. *Frontiers in Oncology* 3:30–
439 34. DOI: 10.3389/fonc.2013.00202.

Givnish TJ. 2015. New evidence on the origin of carnivorous plants. *Proceedings of the National Academy of Sciences* 112:10–11. DOI: 10.1073/pnas.1422278112.

Givnish TJ., Burkhardt EL., Happel RE., Weintraub JD. 1984. Carnivory in the Bromeliad *Brocchinia reducta*, with a cost/benefit model for the general restriction of carnivorous plants to sunny, moist, nutrient-poor habitats. *The American Naturalist* 124.

Gorb E., Kastner V., Peressadko A., Arzt E., Gaume L., Rowe N., Gorb S. 2004. Structure and properties of the glandular surface in the digestive zone of the pitcher in the carnivorous plant *Nepenthes ventrata* and its role in insect trapping and retention. *Journal of Experimental Biology* 207:2947–2963. DOI: 10.1242/jeb.01128.

Hatano N., Hamada T. 2008. Proteome analysis of pitcher fluid of the carnivorous plant *Nepenthes alata*. *Journal of Proteome Research* 7:809–816. DOI: 10.1021/pr700566d.

Higashi S., Nakashima A., Ozaki H., Abe M., Uchiumi T. 1993. Analysis of Feeding Mechanism in a Pitcher of *Nepenthes hybrida*. *Journal of Plant Research* 106:47–54.

Houde A., Kademi A., Leblanc D. 2004. Lipases and Their Industrial Applications: An Overview. *Applied Biochemistry and Biotechnology* 118:155–170. DOI: 10.1385/ABAB:118:1-3:155.

Ishisaki K., Arai S., Hamada T., Honda Y. 2012a. Biochemical characterization of a recombinant plant class III chitinase from the pitcher of the carnivorous plant *Nepenthes alata*. *Carbohydrate Research* 361:170–174. DOI: 10.1016/j.carres.2012.09.001.

Ishisaki K., Honda Y., Taniguchi H., Hatano N., Hamada T. 2012b. Heterogonous expression and characterization of a plant class IV chitinase from the pitcher of the carnivorous plant *Nepenthes alata*. *Glycobiology* 22:345–351. DOI: 10.1093/glycob/cwr142.

Jaffe K., Michelangeli F., Gonzalez JM., Miras B., Ruiz MC. 1992. Carnivory in pitcher plants of the genus *Heliamphora* (Sarraceniaceae). *New Phytologist* 122:733–744. DOI: 10.1111/j.1469-8137.1992.tb00102.x.

Jentsch J. 1972. Enzymes from carnivorous plants (*nepenthes*). Isolation of protease nepenthacin. *FEBS Letters* 21:273–276.

Jopcik M., Moravcikova J., Matusikova I., Bauer M., Rajninec M., Libantova J. 2017. Structural and functional characterisation of a class I endochitinase of the carnivorous sundew (*Drosera rotundifolia* L.). *Planta* 245:313–327. DOI: 10.1007/s00425-016-2608-1.

Kadek A., Mrazek H., Halada P., Rey M., Schriemer DC., Man P. 2014a. Aspartic protease nepenthesin-1 as a tool for digestion in hydrogen/deuterium exchange mass spectrometry. *Analytical Chemistry* 86:4287–4294. DOI: 10.1021/ac404076j.

Kadek A., Tretyachenko V., Mrazek H., Ivanova L., Halada P., Rey M., Schriemer DC., Man P. 2014b. Expression and characterization of plant aspartic protease nepenthesin-1 from *Nepenthes gracilis*. *Protein Expression and Purification* 95:121–128. DOI: 10.1016/j.pep.2013.12.005.

Karnchanatat A., Tiengburanatam N., Boonmee A., Puthong S., Sangvanich P. 2011. Zingipain, a cysteine protease from *Zingiber ottensii* valeton rhizomes with antiproliferative activities against fungi and human malignant cell lines. *Preparative Biochemistry and Biotechnology* 41:138–153. DOI: 10.1080/10826068.2011.547347.

Koopman MM., Fuselier DM., Hird S., Carstens BC. 2010. The Carnivorous Pale Pitcher Plant Harbors Diverse , Distinct , and Time-Dependent Bacterial Communities □ †. 76:1851–1860. DOI: 10.1128/AEM.02440-09.

