Digestive enzyme discovery in carnivorous plants

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Background. Carnivorous plants have fascinated 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 the latest literature in the study of digestive enzymes in 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. Digestive enzymes from carnivorous plants have been the focus of studies for half a decade since the discovery of nepenthesin. 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 alongside the role of secreted plant proteases and their potential industrial applications.

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Abstract

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Keywords: Carnivorous plants; digestive enzyme discovery; industrial application; protein characterisation; secreted protease

Introduction

Nitrogen is the most crucial mineral nutrient required by plants but its availability is largely limited in many terrestrial ecosystems (Behie & Bidochka, 2013). In adaptation to such unfavourable environment, carnivorous plants have developed the ability to attract, capture and digest preys into simpler mineral compounds which are absorbed for plant growth and reproduction (Ellison, 2006). The very 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 physiological and anatomical traits linked to carnivory (Krol et al., 2012).

There have been a few reviews on the evolution of carnivorous plants and their biotechnological applications (Król et al., 2012; Miguel, Hehn & Bourgaud, 2018) but a systematic review with focus on the 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 not only be interest 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, the interest on carnivorous plants has centred on their axenic culture, ultrastructure of the specialised trapping organs, absorption of nutrients derived from preys through foliar and the enzymology involved in the prey digestion (Adamec, 1997; Gorb et al., 2004; Farnsworth & Ellison, 2008). Thus, this review summarises the previous findings with focus on the 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 anatomy and physiology. 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 does not have any movement mechanics. Independent evolution in several families of carnivorous plants resulted in five distinct trapping mechanisms(Król et al., 2012), including flypaper trap, snap trap, pitfall trap, suction trap, and eel trap (Table 1).

Drosera with flypaper trapping mechanism is commonly known as sundews, which belongs to the family of Droseraceae. Earlier studies have reported the application of sundew plant 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 been functioning as antispasmodic, diuretic and expectorant agent. Additionally, *in vitro* culture extracts of *Drosera* have reported to contain antibacterial and anticancer properties (Banasiuk, Kawiak & Krölicka, 2012). Interestingly, a crystal-like pigment from *D. peltata* has been used as a dye in silk industry (Patel, 2014).

Apart from this, 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. The spectacular trait of *Dionaea* is that it has the fastest trapping signal in the plant kingdom was reported in details over 140 years ago (Darwin, 1875). The secretion of digestive fluid is highly induced by touch on the sticky surface of the trap. Naphthoquinones have been discovered from *in vitro* culture extract of Venus flytrap which has been used as 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. The digestive zone which located in the lowest part of the pitcher contains abundant digestive glands responsible for the secretion of hydrolytic enzymes. Some of the pitchers of *Nepenthes* and *Sarracenia* are so big that larger prey, such as frog and rodent are frequently found partially digested inside the pitcher. This phenomenon caused the plants to be classified as carnivorous instead of insectivorous.

For *Sarracenia*, the pitcher acts as a rainwater storage and at the same time contains plant secretions, such as hydrolytic enzymes and other proteins for prey digestion. The secretions formed at the hood of the pitcher draw the attention of prey, which eventually fall and drown in the pitcher fluid (Ellison & Gotelli, 2003). 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 traditional remedy for childbirth and as diuretic agent (Patel, 2014). Tea made from dried foliage can be used to treat fever and cold. Besides, the roots are consumed as a remedy for lung, liver and smallpox diseases.

Nepenthes Tropical pitcher plants are from one of the most 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 which will be discussed later. 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 an eye drops to treat itchy eyes. 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, beetles and some even kill small animals such as frog and rats.

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

Digestive Enzyme Discovery, Identification and Characterisation

Digestions of prey by carnivorous plants are determined in part by suites of enzymes that are associated with morphologically and anatomically 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 some digestive enzymes which are secreted in a closed pitcher. This indicates plant regulation of enzyme secretion as the production and secretion of enzymes incur energetic costs.

