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ORIGINAL ARTICLE

Regulation of enzyme activities in carnivorous pitcher plants of the genus *Nepenthes*

Michaela Saganová[1] · Boris Bokor[1,2] · Tibor Stolárik[3,4] · Andrej Pavlovič[4]

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Abstract

Main conclusion ***Nepenthes*** **regulates enzyme activities by sensing stimuli from the insect prey. Protein is the best inductor mimicking the presence of an insect prey.**

Carnivorous plants of the genus *Nepenthes* have evolved passive pitcher traps for prey capture. In this study, we investigated the ability of chemical signals from a prey (chitin, protein, and ammonium) to induce transcription and synthesis of digestive enzymes in *Nepenthes* × *Mixta*. We used real-time PCR and specific antibodies generated against the aspartic proteases nepenthesins, and type III and type IV chitinases to investigate the induction of digestive enzyme synthesis in response to different chemical stimuli from the prey. Transcription of nepenthesins was strongly induced by ammonium, protein and live prey; chitin induced transcription only very slightly. This is in accordance with the amount of released enzyme and proteolytic activity in the digestive fluid. Although transcription of type III chitinase was induced by all investigated stimuli, a significant accumulation of the enzyme in the digestive fluid was found mainly after protein and live prey addition. Protein and live prey were also the best inducers for accumulation of type IV chitinase in the digestive fluid. Although ammonium strongly induced transcription of all investigated genes probably through membrane depolarization, strong acidification of the digestive fluid affected stability and abundance of both chitinases in the digestive fluid. The study showed that the proteins are universal inductors of enzyme activities in carnivorous pitcher plants best mimicking the presence of insect prey. This is not surprising, because proteins are a much valuable source of nitrogen, superior to chitin. Extensive vesicular activity was observed in prey-activated glands.

Keywords Carnivorous plant · Chitin · Chitinase · Enzyme · Nepenthesin · Pitcher plant · Protease

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✉ Andrej Pavlovič
andrej.pavlovic@upol.cz

[1] Department of Plant Physiology, Faculty of Natural Sciences, Comenius University in Bratislava, Ilkovičova 6, Mlynská dolina B2, 842 15 Bratislava, Slovakia

[2] Comenius University Science Park, Comenius University in Bratislava, Ilkovičova 8, 841 04 Bratislava, Slovakia

[3] Department of Plant Physiology, Plant Science and Biodiversity Centre, Slovak Academy of Sciences, Dúbravská cesta 9, 845 23 Bratislava, Slovakia

[4] Department of Biophysics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University Olomouc, Šlechtitelů 27, 783 71 Olomouc, Czech Republic

Abbreviation

AP Aspartic protease

Introduction

Carnivorous plants of the genus *Nepenthes* grow in nutrient poor habitat and have evolved modified leaves called pitchers to capture insect prey. Inspired by Charles Darwin, Hooker (1874) was the first to document that pitcher plants of *Nepenthes* are carnivorous. He established the digestive activity of the fluid, stating that egg-white and meat showed unmistakable evidence of disintegration within 24 h. How *Nepenthes* accomplishes this process has been the object of studies for 125 years (for review, see Frazier 2000). Some researchers believed that the digestion was accomplished by bacteria; others believed that pitcher plants secrete their own enzymes. Today, we know that both probably contribute

to prey degradation (Takeuchi et al. 2011). Athauda et al. (2004) were the first to purify to homogeneity two acid proteinases (nepenthesins I and II) from the pitcher fluid of *Nepenthes distillatoria,* and investigated their enzymatic and structural characteristics, confirming endogenous origin of the enzymes. They found that nepenthesins are unique members of aspartic proteases (APs) with low amino acid sequence identity with ordinary vacuolar APs and form a novel subfamily of APs with a high content of cysteine residues and a characteristic insertion, named 'the nepenthesin-type AP-specific insertion'. Since that time, many other digestive enzymes have been identified from the *Nepenthes* digestive fluid. Three other nepenthesin homologues designed nepenthesin III, IV, and V were recently described by Lee et al. (2016). They have also identified a novel class of prolyl-endoprotease called neprosin in the fluid of *N. rafflesiana* (Lee et al. 2016; Rey et al. 2016). Four putative serine carboxypeptidases were described by Rottloff et al. (2016). Although proteins are the dominant source of nitrogen for carnivorous plants, the pitcher plants possess enzymes which are also able to digest another important source of nitrogen in insects—chitin. Three classes of chitinases have been isolated and characterized in *Nepenthes*. Eilenberg et al. (2006) isolated and characterized type I chitinase in *N. khasiana* (see also Renner and Specht 2012), type III and IV were described in different *Nepenthes* species (Rottloff et al. 2011, 2016; Ishisaki et al. 2012a, b; Lee et al. 2016). Phosphatases, peroxidases, galactosidases, glucanases, and nucleases complement the list of endogenous enzymes produced in carnivorous *Nepenthes* plants (Hatano and Hamada 2008, 2012; Lee et al. 2016; Rottloff et al. 2016).

Although the composition of digestive fluid in *Nepenthes* is now well recognized, the regulation of enzyme activity is still poorly understood. The carnivorous plants with active trapping mechanisms (i.e., with moving traps, e.g., Venus flytrap and sundew) rely on mechanical stimulation from insect prey which induce electrical signals as alert for the presence of captured prey, and action potentials induce the synthesis of digestive enzymes (Bemm et al. 2016; Böhm et al. 2016; Krausko et al. 2017; Pavlovič et al. 2017). Later, after the prey struggle ceases, chemical cues from the entrapped prey keep the digestive process running for several days (Libiaková et al. 2014; Bemm et al. 2016; Krausko et al. 2017). No action potentials have been detected in pitcher plants so far, and the unrelated carnivorous pitcher plant *Sarracenia purpurea* showed no induction of enzyme secretion in response to mechanical stimulation (Gallie and Chang 1997). This indicates that pitcher plants have only to rely on chemical cues from entrapped prey.