Król E., Płachno BJ., Adamec L., Stolarz M., Dziubińska H., Trebacz K. 2011. Quite a few reasons for calling carnivores "the most wonderful plants in the world." *Annals of Botany*

109:47–64. DOI: 10.1093/aob/mcr249.
Lakshmi BKM., Hemalatha KPJ. 2016. Eco Friendly Recovery of Silver from Used X-ray Films by Alkaline Protease of *Bacillus Cereus* Strain S8. *Frontiers in Environmental Microbiology* 2:45–48. DOI: 10.11648/j.fem.20160206.14.
Lan T., Renner T., Ibarra-Laclette E., Chang T-H., Cervantes-Pérez SA., Zheng C., Sankoff D., Tang H., W.Purbojati R., Putra A., Drautz-Moses DI., Schuster SC., Herrera-Estrella L., Albert VA. 2017. Long-read sequencing uncovers the adaptive topography of a carnivorous plant genome. *Nature* 114:E5483–E5483. DOI: 10.1073/pnas.1709197114.
Lee L., Zhang Y., Ozar B., Sensen CW., Schriemer DC. Supplementary Carnivorous Nutrition in Pitcher Plants (Nepenthes spp .) via an Unusual Complement of Endogenous Enzymes. :1–9.
Lee L., Zhang Y., Ozar B., Sensen CW., Schriemer DC. 2016. Carnivorous Nutrition in Pitcher Plants (*Nepenthes* spp.) via an Unusual Complement of Endogenous Enzymes. *Journal of Proteome Research* 15:3108–3117. DOI: 10.1021/acs.jproteome.6b00224.
Leushkin E V., Sutormin RA., Nabieva ER., Penin AA., Kondrashov AS., Logacheva MD. 2013. The miniature genome of a carnivorous plant *Genlisea aurea* contains a low number of genes and short non-coding sequences. *BMC Genomics* 14:476. DOI: 10.1186/1471-2164-14-476.
Luciano CS., Newell SJ. 2017. Effects of prey, pitcher age, and microbes on acid phosphatase activity in fluid from pitchers of *Sarracenia purpurea* (Sarraceniaceae). *PLoS ONE* 12:e0181252.
Malone LA., Todd JH., Burgess EPJ., Philip BA., Christeller JT. 2005. Effects of kiwifruit (*Actinidia deliciosa*) cysteine protease on growth and survival of *Spodoptera litura* larvae (*Lepidoptera*: *Noctuidae*) fed with control or transgenic avidin-expressing tobacco. *New Zealand Journal of Crop and Horticultural Science* 33:99–105. DOI: 10.1080/01140671.2005.9514337.
Matušíková I., Salaj J., Moravčíková J., Mlynárová L., Nap JP., Libantová J. 2005. Tentacles of in vitro-grown round-leaf sundew (*Drosera rotundifolia* L.) show induction of chitinase activity upon mimicking the presence of prey. *Planta* 222:1020–1027. DOI: 10.1007/s00425-005-0047-5.
Mazorra-Manzano MA., Ramírez-Suarez JC., Yada RY. 2017. Plant proteases for bioactive peptides release: A review. *Critical Reviews in Food Science and Nutrition*. DOI: 10.1080/10408398.2017.1308312.
Mcnally SF., Stewart A., Wilson UE. 1988. The stimulation of acid phosphatase activity in the stalked gland of *Drosera-rotundifolia*. *Ann. Bot.* 61:289–292.
Michalko J., Socha P., Mészáros P., Blehová A., Libantová J., Moravčíková J., Matušíková I. 2013. Glucan-rich diet is digested and taken up by the carnivorous sundew (*Drosera rotundifolia* L.): Implication for a novel role of plant β-1,3-glucanases. *Planta* 238:715–725. DOI: 10.1007/s00425-013-1925-x.
Miguel S., Hehn A., Bourgaud F. 2018. *Nepenthes* : State of the art of an inspiring plant for biotechnologists. *Journal of Biotechnology* 265:109–115. DOI: 10.1016/j.jbiotec.2017.11.014.
Mithöfer A. 2011. Carnivorous pitcher plants: Insights in an old topic. *Phytochemistry* 72:1678–1682. DOI: 10.1016/j.phytochem.2010.11.024.
Morohoshi T., Oikawa M., Sato S., Kikuchi N., Kato N., Ikeda T. 2011. Isolation and characterization of novel lipases from a metagenomic library of the microbial community in

the pitcher fluid of the carnivorous plant *Nepenthes hybrida*. *Journal of Bioscience and Bioengineering* 112:315–320. DOI: 10.1016/j.jbiosc.2011.06.010.