To date, there are numerous studies reported the discovery of distinct digestive enzymes in carnivorous plants (Table 2). Different carnivorous families shared similar class of enzymes which display various enzymatic properties (Takahashi et al., 2009; Adlassnig, Peroutka & Lendl, 2011). Due to the genome sequencing of *Cephalotus follicularis*, various digestive enzymes have been discovered, namely esterases, proteases, nucleases, phosphatases, glucanases and peroxidases (Takahashi et al., 2009; Adlassnig, Peroutka & Lendl, 2011; Fukushima et al., 2017). Similar classes of enzymes were also detected in other carnivorous families such as Droseraceae (Scala et al., 1969; Amagase, 1972; Matušíková et al., 2005; Morohoshi et al., 2011; Schulze et al., 2012; Michalko et al., 2013; Pavlovic et al., 2014; Butts et al., 2016; Krausko et al., 2017), Lentibulariacea (Sirova, Adamec & Vrba, 2003; Płachno et al., 2006), Sarraceniaceae (Jaffe et al., 1992; Porembski & Barthlott, 2006; Srivastava et al., 2011; Adlassnig, Peroutka &

Lendl, 2011; Morohoshi et al., 2011; Luciano & Newell, 2017), and Nepenthaceae (Higashi et al., 1993; Athauda et al., 2004; Stephenson & Jamie, 2006; Eilenberg et al., 2006; Kadek et al., 2014b; Lee et al., 2016; Rey et al., 2016; Rottloff et al., 2016; Schrader et al., 2017). This indicates the significant role of the common hydrolytic enzymes in prey digestion of various carnivorous plants regardless of distinct trapping mechanisms. However, chitinases and lipases reported in Nepenthaceae and Sarraceniaceae have not been reported for Cephalotaceae and Lentibulariacea. 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 identification of all the proteins found in the pitcher fluid is highly challenged by the unusual amino acid composition of the proteins and limited source of carnivorous plant genomic/protein database (Lee et al., 2016). Lately, the transcriptome sequences for *N. ampullaria* and *N.* x *ventrata* were reported (Wan Zakaria et al., 2016a; Wan Zakaria et al., 2016b), which can serve as reference for the identification of novel digestive enzymes in *Nepenthes*. A combination of proteomics and transcriptomics approach have been used by Schulze et al., (2012) to determine the proteins highly expressed in the digestive fluid of Venus flytrap. They found that there was a synchronised act directed towards the prey with the help of various enzymes such as chitinases, lipases, phosphatases, peroxidases, glucanases and peptidases. Furthermore, Rey et al., (2016) applied a similar approach to address the proteolytic efficiency of the protein secreted in the pitcher fluid of *Nepenthes* spp. In the past, Amagase (1972) utilised zymography technique to determine the protease activity found in fluid of *Nepenthes* spp. and *D. peltata.* He purified and characterised the acid protease and demonstrated how the enzyme from two distinct families resemble to each other. Besides, Hatano & Hamada, (2008) also conducted proteomic analysis on the digestive fluid from *N. alata* where the proteins secreted (chitinase, glucanase and xylosidase) were detected using in-gel trypsin digestion, followed by *de novo* peptide assembly and matched with homology in public databases. According to Buch et al., (2015), fluorescent resonance energy transfer (FRET) based technique was utilised as an efficient and rapid detection of proteolytic activities in the pitcher fluid of various *Nepenthes* spp.