Among many chemical substances found in insect prey, the compound found in the insect exoskeleton, the chitin, has gained increased attention. It is known that addition of colloidal chitin (a chemically modified water-soluble form of chitin, not very typical for the insect exoskeleton) to the pitchers increased transcription of genes encoding type I and III chitinases and nepenthesin I in *N. alata* and *N. khasiana* (Eilenberg et al. 2006; Yilamujiang et al. 2016). However, Yilamujiang et al. (2016) suggested that chitin is only one of probably more insect-derived signaling compounds that are involved in the induction of the digestive process, because fruit flies induced a stronger and more sustained transcription of genes encoding digestive enzymes. In the present study, we focused on these chemical stimuli. We applied live prey and their component separately: proteins (in the form of bovine serum albumin, BSA) and their breakdown products ammonium (in the form of NH_4Cl) and chitin (unmodified) into *Nepenthes* × *Mixta* pitchers. We monitored the pitcher plant reactions of gene expression by quantitative real-time PCR, through protein abundance using specific antibodies to their enzyme activities in the digestive fluid, correlated with changes of the pH in response to different stimuli. We were interested in finding which of the components separately applied was the best in mimicking the insect prey.

Materials and methods

Plant material and experimental setup

We used *Nepenthes* × *Mixta* (horticultural hybrid between *N. northiana* and *N. maxima*) which produces sufficient numbers of big pitchers with high volume of digestive fluid. The plants were cultivated in growth chambers with a photoperiod 14 h light/10 h dark, day/night temperature of 23–25/19–21 °C, and humidity of 50–70%, and produce up to 25 cm high pitchers. Other *Nepenthes* species used in this study were from different geographical region: *N. alata* (Philippines), *N. ampullaria* (throughout in Southeast Asia), *N. bicalcarata* (Borneo), *N. eymae* (Sulawesi), *N. mirabilis* (throughout in Southeast Asia), *N. spathulata* (Sumatra), *N. truncata* (Philippines), and *N. ventricosa* (Philippines). All were grown under greenhouse conditions of the Comenius University in Bratislava.

To prevent entry of prey and microbes (semi-sterile conditions) into pitchers, freshly opened pitchers were immediately plugged, without damaging them, with wads of cotton wool. After few days, 1 mL of digestive fluid was collected before addition of 350 mg of meal worms (*Tenebrio molitor*), 150 mg of protein (bovine serum albumin, BSA, Sigma-Aldrich, St. Louis, MO, USA), 0.5 M NH_4Cl to a final 50 mM concentration, and 150 mg of chitin from shrimp shells (95% deacetylated, Sigma-Aldrich) into different pitchers. Then, 1 mL of digestive fluid was collected at different time points (after 3, 6, and 9 days) from the pitchers. The samples were frozen and stored at − 18 °C.

The removed volume of digestive fluid was replaced with 1 mL of distilled water.

Based on the results from these experiments, we decided to collect the digestive fluid on the 6th day after addition of meal worms from different *Nepenthes* species, when nepenthesin activity was sufficiently high to measure pH dependence and presence of nepenthesins from different species.

Real-time polymerase chain reaction (qPCR)

To study induction of gene expression in the digestive zone of the pitchers, four corresponding genes of well-characterized proteins from the pitcher fluid were chosen, namely nepenthesin I and II (Athauda et al. 2004), chitinase III (Rottloff et al. 2011; Hatano and Hamada 2012; Ishisaki et al. 2012a), and chitinase IV (Hatano and Hamada 2008, 2012; Ishisaki et al. 2012b). Because a clear upregulation of digestive enzymes in the digestive fluid was evident on the third day after feeding, we harvested 100 mg of tissue from the digestive zone for qPCR earlier: 18, 36, and 72 h after application of BSA, chitin, NH_4Cl, and meal worms. The digestive zones from control pitchers which have not obtained any of the studied compounds were also harvested. Samples were stored in − 80 °C before gene expression analyses. Total RNA was extracted and DNase I treated using Spectrum Plant Total RNA kit (Sigma-Aldrich) according to the manufacturer′s instructions. The integrity of RNA was checked by agarose (1%) gel electrophoresis. The concentration and sample purity were measured by NanoDrop™ 1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). The synthesis of first strand of cDNA was performed by ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) using Oligo(dT)$_{15}$ primers and after that, cDNA was purified by DNA Clean & Concentrator™-5 (ZymoResearch, Irvine, CA, USA) using the manufacturer's protocol. The primers (Table 1) for *nepenthesin I* and *II*, *chitinase class III* and *IV,* and reference genes *actin* and *18S rRNA* were designated by Primer3plus tool (http://primer3plus.com/web_3.0.0/primer3web_input.htm) from known sequences of *Nepenthes* species. Gradient PCR was used to determine annealing temperature (T_a) of primers (Table 1). Each amplified product was checked by agarose (2%) gel electrophoresis and subsequently sequenced by the Sanger method to verify product specificity at Department of Molecular Biology, Faculty of Natural Sciences, Comenius University in Bratislava. The stability of reference genes was evaluated by $2^{-\Delta C'_T}$ method (Livak and Schmittgen 2001) and BestKeeper tool (http://www.gene-quantification.info/) and only *actin* gene was suitable for gene expression analysis (data not shown). For real-time PCR, specific gene sequences were amplified by Maxima SYBR Green/ROX qPCR Master Mix (ThermoFisher Scientific). Real-time PCR reactions were performed in 96-well plates on Light Cycler II 480 (Roche, Basel, Switzerland) device and the relative changes in gene expression were estimated according to Pfaffl (2001). All samples for PCR experiments were analysed in three biological, each in three technical replicates.

Enzyme activity measurements

Before enzyme activity measurements, the pH was measured in each collected sample by digital pH meter (Hanna Instruments, Woonsocket, RI, USA).