Mu'izzuddin M., Aqil M., Rosli F., Imadi F., Salleh M., Noor NM., Aizat WM., Goh H. 2017. Iso-Seq analysis of *Nepenthes ampullaria*, *Nepenthes rafflesiana* and *Nepenthes* × *hookeriana* for hybridisation study in pitcher plants. *Genomics Data* 12:130–131. DOI: 10.1016/j.gdata.2017.05.003.

Müller K., Borsch T., Legendre L., Porembski S., Theisen I., Barthlott W. 2004. Evolution of carnivory in lentibulariaceae and the lamiales. *Plant Biology* 6:477–490. DOI: 10.1055/s-2004-817909.

Nakayama S., Amagase S. 1968. Acid Protease in *Nepenthes*. *Proceedings of the Japan Academy* 44:358–362.

Nor Adibah WZ., Wan A., Loke K., Goh H., Noor NM. 2016. RNA-seq analysis for plant carnivory gene discovery in *Nepenthes* × *ventrata*. *Genomics Data* 7:18–19. DOI: 10.1016/j.gdata.2015.11.007.

Patel NR. 2014. Carnivory in pitcher plants : An enigmatic meat eating plant. *Research & Review in BioScience* 8:94–106.

Pavlovic A., Jaksova J., Novak O. 2017. Triggering a false alarm: wounding mimics prey capture in the carnivorous Venus flytrap (*Dionaea muscipula*). *New Phytologist* 216:927–938. DOI: 10.1111/nph.14747.

Pavlovic A., Krausko M., Libiaková M., Adamec L. 2013. Feeding on prey increases photosynthetic efficiency in the carnivorous sundew *Drosera capensis*. *Annals of Botany* 113:69–78. DOI: 10.1093/aob/mct254.

Peiter E. 2014. Chapter 11: Mineral Deficiencies. In: Krauss G-J, Nies DH eds. *Ecological Biochemistry: Environmental and Interspecies Interactions*. Wiley-VCH Verlag GmbH & Co. KGaA, 208–235. DOI: 10.1002/9783527686063.

Płachno BJ., Adamec L., Lichtscheidl IK., Peroutka M., Adlassnig W., Vrba J. 2006. Fluorescence Labelling of Phosphatase Activity in Digestive Glands of Carnivorous Plants. *Plant Biology* 8:813–820. DOI: 10.1055/s-2006-924177.

Porembski S., Barthlott W. 2006. Advances in Carnivorous Plants Research. *Plant Biology* 8:737–739. DOI: 10.1055/s-2006-924669.

Rao MB., Tanksale AM., Ghatge MS., Deshpande V V. 1998. Molecular and Biotechnological Aspects of Microbial Proteases. *Microbiology and Molecular Biology Reviews* 62:597–635. DOI: papers2://publication/uuid/E58ABF6D-8C97-4209-810D-A452EE30B2CD.

Renner T., Specht CD. 2013. Inside the trap: Gland morphologies, digestive enzymes, and the evolution of plant carnivory in the Caryophyllales. *Current Opinion in Plant Biology* 16:436–442. DOI: 10.1016/j.pbi.2013.06.009.

Rey M., Yang M., Lee L., Zhang Y., Sheff JG., Sensen CW., Mrazek H., Halada P., Man P., Mccarville JL., Verdu EF., Schriemer DC. 2016. Addressing proteolytic efficiency in enzymatic degradation therapy for celiac disease. *Scientific Reports* 6:30980. DOI: 10.1038/srep30980.

Risør MW., Thomsen LR., Sanggaard KW., Nielsen TA., Thøgersen IB., Lukassen M V., Rossen L., Garcia-Ferrer I., Guevara T., Scavenius C., Meinjohanns E., Gomis-Rüth FX., Enghild JJ. 2016. Enzymatic and structural characterization of the major endopeptidase in the Venus flytrap digestion fluid. *Journal of Biological Chemistry* 291:2271–2287. DOI: 10.1074/jbc.M115.672550.