On the other hand, purification of the digestive enzymes from carnivorous fluid is extremely challenging due to low quantity of the fluid and very poor concentration of the

enzymes secreted. Apart from that, pitcher fluids are often diluted by rainwater and even contaminated by prey. Nevertheless, there are also studies which manage to partially or fully purify and characterise few digestive enzymes form carnivorous plants. Based on the purification and characterisation studies reported (Table 3), protease is one of the most abundant enzyme found in the digestive fluid of carnivorous plant which has been purified and well characterised (Amagase, Nakayama & Tsugita, 1969; Jentsch, 1972; Tokes, Woon & Chambers, 1974; An, Fukusaki & Kobayashi, 2002; Athauda et al., 2004). All the secreted proteases that have been purified to date are originated from the genus *Nepenthes*. The very first purification of protease from *Nepenthes* spp. was performed by Steckelberg, Luttge & Weigl (1967) using Ecteola column chromatography and the optimum activity was detected at pH 2.2 and stable at 50^{o}C. To date, the common purification strategies applied by various studies are column chromatography, affinity chromatography, ultrafiltration and dialysis. Although there are numerous studies identified the digestive enzymes from carnivorous plants, only few studies have purified and characterised the enzymes. Therefore, more studies are needed to purify and characterise the reported enzymes.

Most of the secreted enzymes characterised to date exhibit high versatility towards various substrates. Besides, the activities of the same class of enzymes from different genus of carnivorous plants are not very distinct in terms of the optimum pH, temperature and substrate. For instance, most of the proteases that have been characterised from different families function optimally at acidic condition (Table 3). Interestingly, there are few proteases reported to function extremely well at high temperature ranging from 40-60^{o}C. Additionally, the secreted plant enzymes demonstrate high stability against various chemicals and denaturing agents compared to enzymes from other sources. This is because plants require extremely active digestive enzymes that allow digestion of prey for a longer time span under mild chemical condition (Butts, Bierma & Martin, 2016). Subtle variations in enzymatic characteristics of digestive enzymes from different carnivorous plants remain to be explored with future studies.

On the other hand, feeding or chitin induction facilitates the secretion of digestive enzymes in the fluid. Clancy & Coffey, (1977) have reported the maximal secretion of digestive enzyme specifically phosphatases and proteases in model plants *Venus flytrap* and *Drosera* within 3 to 4 days after feeding. Thus, the plant secretes the digestive enzyme to the maximum

level to digest the prey completely and absorb the nutrients for the growth. Apart from that, mechanical irritation also stimulates the increase in activity of phosphatases and phosphodiesterases in *Drosera* (Mcnally et al., 1988). Moreover, the quantity of enzymes secreted often associates to the size of the prey (Darwin, 1875; An, Fukusaki & Kobayashi, 2002). In other words, a signal transduction mechanism stimulates the expression of digestive enzymes in the plant. Consequently, the plant responds toward the prey and counterbalances the cost-benefit ratio efficiently (Chang & Gallie, 1997).

On the contrary, there been a continuous debate regarding the origin of the digestive enzyme found in the pitcher fluid. The main question of interest arise was the enzymes secreted by the plants or originated from the microbial community found in the digestive fluid. As a perfect clarification, there was a study found the genes responsible for the digestive enzyme which highly expressed in the lower part (digestive zone) of the pitcher trap. This study become one of the significant proves which showed there is a symbiotic interaction between the microorganism and plants in prey digestion (Koopman et al., 2010). Some plants even save the investment cost and energy by not secreting the enzymes meanwhile utilising the microorganism in the fluid to digest the prey. Looking from a different perspective, synthesis of digestive enzymes by carnivorous plants which already contain external microbial power source for digesting the prey seems to be an unnecessary cost for the plant. The mutualistic interaction between the microbial community in the digestive fluid and the plant will boost the process of digestion and nutrients absorption.

There are numerous results available on the properties of digestive fluid of carnivorous plants but still fragmented for a complete understanding. Therefore, further extensive biochemical and morphological studies on carnivorous plants will be needed to help in further understanding regulation of hydrolytic enzyme secretion.