Proteolytic activity of pitcher fluid was determined by incubating 150 µL of a sample with 150 µL of 2% (w/v) bovine serum albumin (BSA) in 200 mM glycine-HCl (pH 3.0) at 37 °C for 1 h. The reaction was stopped by the addition of 450 µL of 5% (w/v) trichloroacetic acid. Samples were incubated on ice for 10 min, and centrifuged at 20,000*g* for 10 min at 4 °C. Absorbance of the supernatant at 280 nm was measured by a spectrophotometer Jenway 6705 UV/Vis

Table 1 Primer sequences and properties

Primer	Product size (bp)	Primer sequence (5′- 3′ direction)	T_a (°C)
ACTIN	100	Forward: CTCTTAACCCCAAAGCAAACAGG Reverse: GTGAGAGAACAGCCTGGATG	59
18S rRNA	100	Forward: CTTGATTCTATGGGTGGTGGTG Reverse: GTTAGCAGGCTGAGGTCTC	59
Nepenthesin I (*NepI*)	193	Forward: CCAACTCTGTCAAGCCCTTC Reverse: CCGAATGTGATATTAGGGATGG	59
Nepenthesin II (*NepII*)	210	Forward: TTCCTTGCGAGAGCCAGTAT Reverse: CCGAATCCCTGGTTGTCTT	59
Chitinase, class III (*ChitIII*)	213	Forward: GCTCCGGCATAGCAGTCTAC Reverse: CTTGGTTTTGGCATGAGGTT	60
Chitinase, class IV (*ChitIV*)	219	Forward: ATGTCACGCATGAGACTGGA Reverse: CCACCGTTTGAGGTGAGTTT	59

T_a annealing temperature

(Bibby Scientific Ltd, Essex, UK). For inhibitory studies, prior to incubation, 3 µL of 150 µM pepstatin were added to 150 µL of digestive fluid and incubated and measured as described above. For determination of pH effects on the protease activity of the pitcher fluid, the standard activity assay was modified using 2% (w/v) BSA as a substrate in different buffers. 200 mM glycine-HCl buffer and standard McIlvaine's citrate-phosphate buffer were used for pH 1.0 and pH 2.0–8.0 range, respectively.

To measure the activity of acid phosphatases, we used chromogenic substrate 4-nitrophenyl phosphate (Sigma-Aldrich). The substrate was prepared in 50 mM (pH 5.0) acetate buffer, and the concentration was 5 mM. 75 µL of collected fluid was added to 475 µL of 50 mM acetate buffer (pH 5.0), and mixed with 400 µL of the substrate. For control, 400 µL of the substrate solution was mixed with 550 µL of the acetate buffer. Mixed samples were incubated at 25 °C for 20 min, and then, 160 µL of 1.0 N NaOH were added to terminate the reaction. Absorbance was measured at 405 nm with a spectrophotometer Jenway 6705 UV/Vis (Bibby Scientific Ltd).

Endochitinase activity was measured using chitinase assay kit with 4-nitrophenyl β-D-*N*,*N*′,*N*″-triacetylchitotriose as a substrate (Sigma-Aldrich). 15 µL of collected fluid was added to 135 µL of substrate at a concentration of 0.2 mg mL^{-1}. Mixed samples were incubated at 37 °C for 5 h, and then, 400 µL of stop solution (sodium carbonate) was added to terminate the reaction. For control, 150 µL of the substrate solution was also incubated. Absorbance was measured at 405 nm with a spectrophotometer Jenway 6705 UV/Vis (Bibby Scientific Ltd).

SDS-PAGE and western blots

For detection and quantification of AP (nepenthesins) and type III and IV chitinase, polyclonal antibodies against these proteins were raised in rabbits by Genscript (Piscataway, NJ, USA). For detection of AP nepenthesin, the following amino acid sequences (epitopes) was synthesized: (NH_2–) SAIMDTGSDLIWTQC (–$CONH_2$). For detection of type III and IV chitinases, the following amino acid sequences (epitopes) were synthesized: (NH_2–) CWSKYYDNGYS-SAIKD (–$CONH_2$) and (NH_2–) CNGGNPSAVDDRVGYY (–$CONH_2$), respectively. They were coupled to a carrier protein (keyhole limpet haemocyanin, KLH) and each injected into two rabbits. The terminal cysteine of the peptide was used for conjugation. The rabbit serum was analysed for the presence of antigen-specific antibodies using an ELISA test.

The digestive fluid collected for the enzyme assays was subjected to Western blotting. The samples were heated and denatured for 10 min at 70 °C, and mixed with modified Laemmli sample buffer to a final concentration of 50 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, and 0.02% bromophenol blue. The same volume of digestive fluid was electrophoresed in 15% (v/v) SDS-polyacrylamide gel. The proteins in gels were transferred from the gel to a nitrocellulose membrane (Bio-Rad, München, Germany) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). After blocking in TBS-T containing 5% BSA overnight, the membranes were incubated with the primary antibody for 1 h at room temperature. After washing, the membranes were incubated with the secondary antibody; the goat antirabbit IgG (H+L)–horseradish peroxidase conjugate (Bio-Rad). Blots were visualized using Immobilon Western chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) and medical X-ray film (FOMA BOHEMIA, Hradec Králové, Czech Republic).

Extracellular recording of membrane potentials

The extracellular electrical potential was recorded inside a Faraday cage using a non-invasive device according to Ilík et al. (2010) and Libiaková et al. (2014) under the standard laboratory conditions (room temperature of 23 ± 1 °C and relative air humidity of 50 ± 5%). The electrical signals were measured on several cells of digestive glands with non-polarizable Ag–AgCl surface electrodes (Scanlab Systems, Praha, Czech Republic). A small opening on the opposite wall of the pitcher was cut to get access to the digestive zone. A stimulus was applied in the form of 50 µL of 50 mM NH_4Cl solution or BSA (3 mg mL^{-1}). The reference electrode was submerged in a dish filled with 1–2 cm of water beneath the pot. The electrodes were connected to two channels of an amplifier that had been made in house (gain: 1–1000, noise: 2–3 mV, bandwidth [− 3 dB]: 10^5 Hz, response time: 10 µs, and input impedance: 10^{12} Ω). The signals from the amplifier were transferred to an analog–digital PC data converter (12-bit converter, ± 10 V, PCA-7228AL supplied by TEDIA, Plzeň, Czech Republic), and the data were collected every 6 ms. The sensitivity of the device was 13 µV. Moistened electrodes were equilibrated on the leaves for approximately 0.5 h before measurement. At least five measurements were performed.