Rottloff S., Miguel S., Biteau F., Nisse E., Hammann P., Kuhn L., Chicher J., Bazile V., Gaume

578 L., Mignard B., Hehn A., Bourgaud F. 2016. Proteome analysis of digestive fluids in
579 *Nepenthes* pitchers. *Annals of botany* 117:479–495. DOI: 10.1093/aob/mcw001.
580 Rottloff S., Stieber R., Maischak H., Turini FG., Heubl G., Mithöfer A. 2011. Functional
581 characterization of a class III acid endochitinase from the traps of the carnivorous pitcher
582 plant genus, Nepenthes. *Journal of Experimental Botany* 62:4639–4647. DOI:
583 10.1093/jxb/err173.
584 Rudenskaya GN., Bogdanova EA., Revina LP., Golovkin BN., Stepanov VM. 1995.
585 Macluralisin - a serine proteinase from fruits of *Maclura pomifera* (Raf.) Schneid. *Planta*
586 196:174–179. DOI: 10.1007/BF00193231.
587 Runeberg-Roos P., Saarma M. 1998. Phytepsin, a barley vacuolar aspartic proteinase, is highly
588 expressed during autolysis of developing tracheary elements and sieve cells. *The Plant*
589 *Journal* 15:139–145. DOI: 10.1046/j.1365-313X.1998.00187.x.
590 Scala J., Iott K., Schwab DW., Semersky FE. 1969. Digestive Secretion of Dionaea muscipula
591 (Venus's Flytrap). *Plant Physiology* 44:367–371. DOI: 10.1104/pp.44.3.367.
592 Schrader CU., Lee L., Rey M., Sarpe V., Man P., Sharma S., Zabrouskov V., Larsen B.,
593 Schriemer DC. 2017. Neprosin, a Selective Prolyl Endoprotease for Bottom-up Proteomics
594 and Histone Mapping. *Molecular & Cellular Proteomics* 16:1162–1171. DOI:
595 10.1074/mcp.M116.066803.
596 Schulze WX., Sanggaard KW., Kreuzer I., Knudsen AD., Bemm F., Thøgersen IB., Bräutigam
597 A., Thomsen LR., Schliesky S., Dyrlund TF., Escalante-Perez M., Becker D., Schultz J.,
598 Karring H., Weber A., Højrup P., Hedrich R., Enghild JJ. 2012. The Protein Composition of
599 the Digestive Fluid from the Venus Flytrap Sheds Light on Prey Digestion Mechanisms.
600 *Molecular & Cellular Proteomics* 11:1306–1319. DOI: 10.1074/mcp.M112.021006.
601 Schwallier R., Boer HJ De., Visser N., Vugt RR Van., Gravendeel B. 2015. Traps as treats : a
602 traditional sticky rice snack persisting in rapidly changing Asian kitchens. *Journal of*
603 *Ethnobiology and Ethnomedicine* 11:24. DOI: 10.1186/s13002-015-0010-x.
604 Simões I., Faro C. 2004. Structure and function of plant aspartic proteinases. *European Journal*
605 *of Biochemistry* 271:2067–2075. DOI: 10.1111/j.1432-1033.2004.04136.x.
606 Sirova D., Adamec L., Vrba J. 2003. Enzymatic activities in traps of four aquatic species of the
607 carnivorous genus *Utricularia*. *New Phytologist* 159:669–675. DOI: 10.1046/j.1469-
608 8137.2003.00834.x.
609 Srivastava A., Rogers WL., Breton CM., Cai L., Malmberg RL. 2011. Transcriptome analysis of
610 *Sarracenia*, an insectivorous plant. *DNA Research* 18:253–261. DOI:
611 10.1093/dnares/dsr014.
612 Steckelberg R., Lüttge U., Weigl J. 1967. Nepenthes.Kannensaft. *Planta* 76:238–241.
613 Stephenson P., Hogan J. 2006. Cloning and Characterization of a Ribonuclease, a Cysteine
614 Proteinase, and an Aspartic Proteinase from Pitchers of the Carnivorous Plant *Nepenthes*
615 *ventricosa* Blanco. *International Journal of Plant Sciences* 167:239–248.
616 Takahashi K., Matsumoto K., Nishii W., Muramatsu M., Kubota K. 2009. Comparative studies
617 on the acid proteinase activities in the digestive fluids of *Nepenthes*, *Cephalotus*, *Dionaea*,
618 and *Drosera*. *Carnivorous Plant Newsletter* 38:75–82.
619 Takahashi K., Tanji M., Shibata C. 2007. Variations in the content and isozymic composition of
620 Nepenthesin in the pitcher fluids among *Nepenthes* species. *Carnivorous Plant Newsletter*
621 36:73–76.
622 Takeuchi Y., Chaffron S., Salcher MM., Shimizu-inatsugi R., Kobayashi MJ., Diway B., Mering
623 C Von., Pernthaler J., Shimizu KK. 2015. Bacterial diversity and composition in the fluid of

pitcher plants of the genus *Nepenthes*. *Systematic and Applied Microbiology* 38:330–339. DOI: 10.1016/j.syapm.2015.05.006.