Secreted Proteases in Different Families of Carnivorous Plants

The capability of carnivorous plants to trap and digest their prey using specialised trapping organs contains digestive fluid has been a matter of great interest for over a century. Carnivorous plants attain substantial amount of nitrogen from their prey. They accumulate acidic fluid

containing proteases in their trapping organs, signifying that the plant utilises prey protein as a nitrogen source. The earliest reports of digestive enzymes involved in carnivorous plants initiated by Sir Joseph Hooker's studies of protease activity in the pitcher fluid of *Nepenthes* plants. The occurrence of diverse protease classes linked with carnivorous traps and digestive fluid may directs the synergistic roles of the enzyme in prey digestion, which might be triggered by the differential expression patterns. Besides, the evolution of the trap mechanism of carnivorous plant with extreme condition and limited nutrients may results in synthesis of distinct proteins or enzymes with some novel traits in order to continue survive. For instance, the novel prolyl endopeptidase (Npr1 & Npr2) that been discovered from *Nepenthes* possibly due to the evolution of the plant. Thus, in future this would lead us towards discovery of various novel proteases with extremely unique properties from carnivorous plant which can replace the existing sources in the industries.

Aspartic protease (AP) is one of the most abundant enzymes found in the digestive fluid and well characterised in previous reports (An, Fukusaki & Kobayashi, 2002; Rottloff et al., 2016). Moreover, AP have been purified and characterised from sterile pitcher fluid of several *Nepenthes* spp. (Jentsch, 1972; Tokes, Woon & Chambers, 1974). These studies strongly provide evidence that APs are secreted into pitcher fluid. However, less information on the sequence and expression of AP genes from *Nepenthes* that has been presented. It is very crucial to gather information about the AP genes for a better understanding of the nitrogen-acquisition mechanism of *Nepenthes* plants. Besides, An, Fukusakhi & Kobayashi, (2002) have cloned homologous APs genes and examined their expression in *N. alata* as a model plant to detect the genes encoding for APs secreted in pitcher fluid. The protease secreted in the pitcher fluid resembles a pepsin-like characteristic where it digests proteins at acidic condition. Amagase, (1972) have investigated the similar properties of aspartic protease found in digestive fluid of *Nepenthes* species (*N. ampullaria, N. mixta, N. rafflesiana, N. maxima and N. dyeriana*) compared to the one from the leaf extract of *Drosera peltata*. Surprisingly, they discovered that both the purified proteases from *Nepenthes* and *Drosera* share the common characteristics. In a study conducted by (Nakayama & Amagase, 1968) the protease from the mixture of pitcher fluid of Nepenthes species mainly *N. mixta* and *N. maxima* was partially purified and characterised due to insufficient amount of digestive fluid. Lately, acid protease from *Nepenthes* and *Drosera* genus are partially purified and characterised by Takahashi, Tanji & Shibata, (2007); Tokes, Woon &

Chambers, (1974). Although they have been categorised as APs, (Rudenskaya et al., 1995) none of the enzymes secreted in carnivorous plants have been purified to the homogeneity, mainly due to the difficulty in obtaining sufficient amount of pitcher fluid. In a way to unravel this scenario, Athauda et al., (2004) for the first time have purified and characterised two APs namely Nep1 and Nep2 from pitcher fluid *N. distillatoria.* They also have found the amino acid sequences of the enzymes by cloning the cDNAs from pitcher tissue of *N. gracilis.* Besides, Rey et al., (2013) have stated that Nep is secreted by specific cells located at the bottom part of the pitchers which essentially used for the digestion of the prey trapped by the plant. So far they reported to be the only APs found in the pitcher fluid and can be enhanced from crude fluid.