Transmission electron microscopy (TEM)

Control and traps fed with meal worms were collected 3 days after feeding. The digestive zone of the trap was cut into 1.5 mm-long sections by a razor and immediately fixed in 5% (v/v) glutaraldehyde and postfixed in 1% osmium tetroxide at room temperature. Fixed samples were gradually dehydrated in ethanol series and the dehydratation was finished by pure propylene oxide. The samples were embedded in Spurr Low-Viscosity Embedding Kit epoxide resin (Sigma-Aldrich). Semithin sections were stained by 0.5% (w/v) toluidine blue

and examined by light-microscopy. Ultrathin sections were contrasted by 2% (w/v) uranyl acetate and 2% (w/v) lead (II) citrate, and observed by electron microscope (Jeol JEM 2010, Tokyo, Japan).

Statistical analyses

Data from enzyme activities were evaluated by paired Student *t* test (Microsoft Excel). We compared day 0 (before treatment) with days 3, 6, and 9 after addition of different matters, because different pitchers had different enzyme activities at different period of year. Data from qPCR were evaluated by Student's *t* test (Microsoft Excel) by comparison to control pitcher sampled in the same time. For Western blots, representative results from three to four biological replicates are shown.

Results

Acidification of pitcher fluid

Prey capture resulted in a decrease in pH, and the lowest values within 9 days were reached in the range from pH 1.8 to 3.5 in different pitchers. Protein addition also decreased the pH to the lowest values in the range of 2.6–3.9. Addition of NH_4Cl strongly lowered the pH between 1.2 and 1.5. Addition of the same amount of NH_4Cl to the digestive fluid in a Falcon tube resulted in no change in pH, indicating the participation of pitcher tissue in acidification (data not shown). On the contrary, chitin lowered the pH only very slightly (lowest values reached in the range 4.1–5.1) in comparison to control plants (addition of water pH 5.5–7.2) (Fig. 1a).

Enzyme activity measurements

In control pitchers which after sample collection obtained the same volume of water, a small decrease of enzyme activities was observed probably caused by dilution of the digestive fluid. The live prey and BSA were the best inductors of proteolytic, phosphatase and endochitinase activities. Chitin slightly upregulated proteolytic activity on the third day, phosphatase and endochitinase activities were stimulated weakly but not significantly. The pitcher fluids, to which chitin had been added, got red probably caused by secretion of antifungal naphthoquinones as documented before by Eilenberg et al. (2010). Ammonium chloride stimulated only proteolytic activity. Acid phosphatase and endochitinase activities were strongly suppressed (Fig. 1b–d).

Proteolytic activity was clearly inhibited by pepstatin indicating that a major component of measured activity was AP nepenthesin (Fig. S1). We measured the proteolytic activities at pH 3, what was approx. the average acidity

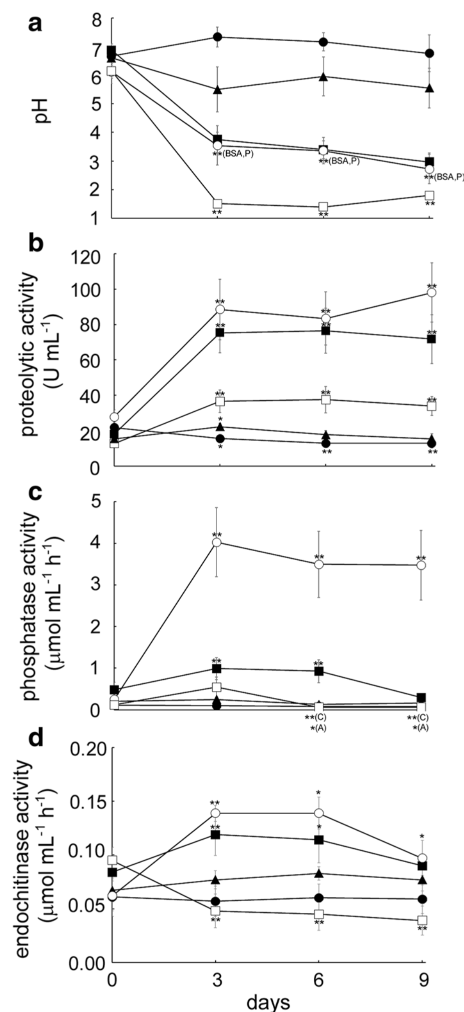


Fig. 1 Digestive enzyme activities and pH in response to different chemical stimuli in *Nepenthes* × *Mixta*. pH (**a**), proteolytic activity (**b**), phosphatase activity (**c**), and endochitinase activity (**d**). Control (closed circles), live prey (open circles), BSA (closed squares), ammonium chloride (open squares), and chitin (closed triangles). The results are the means of four-to-five biological replicates each in three technical measurements. Statistical significant differences between control (day zero) and treated pitchers are denoted as ** at $P<0.01$ and * at $P<0.05$. Significant upregulation is denoted as asterisks above the symbols, downregulation is indicated as asterisks below the symbols. If the symbols are overlapping, (C) indicates control, (BSA) indicates protein, (P) indicates prey, and (A) indicates ammonium. Paired Student's *t* test, $n=4$–5, mean ± SD

reached after protein and insect prey digestion in *N.* × *Mixta* (Fig. 1a). From pH dependence of the proteolytic activity of the digestive fluid (Fig. 2), it was evident that the reached pH after chitin addition was suboptimal for nepenthesins digestion, and thus, proteolytic activity measured at pH 3 for chitin is overestimated. On the other hand, the pH value reached after addition of NH_4Cl was nearest to the optimal pH value for nepenthesins, and proteolytic activity measured at pH 3 is thus slightly underestimated (Fig. 2). Because