Takeuchi Y., Salcher MM., Ushio M., Shimizu-Inatsugi R., Kobayashi MJ., Diway B., von Mering C., Pernthaler J., Shimizu KK. 2011. *In Situ* Enzyme Activity in the Dissolved and Particulate Fraction of the Fluid from Four Pitcher Plant Species of the Genus Nepenthes. *PLoS ONE* 6. DOI: 10.1371/journal.pone.0025144.

Thornhill AH., Harper IS., Hallam ND. 2008. The Development of the Digestive Glands and Enzymes in the Pitchers of Three Nepenthes Species: *N. alata*, *N. tobaica*, and *N. ventricosa* (Nepenthaceae). *International Journal of Plant Science* 169:615–624. DOI: 10.1086/533599.

Tokes ZA., Woon WC., Chambers SM. 1974. Digestive enzymes secreted by the carnivorous plant *Nepenthes macferlanei L*. *Planta* 119:39–46. DOI: 10.1007/BF00390820.

Unhelkar MH., Duong VT., Enendu KN., Kelly JE., Tahir S., Butts CT., Martin RW. 2017. Structure prediction and network analysis of chitinases from the Cape sundew, *Drosera capensis*. *Biochimica et Biophysica Acta - General Subjects* 1861:636–643. DOI: 10.1016/j.bbagen.2016.12.007.

Usharani B., Muthuraj M. 2010. Production and Characterization of Protease Enzyme from *Bacillus laterosporus*. *Journal of Microbiology Research* 4:1057–1063.

Wan Zakaria W-N-A., Loke K., Zulkapli M., Mohd Salleh F-'Imadi., Goh H., Mohd Noor N. 2016. RNA-seq Analysis of *Nepenthes ampullaria*. *Frontiers in Plant Science* 6:1229. DOI: 10.1086/375422.

Yang M., Hoeppner M., Rey M., Kadek A., Man P., Schriemer DC. 2015. Recombinant nepenthesin II for hydrogen/deuterium exchange mass spectrometry. *Analytical Chemistry* 87:6681–6687. DOI: 10.1021/acs.analchem.5b00831.

Yilamujiang A., Zhu A., Ligabue-Braun R., Bartram S., Witte CP., Hedrich R., Hasabe M., Schöner CR., Schöner MG., Kerth G., Carlini CR., Mithöfer A. 2017. Coprophagous features in carnivorous *Nepenthes* plants: A task for ureases. *Scientific Reports* 7:11647. DOI: 10.1038/s41598-017-11999-z.

Table 1(on next page)

Different carnivorous plant families and trapping mechanisms

Modified from Król et al. (2011) and Givnish (2015) .

Table 1. Different carnivorous plant families and trapping mechanisms. Modified from Król et al. (2011) and Givnish (2015).

Order	Family	Genus	Trap
Caryophyllales	Dioncophyllaceae	*Triphyophyllum*	Flypaper
	Drosophyllaceae	*Drosophyllum*	Flypaper
	Droseraceae	*Drosera*	Flypaper
		Aldrovanda	Snap
		Dionaea	Snap
	Nepenthaceae	*Nepenthes*	Pitfall
Ericales	Roridulaceae	*Roridula*	Flypaper
	Sarraceniaceae	*Darlingtonia*	Pitfall
		Heliamphora	Pitfall
		Sarracenia	Pitfall
Lamiales	Plantaginaceae	*Philcoxia*	Flypaper
	Byblidaceae	*Byblis*	Flypaper
	Lentibulariaceae	*Pinguicula*	Flypaper
		Utricularia	Suction
		Genlisea	Eel
Oxalidales	Cephalotaceae	*Cephalotus*	Pitfall
Poales	Bromeliaceae	*Brocchinia*	Pitfall
		Catopsis	Pitfall
	Eriocaulaceae	*Paepalanthus*	Pitfall

Table 2(on next page)

Digestive enzyme discovery from different carnivorous plant families.

Modified from Adlassnig, Peroutka & Lendl (2010) and Peiter (2014) .

Table 2. Digestive enzyme discovery from different carnivorous plant families. Modified from Adlassnig, Peroutka & Lendl (2010) and Peiter (2014).