Apart from aspartic proteases, there is also presence of cysteine protease in carnivorous plants. Lately, it also has been found that cysteine protease is the primary protease found in digestive fluid of Venus flytrap. Prey proteins found in the digestive fluid of Venus flytrap are degraded by cysteine endopeptidases in association with serine carboxypeptidases. This is highly distinct to the digestive fluid found in *Nepenthes* and *Drosera* which strongly rely on aspartic proteases (Athauda et al., 2004). However, there is also presence of both aspartic protease and cysteine protease in *N. ventricosa* as reported by (Stephenson & Jamie, 2006). Besides, Takahashi, Tanji & Shibata, (2007) have conducted comparative enzymatic characteristics studies of acid proteases from crude digestive fluid of various carnivorous plants such as *Nepenthes*, *Chepalotus*, *Drosera* (Sundew) and *Dionaea* (Venus flytrap) which have distinct trapping mechanisms. The study proved that there are significant variances between them which eventually reflecting the phylogenetic diversity of these carnivorous plants. Eventually, it might be caused by the presence of different class of proteases in the families.

Moreover, there are also few attempts on the recombinant production and expression of the enzymes from carnivorous plants (Morohoshi et al., 2011; Ishisaki et al., 2012; Kadek et al., 2014b) in order to enhance the protein yield. Initially, Kadek et al., (2014b) reported an efficient way to obtain huge amount of Nepenthesin I (Nep1) from *N. gracilis* through heterologous production in *Escherichia coli.* The characteristics of the recombinant protein obtained similar to the native protein isolated from the pitcher fluid. Later, the recombinantly produced Nep1 from *N. gracilis* was successfully purified and crystallised (Fejfarová et al., 2016). Apart from that, Schrader et al., (2017) also have discovered neprosin from the digestive fluid of carnivorous

pitcher plant and it was characterised to be proline cleaving enzyme. They also produced neprosin through recombinant approach and demonstrated that it has the potential to be utilised for whole proteomic profiling and histone mapping. This is supported by the facts that neprosin is a legitimate low molecular weight prolyl endopeptidase (PEPs) and extremely active at low concentration and low pH. Surprisingly, the combined actions of a novel prolyl endopeptidase and an aspartic protease from the pitcher fluid of *Nepenthes* species have demonstrated effective gluten detoxification potential. These discoveries broaden the prospects for treating celiac disease through enzymes supplementation approach (Rey et al., 2016)

Although the proteolytic activity found in the digestive fluid of pitcher plant became the interesting topic of study among researchers, the low yield of enzymes secreted by the plant made it more challenging. Nevertheless, there is no adequate studies have been conducted specifically on the enzymatic characteristic of the purified acid proteases. Hence, further researches are crucial in order to fill the gap of knowledge on the essence of acid proteases present in the digestive secretion of carnivorous plants. Additionally, as the prey digestion occurs in the raw digestive fluid, it is vital to characterise the secreted protease activity in overall as well as to purify and characterise the individual proteases.

Applications of proteases from carnivorous plants

Protease plays a major role in worldwide enzyme market with a long array of applications. They contribute an invincible role in industrial biotechnology, primarily in detergent, food and pharmaceutical arena (Rao et al., 1998; Lakshmi & Hemalatha, 2016). Microbes and animals are the major source of protease in current industries followed by few commercialised plant proteases. Interest has been growing in plant proteases which have significant commercial values due to their high stability in extreme condition (Canay, Erguven & Yulug, 1991; Houde, Kademi & Leblanc, 2004; Malone et al., 2005; Karnchanatat et al., 2011; Amri & Mamboya, 2012; Rey et al., 2016; Mazorra-Manzano, Ramírez-Suarez & Yada, 2017). The plant sources would be the possible alternatives for microbial and animal proteases (Chanalia et al., 2011; Akhtaruzzaman et al., 2012; Zhou et al., 2012; Gurung et al., 2013; Khan & Sathya, 2017)

Proteases are one of the largest groups of hydrolytic enzymes that cleave the peptide bonds in the polypeptide chains. Exopeptidase and endopeptidase are the two major groups of proteases which are classified based on the ability to cleave the N or C terminal peptide bond. The metabolic activity of almost all the organisms includes plants, animals, fungi, bacteria and viruses are influenced by the proteolytic enzymes. Proteases become an ideal topic of research in enzyme technology field due to their massive structural importance and wide contribution in research and economical activities. Moreover, proteases are the dominant class industrial enzymes that are used in diverse industrial applications such as pharmaceutical, leather products, detergents, meat tenderizers, food products and also in waste processing industry (Table 4). The four major classes of proteases enzyme that widely used in industries are aspartic proteases, serine proteases, cysteine proteases and metalloproteases. Merely, 60% of the total worldwide production of the enzymes are covered by proteases (Usharani & Muthuraj, 2010)