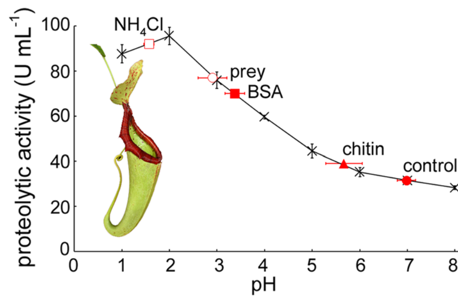
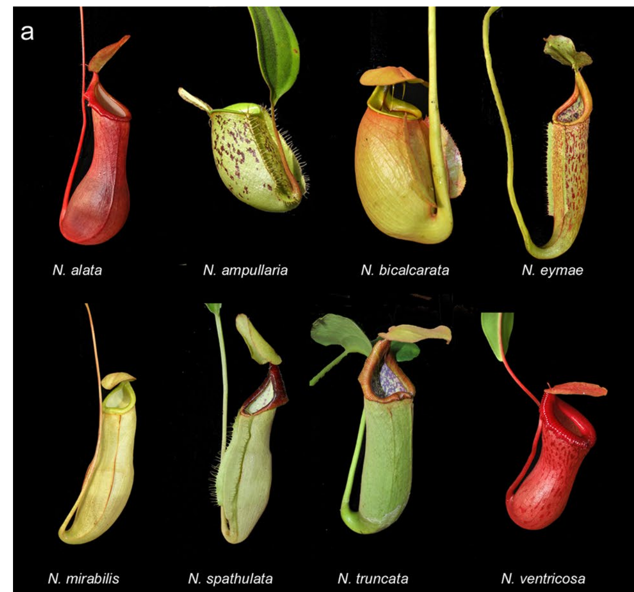


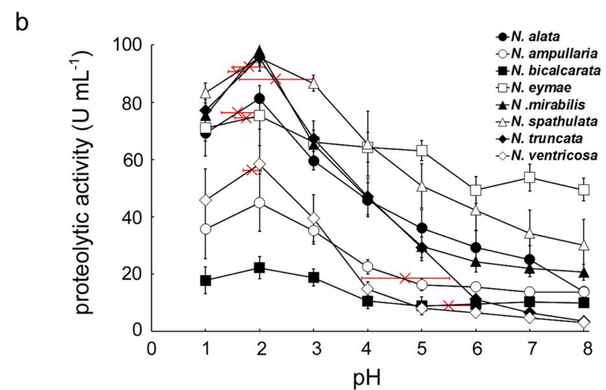
Fig. 2 Proteolytic activity at various pH in *Nepenthes* × *Mixta*. Induced digestive fluid after addition of prey was collected and measured at a different pH. The red symbols represent the average pH values on day 3, 6 and 9 in control traps (closed circle), after addition of live prey (open circle), proteins (closed square), ammonium chloride (open square), and chitin (closed triangle). Mean ± SD, $n = 12–18$

different species keep different pHs in the pitchers (Moran et al. 2010), we measured pH and pH dependence of proteolytic activities in eight *Nepenthes* species after addition of meal worms to find out whether a pH decrease could create the optimal value for nepenthesin digestion (Fig. 3a). After addition of meal worms to *N. alata*, *N. eymae*, *N. mirabilis*, *N. spathulata*, *N. truncata,* and *N. ventricosa*, the average pH value was almost the same with maximal activity of the nepenthesins at pH 2. However, two species (*N. ampullaria* and *N. bicalcarata*) acidify the pH of the digestive fluid only slightly, to 4.7 ± 0.8 and 5.5 ± 0.5 (mean ± SD, $n = 5$), respectively; the reached pH is out of the optimal values for their nepenthesins digestion activity (Fig. 3b).

Induction of gene expression

Based on the enzyme activity measurements, we wanted to reveal the molecular background behind enzyme activity measurements. We quantified the gene expression of corresponding genes responsible for proteolytic and endochitinase activities using qPCR. We noticed an extremely high variability among our three biological replicates. Within the same treatment, some pitchers showed high upregulation of gene expression, others only very low if at all. Therefore, statistical significance was not always obvious. The highest and most significant induction of *NepI* gene expression was achieved after addition of NH_4Cl, the lowest after addition of chitin. Live prey and BSA also significantly upregulated the *NepI* gene expression (Fig. 4a). The *NepII* gene expression showed a similar pattern (Fig. 4b). On the contrary, chitin induced the best *ChitIII* expression after 18 h, however, later (36–72 h) the live prey; BSA and NH_4Cl also upregulated the expression (Fig. 4c). Gene expression of *ChitIV* was significantly upregulated later by addition of live prey, BSA, and ammonium but not by chitin (Fig. 4d).





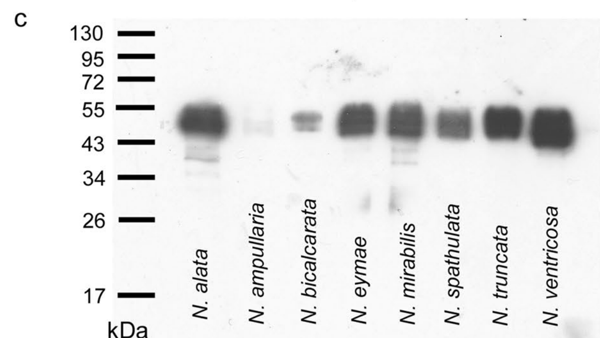


Fig. 3 Comparison of proteolytic activity in different *Nepenthes* species. **a** Pitchers of investigated species. **b** Proteolytic activity at various pH. Red crosses depict average pH values of given *Nepenthes* species, $n = 3$, mean ± SD. **c** Western blots against nepenthesins in different species. The result shown is representative for three experiments

Enzymes in digestive fluids

To semiquantify the amount of digestive enzymes released into the digestive fluid, we used antibodies against nepenthesins and type III and IV chitinases. Different pitchers had