Family	Species	Enzyme Category														Reference
		Phosphatase	Protease	Chitinase	Glucanase	Esterase	Peroxidase	Nuclease	Glucosaminidase	Glucosidase	Amylase	Lipase	Ribonuclease	Phosphoamidase	Xylosidase	Urease	
Cephalotaceae	*C. follicularis*	*	*	*	*	*	*	*			*		*				1
Droseraceae	*D. muscipula*	*	*	*	*	*	*	*			*						2, 3
	D. capensis	*	*	*													4 - 6
	D. rotundifolia		*	*	*												7 - 9
	D. villosa											*					10
	D. peltata			*													11
Lentibulariacea	*Utricularia* spp.	*	*	*		*				*							12
	G. aurea	*	*			*											13
	U. multifida	*															13
	U. foliosa	*															12
	U. australis	*															12
Sarraceniaceae	*S. purpurea*	*	*	*	*							*	*				1, 14
	Sarracenia spp.					*					*						15
	D. californica		*														16
	H. tatei		*														17, 18
	S. psittacina							*									19
Nepenthaceae	*N. alata*	*	*	*	*	*	*								*		20, 21
	N. bicalcarata	*	*		*		*		*	*							21, 22
	N. x *ventrata*	*	*	*	*		*	*									23, 24
	N. albomarginata	*		*	*				*	*							21, 22
	N. gracilis	*	*	*					*	*							22, 25

N. ampullaria	*		*						*	*								22
N. hybrida	*				*		*							*				10, 26
N. mirabilis		*	*	*		*												21, 27
N. sanguinea		*	*	*		*												21
N. ventricosa		*	*															28
N. distillatoria		*																29
N. hemsleyana																	*	30
N. khasiana			*															31, 32
N. macfarlanei												*						20, 33
N. rafflesiana			*															21
N. tobaica	*																	34

* Present

References: [1] Fukushima et al., 2017; [2] Schulze et al., 2012; [3] Pavlovic, Jaksova & Novak, 2017; [4] Pavlovic et al., 2013; [5] Butts, Bierma & Martin, 2016; [6] Unhelkar et al., 2017; [7] Matušíková et al., 2005; [8] Michalko et al., 2013; [9] Jopcik et al., 2017; [10] Morohoshi et al., 2011; [11] Amagase, 1972; [12] Sirova, Adamec & Vrba, 2003; [13] Płachno et al., 2006; [14] Luciano & Newell, 2017; [15] Porembski & Barthlott, 2006; [16] Adlassnig, Peroutka & Lendl, 2010; [17] Jaffe et al., 1992; [18] Mithöfer, 2011; [19] Srivastava et al., 2011; [20] Hatano & Hamada, 2008; [21] Rottloff et al., 2016 ; [22] Takeuchi et al., 2011; [23] Lee et al., 2016; [24] Schrader et al., 2017; [25] Kadek et al., 2014; [26] Higashi et al., 1993; [27] Buch et al., 2015; [28] Stephenson & Hogan, 2006; [29] Athauda et al., 2004; [30] Yilamujiang et al., 2017; [31] Eilenberg et al., 2006; [32] Renner & Specht, 2012; [33] Tokes, Woon & Chambers, 1974; [34] Thornhill, Harper & Hallam, 2008

Table 3(on next page)

Characterisation and purification of digestive enzymes from carnivorous plants.

1 **Table 3**. Characterisation and purification of digestive enzymes from carnivorous plants.