The 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 enzyme from plant sources. There are extensive studies have been performed on aspartic proteinases that found in mammalian, microbial and viral cells. However, the ethical, spiritual reasons or even regulatory limitations which restrict the application of non-plant proteases (animal and recombinant sources) in certain countries offer great chances for the use of novel plant proteases. In addition, aspartic proteases also widely distributed in the seed, flowers, leaves of various plant species and as well as in the pitcher fluid of carnivorous plants. Several plant aspartic proteases such as those from rice, barley and cardosins have been purified and well characterized in previous studies. The most significant trademark of the aspartic proteases found in the digestive fluid of carnivorous plant is they are the only extracellular proteinase from plant origin. The rest known to be intracellular vacuolar enzyme. Athauda et al., (2004) have for the first time successfully purified and characterised plant aspartic proteases (Nep1 and Nep2) from the pitcher fluid of *N. distillatoria*.

Protein hydrogen/deuterium exchange coupled to mass spectrometry (HXMS) is one of the important analyses practised in biopharmaceutical industry which involves the enzymatic digestion of proteins to track the information about the new exchanged patterns in protein structure. Protease has the abilities to digest the protein into small peptides and overlapping

fragments and provide necessary coverage of protein sequences which are vital for focusing region of interest. Kadek et al., (2014a) successfully immobilised the Nep1 from pitcher fluid of genus *Nepenthes* and used as a tool for digestion in Hydrogen / Deuterium Exchange Mass Spectrometry (HXMS). This is because Nep1 exhibits wide substrate cleavage specificities and high stability towards denaturing reagents compared to pepsin. The hunt for the valuable protease with unique specificity is always a continuous challenge for diverse industrial application. In addition, the combined actions of a novel prolyl endopeptidase and an aspartic protease from the pitcher fluid of *Nepenthes* species have demonstrated effective gluten detoxification potential. These findings broaden the prospects for treating celiac disease through enzymes supplementation approach (Rey et al., 2016).

Carnivorous plants signify a crucial and gifted source of proteases for various biotechnological applications. The proteases discovered in the secretion of the trap are distinct and provide huge range of temperature, stability and pH activity profiles. Furthermore, the high substrate specificity among the proteases enhances their capability for multidisciplinary use. Although, the pre-existing plant proteases such as bromelain and papain have been extensively used in industries, yet they denote the small portion from the huge portion of plant proteases that have not been discovered. Thus, more studies towards discovering novel plant proteases are required.

Conclusions

The search for new industrially viable plant enzymes is a continuous effort in which carnivorous plants serve as great resources for exploration. Thus, successful purification and characterisation of the secreted enzymes will encourage their exploitation for industrial applications. Future research efforts are still needed in studying the regulatory mechanisms of the digestive enzymes or metabolites responsible for attracting prey. With the advent of omics technologies, more can be discovered to provide a holistic understanding on the molecular mechanisms of carnivory in various carnivorous plants. Comparative genomics approach will help in understanding the evolutionary history of these fascinating plants.

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Table 1(on next page)

Different carnivorous plant families grouped according to different trapping mechanisms.

1 **Table 1**. Different carnivorous plant families grouped according to different trapping mechanisms.