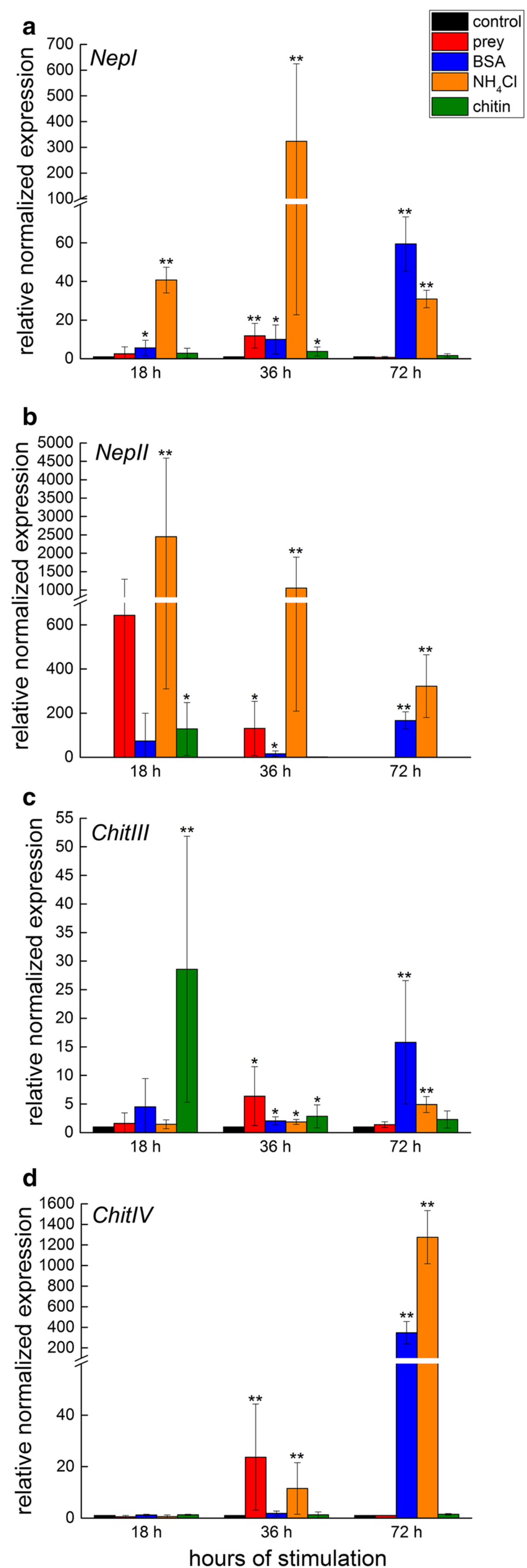


Fig. 4 Gene expression analyses in response to different stimuli after 18, 36, and 72 h in *Nepenthes* × *Mixta*. Nepenthesin I (**a**), nepenthesin II (**b**), chitinase III (**c**), and chitinase IV (**d**). Gene expression for control was set as 1.0. Statistically significant differences between control and treated pitchers were analysed by Student's *t* test, and are denoted as $*P<0.05$ and $**P<0.01$. Mean expression ± SD from three biological replicates ($n=3$)

different initial levels of nepenthesins before induction and Fig. 5a shows these where the initial level of nepenthesins was low (day 0). Nepenthesins occurred in control non-induced pitchers (day 0), but to prevent overexposure of the signal in response to different treatments (days 3, 6, and 9), their presence was not always visible. In the case where the initial level of nepenthesin in non-induced pitcher was relatively high, the induction was also evident (Fig. S2). In both cases, Western blots against nepenthesins confirmed the enzyme activity measurements. There was a clear relationship between induction of nepenthesins in the digestive fluid (Fig. 5a), gene expression (Fig. 4a, b), and the proteolytic activities (Fig. 1b). We detected two bands, which at a high signal intensity often merged to one band between 55 and 43 kDa under reducing SDS conditions, and probably belong to nepenthesin I and II, respectively (Fig. 5a). The specificity of the antibody against AP was checked by mass spectrometry in our previous study (Krausko et al. 2017). In the case of higher abundance of nepenthesins, two additional bands were detected (Fig. S2). The clear and strong upregulation was evident after addition of BSA, prey, and NH_4Cl, yet very weak, almost negligible, after addition of chitin (Fig. 5a, S2). The autoactivation of nepenthesins, involving cleavage of the propeptide sequence of zymogen at acidic pH (Kadek et al. 2014), must occur early after secretion into the digestive fluid even at only slightly acidic pH, as we have not observed any shift in bands from the collected digestive fluid (pH 6.7) of freshly opened pitchers after acidification to pH 3 for 24 h in the test tube (Fig. S3). The nepenthesins were immunodetected in all investigated species (Fig. 3c) after prey capture and their abundance in the digestive fluid was related to enzyme activity measurements (but we realized that the affinity of antibodies against nepenthesin may differ in different species, Fig. 3b).

Type III and IV chitinases were detected at approx. 30 kDa under reducing conditions (Fig. 5b, c). In control, non-induced pitchers, the chitinases were barely detectable. Their abundance in the digestive fluid correlated with endochitinase activity measured as hydrolysis of 4-nitrophenyl β-D-*N*,*N*′,*N*″-triacetylchitotriose as substrate (Fig. 1d). The highest induction of type III and type IV chitinases was found after addition of live prey or proteins. Addition of chitin increased the concentration of type III chitinase in the digestive fluid slightly on the third day. The specific band for both chitinases has never been observed after addition of NH_4Cl, which is related to endochitinase activity (Fig. 1d),