Enzyme	Species	Protein purification method	Substrate	Condition		Reference
				pH	T (°C)	
Proteinase	*N. mixta*, *N. dormanniana*, *N.neuvilleana*	Ecteola cellulose column chromatography	Casein	2.2	50	Steckelberg et al. 1967
Nepenthesin	*Nepenthes* sp.	DEAE-Sephadex A-50	Casein	2.8	40	Amagase, Nakayama & Tsugita, 1969
Proteinase	*D. muscipula*	Sephadex G-150 column	Congocoll	5.5	37	Scala et al., 1969
Nepenthesin	*N.maxima*, *N. rafflesiana*, *N. ampullaria*	Sephadex G-75, Sephadex G-200	Casein	3.0	40	Amagase, 1972
	N. dyeriana, *N. mixta*, *D. peltata*					
Nepenthesin	*Nepenthes* sp.	Sephadex G-75 & G-50, DEAE-Sephadex A-50	Casein	2.9	40	Jentsch, 1972
Nepenthesin	*N. macfarlanei*	Sephadex G-75 gel filtration	Bovine fibrin	NA	37	Tokes, Woon & Chambers, 1974
			Bovine serum albumin	NA	37	
			Horse-heart cytochrome c	2.2	37	
Aspartic protease	*N. alata*	Not purified	Bovine serum albumin	3.0	37	An, Fukusaki & Kobayashi, 2002
Nepenthesin I & II	*N. distillatoria*	DEAE cellulose column, Sephacryl S-200	Acid-denatured haemoglobin	2.8	50	Athauda et al., 2004
		Pepstatin–Sepharose column, Mono Q column				
*Cysteine protease *Aspartic protease	*N. ventricosa*	Not purified	Gelatin	3.0	NA	Stephenson & Hogan, 2006
*Cysteine protease	*D. muscipula*	Hi-Trap Column	7-amino-4-methylcoumarin	3.6	60	Risør et al., 2016
Nepenthesin I & II	*N. alata*, *C. follicularis*,	Not purified	Haemoglobin	2.5	47 - 57	Takahashi et al., 2009
	D. muscipula		Haemoglobin	3.0	60	
	D. capensis		Oxidised insulin B chain	3.5	47	
*Nepenthesin I & II	*N. mirabilis*, *N. alata*	Dialysis	PFU-093 (FRET peptide)	8.0	42	Buch et al., 2015
Nepenthesin I & II	*N. reinwardtiana*, *N. distillatoria*, *N. eymae*, *N. wittei*, *N. hookeriana*, *N. boschiana*, *N. maxima*	Not purified				

Neprosin	*Hybrid N. alata x N. ventricosa N. ventrata*	Reversed phase chromatography	Haemoglobin Gliadin	2.5	NA	Rey et al., 2016
Chitinase I & II	*N. khasiana*	Not purified	*N*-acetylglucosamine (GlcNAc)	3.0	37	Eilenberg et al., 2006
			Glycol-chitin	8.3	37	
*Chitinase III	*N. rafflesiana*	QIAexpressionist Kit - affinity chromatography	CM-chitin-RBV	3.0	41	Rottloff et al., 2011
*Chitinase III	*N. alata*	TALON metal affinity	2-acetamido-2-deoxy- D-glucose	3.9	65	Ishisaki et al., 2012a
			Ethylene glycol chitin			
*Chitinase IV	*N. alata*		β-1,4-linked GlcNAc	5.5	60	Ishisaki et al., 2012b
Lipase	*N. macfarlanei*	Not purified	Glycerol trioleate	6.0	37	Tokes, Woon & Chambers, 1974
			Glycerol tripalmitate	2.6		
			Lecithin	2.2		
	*N. hybrida**	MBPTrap affinity chromatography column	P-nitrophenyl (pNP) palmitate	7.0	37	Morohoshi et al., 2011
			PNP-butyrate	7.0		
			Tributyrin	5.0		
			Triorein	5.0		
Phosphatase	*D. muscipula*	Sephadex G-150 column	P-nitrophenyl phosphate	4.5	37	Scala et al., 1969
	Utricularia foliosa, U. australis, Genlisea lobata, U. multifida D. muscipula, C. follicularis, D. binata, N. tobaica	Not purified	4-methylumbelliferyl (MUF) phosphate ELF 97 phosphatase substrate	5.5	NA	Sirova et al., 2013 Plachno et a., 2006

* Recombinant enzyme; NA: not available; T: optimal temperature

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26940v1 | CC BY 4.0 Open Access | rec: 17 May 2018, publ: 17 May 2018

Table 4(on next page)

Applications of plant proteases from different sources.

Table 4. Applications of proteases from different plant sources.