Trapping mechanism	Family	Genus	Reference
Flypaper	Byblidaceae	*Byblis*	Hatano et al., 2008
	Dioncophyllaceae	*Triphyophyllum*	Ellison et al., 2009
	Drosophyllaceae	*Drosophyllum*	
	Droseraceae	*Drosera*	
	Roridulaceae	*Roridula*	
	Lentibulariaceae	*Pinguicula*	
Snap	Droseraceae	*Aldrovanda*	Bauer et al., 2015
		Dionaea	
Pitfall	Cephalotaceae	*Cephalotus*	Krol et al., 2011
	Nepenthaceae	*Nepenthes*	
	Sarraceniaceae	*Darlingtonia*	
		Heliamphora	
		Sarracenia	
Suction/ Bladder	Lentibulariaceae	*Utricularia*	Bauer et al., 2015
Eel/ Lobster-pot	Lentibulariacea	*Genlisea*	Adamec et al., 2007
	Sarraceniaceae	*Sarracenia*	

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Table 2(on next page)

Digestive enzyme discovery from different carnivorous plant families.

1 **Table 2**. Digestive enzyme discovery from different carnivorous plant families.

Family	Species	Enzyme	Reference
Lentibulariacea	*G. aurea*	Phosphatase	Plachno et al 2006
Droseraceae	*D. capensis*	Protease, Phosphatase	Pavlovic et al.,2013; Butts et al., 2016
	D. muscipula	Chitinase, Nuclease, Protease	Schulze et al., 2012; Palovic et al., 2017
	D. rotundifolia	Chitinase, Glucanase, Protease	Matusikova et al., 2005; Michalko et al., 2012; Martin et al., 2016
	D. villosa	Lipase	Morohoshi et al., 2010
Cephalotaceae	*C. follicularis*	Esterase, Glucanase, Nuclease, Peroxidase, Phosphatase, Protease	Barthlott et al., 2004; Takahashi et al., 2009, Adlassnig et al.,2010, Fukushima et al., 2017
Sarraceniaceae	*D. californica*	Protease	Adlassnig et al.,2010
	H. tatei	Protease	Jaffe et al 1992
	Sarracenia spp.	Amylase, Esterase	Barthlott et al., 2007
	S. psittacina	Nuclease	Srivastava et al., 2011
	S. purpurea	Lipase, Protease, Phosphatase	Adlassnig et al.,2010; Morohoshi et al., 2011; Newell et al., 2017
Nepenthaceae	*N. alata*	Chitinase, Esterase, Glucanase, Peroxidase, Phosphatase, Protease	Hatano & Hamada., 2008; Thornhill et al., 2008; Morohoshi et al., 2011; Buch et al., 2015; Rottloff et al., 2016
	N. albomarginata	Chitinase, Glucanase	Rottloff et al., 2016
	N. bicalcarata	Glucanase, Peroxidase, Protease	Rottloff et al., 2016
	N. distillatoria	Protease	Athauda et al.,2004
	N. gracilis	Protease	Kadek et al., 2014
	N. hemsleyana	Urease	Yilamujiang et al., 2017
	N. hybrida	Esterase, Nuclease	Higashi et al., 1993; Morohoshi et al., 2011
	N. khasiana	Chitinase	Eilenberg et al., 2006
	N. macfarlanei	Lipase	Hatano & Hamada., 2008
	N. mirabilis	Chitinase, Glucanase, Peroxidase, Protease	Buch et al., 2015; Rottloff et al., 2016
	N. sanguinea		
	N. tobaica	Phosphatase	Thornhill et al., 2008
	N. ventricosa	Protease	Stephenson et al., 2006
	N. ventrata	Chitinase, Glucanase, Nuclease, Peroxidase, Phosphatase, Protease	Lee et al., 2016; Schra et al., 2017

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Table 3(on next page)

Enzyme activity characterisation of secreted proteins from carnivorous plants.

1 **Table 3**. Enzyme activity characterisation of secreted proteins from carnivorous plants.