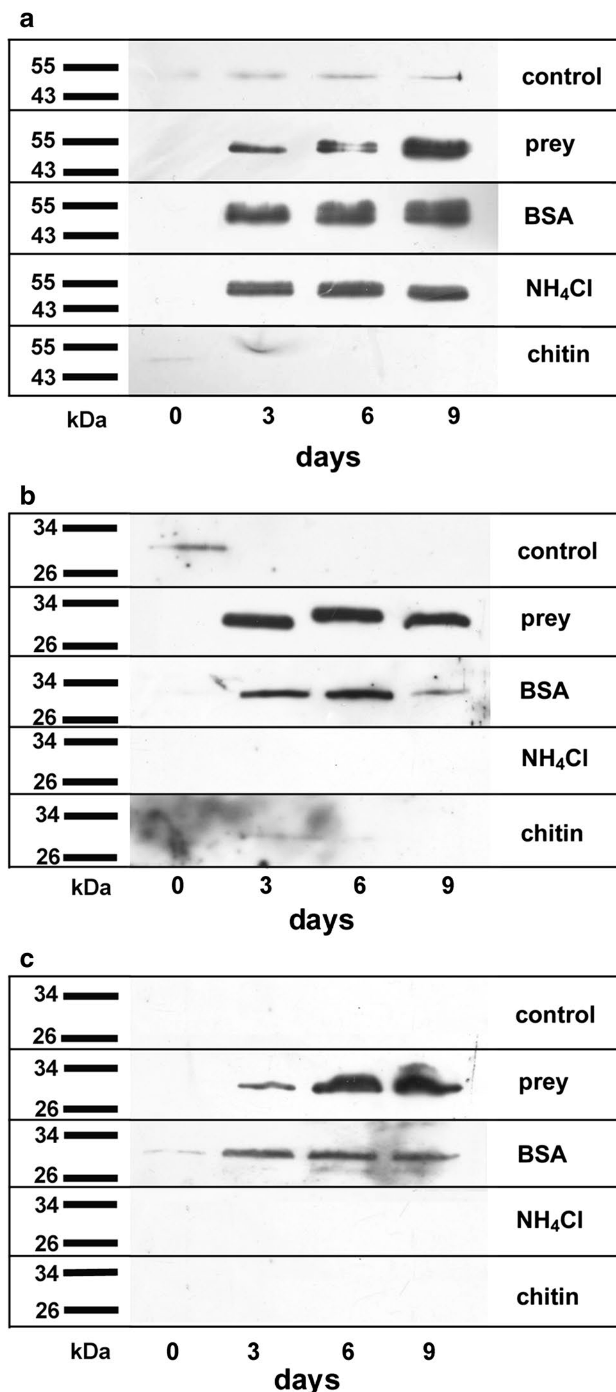


Fig. 5 Abundance of digestive enzymes in the digestive fluid of *Nepenthes × Mixta* by Western blots using specific antibodies before (0) and 3, 6, and 9 days after feeding. **a** Nepenthesins. **b** Chitinase III. **c** Chitinase IV. The same volume of digestive fluid was electrophoresed. Representative results from four biological replicates are shown

despite upregulation of gene expression (Fig. 4c, d). Because chitinases are enzymes active at only slightly acidic conditions (Ishisaki et al. 2012a, b), a low pH induced by NH_4Cl might degradate the proteins in the digestive fluid. To investigate this hypothesis, we collected the prey-induced digestive fluid on the third day and the volume was divided. The same volume of 200 mM glycine-HCl buffer at pH 1.5 and 4.0 was added to the digestive fluid and Western blots were performed after 24 and 72 h of incubation at room temperature in a Falcon tube. The signal intensity was strongly reduced for CHITIII and partially for CHITIV at pH 1.5, whereas nepenthesins were stable at both pHs (Fig. S4).

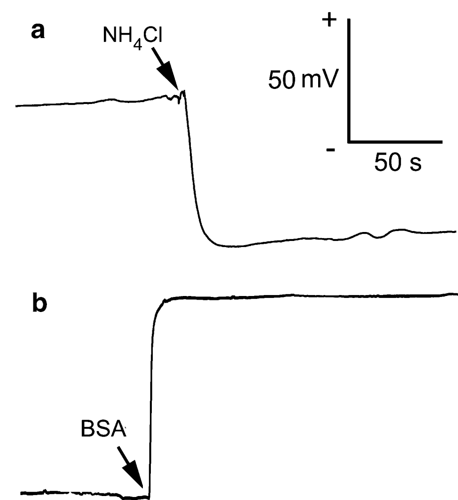


Fig. 6 Extracellular recording of the membrane potential from a digestive glands of *Nepenthes × Mixta*. Application of 50 mM NH_4Cl (**a**) or 3 mg mL^{-1} BSA (**b**)

Measurement of membrane potentials

Because the strongest upregulation of gene expressions was after NH_4Cl treatment, we decided to measure the extracellular membrane potentials in the digestive zone of the trap in response to 50 mM NH_4Cl. The treatment resulted in a rapid depolarization of membrane potentials (negative voltage shift recorded extracellularly, representing intracellular depolarization, Fig. 6a). Addition of water or BSA resulted in an opposite reaction (positive voltage shift recorded extracellularly, representing intracellular hyperpolarization, Fig. 6b).

Gland ultrastructure

To detect changes in the ultrastructure of digestive glands induced by prey capture, we investigated the resting as well as prey-activated glands using transmission electron microscopy. Digestive glands occupy almost the whole inner surface of the pitcher (Fig. 7a, b) and are enclosed within a pit formed by partly overlapping flanges of epidermis—a hood (Fig. 7c). The cells of digestive glands are arranged in discrete layers, with a top layer of elongate columnar cells overlying two