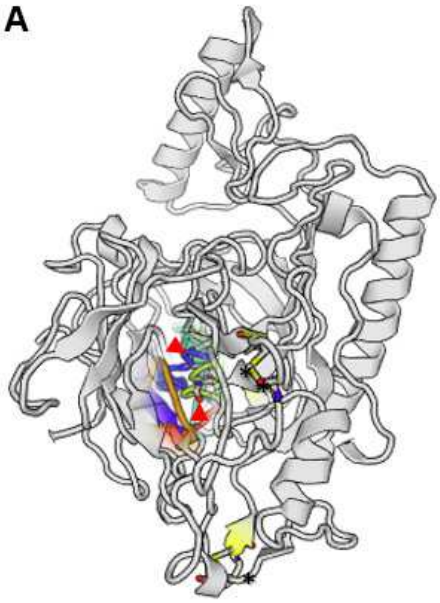
Source	Protease	Application/ Functional Properties	Reference
Nepenthes	Nepenthesin I & II	Tool for digestion in H/D Exchange Mass Spectrometry	Kadek et al., 2014; Yang et al., 2015
	Neprosin	Proteomic analysis / Histone mapping	Schrader et al., 2017
		Gluten digestion	Rey et al., 2016
Papaya	Papain	Meat tenderiser	Amri & Mamboya, 2012
		Denture cleaner	Canay, Erguven & Yulug, 1991
		Detergent, healing burn wound, textiles, cosmestics industry	Choudhury et al., 2009
	Caricain	Gluten-free food processing	Buddrick, Cornell & Small, 2015
Pineapple	Bromelain	Anti-inflammatory and anti-cancer agent	Chanalia et al., 2011
Fig (*Ficus carica*)	Ficin	Pharmaceutical industry	Mazorra-Manzano, Ramírez-Suarez & Yada, 2017
Kiwifruit, Banana, Pineapple, Mango	Actinidin	Dietary supplement	Malone et al., 2005
Zinger	Zingipain	Anti-proliferative agent	Karnchanatat et al., 2011
Musk melon	Cucumisin	Hydrolysis of protein	Feijoo-Siota & Villa, 2011
Cardoon	Cardosin A	Milk clotting, manufacturing of traditional cheese	Frazao et al., 1999
Rice	Oryzasin	Milk clotting	Simões & Faro, 2004
Barley	Phytepsin	Milk clotting	Runeberg-Roos & Saarma, 1998

Figure 1

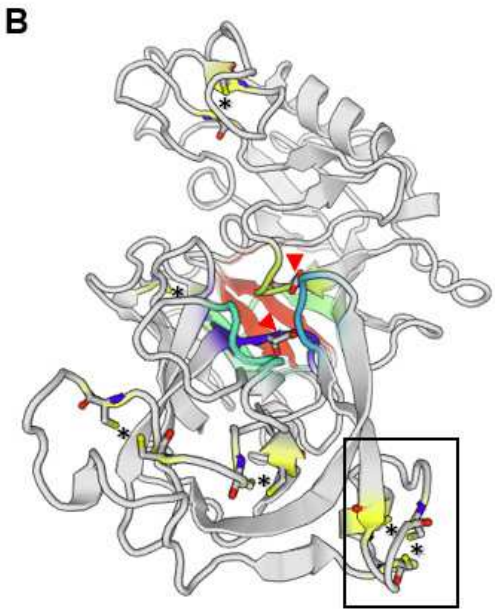
Comparison of the aspartic protease structures

(A) porcine pepsin (P00791), (B) Nepenthesin I (Q766C3) and (C) Nepenthesin II (Q766C2) of Nepenthes gracilis. Active site (colour-shaded) is shown with conserved catalytic Asp residues (arrowheads). Disulfide bonds are marked with asterisks. Box showing the conserved nepenthesin-type aspartic protein (NAP)-specific region with four conserved cysteine residues. Models generated in SWISS-MODEL.

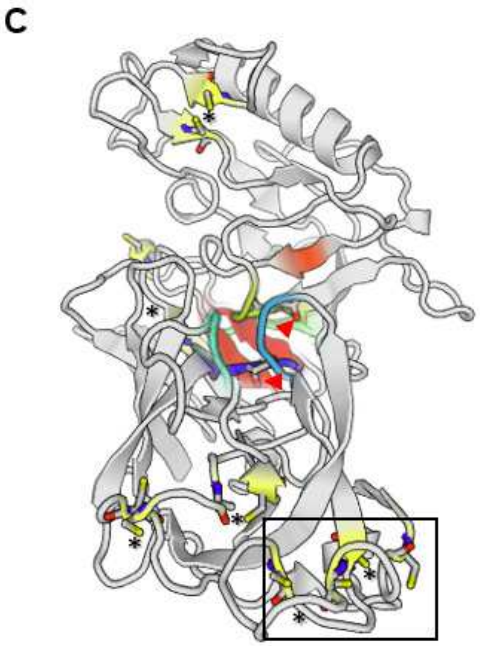
PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26940v1 | CC BY 4.0 Open Access | rec: 17 May 2018, publ: 17 May 2018



Porcine pepsin



Nepenthesin I



Nepenthesin II