Enzyme	Species	Purification method/ Column	Substrate	Condition		Reference
				pH	T (°C)	
Protease	*N. distillatoria*	Mono Q column (FPLC), Pepstatin-Sepaharose	Casein	2.9	40	Jentsch, 1972; Athauda et al., 2004
	N. alata	Sephacryl S-200, DEAE cellulose column	Bovine serum albumin	3.0	37	Fukusaki et al., 2002
		Dialysis, Pressure ultrafiltration	Acid denatured haemoglobin	3.0	37	
	N. mirabilis	His Trap HP column, Dialysis		2.2	37	Athauda et al., 2004
	N. macfarlanei	Sephadex G-75	Bovine serum albumin	NA	37	Tokes et al., 1974
	N. mixta, *N. dormanniana, N.neuvilleana*	Ecteola cellulose column chromatography	Casein	2.2	50	Steckelberg et al., 1967
	Nepenthes sp.	DEAE-Sephadex A-50	Casein	NA	60	Nakayama & Amagase, 1968
	N. alata, C. follicularis,	Not purified	Haemoglobin	2.5	47-57	Takahashi et al., 2007
	D. muscipula		Haemoglobin	3.0	60	
	D. capensis		Oxidised insulin B chain	3.5	47	
	N. gracilis	Dialysis, Pressure ultrafiltration	Haemoglobin	2.5	37	Kadek et al., 2014
Chitinase	*N. khasiana*	Not purified	*N*-acetylglucosamine (GlcNAc)	3.0	37	Eilenberg et al., 2006
			glycol-chitin	8.3	37	
	N. alata	TALON metal affinity resin	2-acetamido- 2-deoxy-D-glucose)	5.5	37	Ishisaki et al., 2011
			Ethylene glycol chitin			
Lipase	*N. macfarlanei*	Not purified	glycerol trioleate	6.0	37	Tokes et al., 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	*Utricularia foliosa* *Utricularia australis*	Not purified	4-methylumbelliferyl (MUF) phosphate	5.5	NA	Sirova et al., 2013
	Genlisea lobata, U. multifida *D. muscipula,C. follicularis* *D. binata, N. tobaica*		ELF 97 phosphatase substrate	NA		Plachno et a., 2006

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Table 4(on next page)

Applications of proteases from different sources.

1 **Table 4**. Applications of proteases from different sources.

Source	Protease	Application	Reference
Plant	Neprosin	Proteomic analysis / Histone mapping	Rey et al., 2016
		Gluten digestion	Linda et al., 2017
	Papain	Meat tenderizer	Amri et al., 2012
		Denture cleaners	Ogunbiyi et al., 1986
		Detergent	Chaudhuri et al., 2017
		Cosmetics industry	
		Healing burn wound	
		Textiles	
	Bromelain	Anti-inflammatory agent	Chanalia et al., 2011
		Anti-cancerous agent	
	Ficin	Pharmaceutical industry	Mazorra et al., 2017
	Actinidin	Dietary supplement	Malone et al., 2010
	Caricain	Gluten-free food processing	Buddrick et al., 2015
	Zingipain	Anti-proliferative agent	Karnchanatat et al., 2011
Animal	Chymotrypsin	Food industry, Leather industry	Zhou et al., 2012
	Pepsin	Cheese making in dairy industry	Chaudhuri et al., 2017
		Dehairing in leather industry	Gurung et al., 2013
	Rennin	Cheese making in dairy industry	Khan et al., 2017
	Trypsin	Dehairing and bating in leather industry	Chaudhuri et al., 2017
Microbial	Carboxypeptidase	Debittering of protein hydrolysates	Mala et al., 1998
	Aminopeptidase		
	Collagenase / subtilisin	Treatment of burns and wounds	Chanalia et al., 2011
	Serine protease	Laundry detergent	Mala et al., 1998
	Alkaline protease	Recovery of silver from waste X-ray	Lakshmi et al., 2016
		Replace trypsin in animal cell cultures	
		Laundry detergent	Mala et al., 1998
	Thermolysin	Synthesis of aspartame	
	Matrix metalloprotease	Therapeutic agent for cancer and arthritis	

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