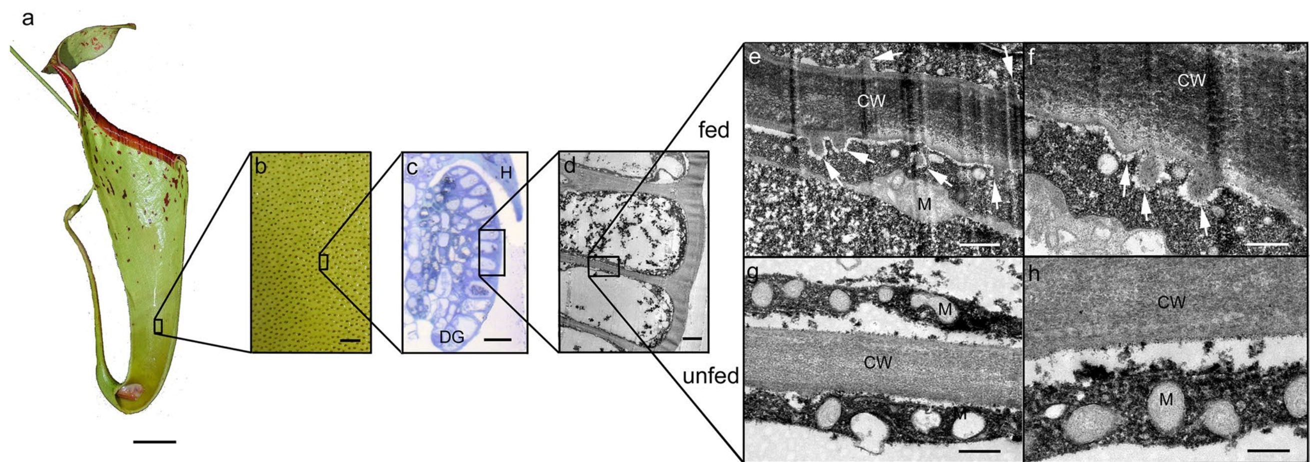


Fig. 7 Vesicular activity in response to prey capture in *Nepenthes*×*Mixta*. **a** Cross section of *N.*×*Mixta* pitcher. Note the prey at the bottom. Bar=2 cm. **b** Digestive glands in the lower part of the pitcher which are submerged in the digestive fluid. Bar=2 mm. **c** Light microscopical view of a semithin section through a digestive gland. Bar=20 µm. **d** Ultrathin section through columnar cells observed by transmission electron microscopy. Bar=3 µm. **e** Exo/endocytosis in prey-activated glands. Bar=700 nm. **f** Different stages of vesicle fusion with the plasma membrane (arrows). Bar=400 nm. **g** No vesicles were observed in control unfed pitchers. Bar=700 nm. **h** No vesicle fusion was visible in control unfed pitchers. Bar=400 nm. *DG* digestive gland, *H* hood, *M* mitochondria, *CW* cell wall, and arrows denote vesicles

or three layers of rounded to flattened cells. Below is a cell layer which appears continuous with the epidermis. At the base of the glands are two layers of highly vacuolated hypodermal cells with large osmiophilic deposits adjacent to the end of tracheary elements. All gland cells in the resting as well-active state exhibited numerous mitochondria with well-developed cristae and endoplasmic reticulum indicating high energy demand for secretory and/or nutrient uptake activity, respectively.

The most prominent differences between resting and prey-activated glands were visible in the top layer of elongate columnar cells (Fig. 7d). The outer wall of the first raw of columnar secretory cells is thick with a thin cuticle and a cutinized layer of the cell wall. The organelles are placed in a narrow strip of electron-dense cytoplasm along the cell wall. A large more or less osmiophilic vacuole, probably due to pigment accumulation and precipitation during fixation, occupies the major volume of the cell. In the prey-activated glands, an increase of exo/endocytosis on periclinal and anticlinal cell walls was observed (Fig. 7e, f). Numerous smooth and coated vesicles in different stages of fusion with the plasma membrane were observed indicating a secretion of enzymes into the cell wall and/or nutrient uptake. Such activity was not observed in non-stimulated glands (Fig. 7g, h).

Discussion

The traps of carnivorous *Nepenthes* plants, in contrast to sundew (*Drosera*) or Venus flytrap (*Dionaea muscipula*), cannot move and are considered passive, holding the digestive fluid in the pitcher and waiting for potential prey. In the famous monograph of Juniper et al. (1989), the authors were not able to conclude if *Nepenthes* responded to prey or at least if it was able to monitor the status of the digestive fluid. It was only known that *Nepenthes* could drop its pH after prey addition. Although the enzyme production is certainly under developmental control and young closed and freshly opened pitchers contain less enzymes than several days after opening (Hatano and Hamada 2008; Biteau et al. 2013), the results from our and recent studies (Eilenberg et al. 2006; Buch et al. 2015; Yilamujiang et al. 2016) have shown that *Nepenthes* can regulate enzyme activity as well as do the carnivorous plants with active trapping mechanisms. In contrast to Venus flytrap, where the digestive fluid is secreted in response to prey capture, the digestive fluid with few enzymes in *Nepenthes* is preformed without any stimuli from prey and is present even in closed pitchers. However, here, we showed that

regulation of enzyme secretion is under the strong control of prey stimuli.

Although the previous studies have been focused mainly on chitin as inductor for enzyme secretion (Eilenberg et al. 2006; Hatano and Hamada 2012; Yilamujiang et al. 2016), we demonstrate here that proteins and ammonium are more powerfull inducers of proteolytic activities. Ammonium is produced in digestive fluid by deamination of aminoacids and proteins in the Venus flytrap (Scherzer et al. 2013). The authors found glutamine deaminase activity in the fluid; however, the enzyme has not been characterized so far. A similar process may occur also in *Nepenthes alata* digestive fluid. Higashi et al. (1993) and An et al. (2001) found that the ammonium concentration raised after feeding. Formed ammonium upregulated the expression of *NepI* and *NepII* 323 and 2450-fold, respectively (Fig. 4a, b) and triggered the secretion of AP nepenthesins in the digestive fluid (Fig. 5a) which are responsible for proteolytic activity (Figs. 1b, 2). Although the transcriptional activation of *Nep* by ammonium was the highest, the overall proteolytic activity in the digestive fluid was lower in comparison to protein and prey addition, indicating that the presence of protein, not of ammonia alone, is also important (Fig. 1b). The ammonium was also the best inductor of acidification of the digestive fluid creating the optimal conditions for proteolytic activity (Fig. 1a). Acidic conditions are also important for the autoactivation of nepenthesins: autocatalytic removal of the propeptide at pH 2.5 activates the enzyme in the range of minutes and hours (Kadek et al. 2014). Because we have not observed changes in electrophoretic mobility of the enzyme in acidification experiments (Fig. S2), the enzyme is present in an active form already in control pitchers, indicating that even slightly acidic conditions in non-stimulated plants are sufficient for nepenthesin autoactivation. Another possibility is a cleavage of nepenthesin in closed developing pitcher when the pH is lower than in open pitchers (for *N.* × *Mixta* in closed pitchers is pH ~ 5.5 in open pH ~ 6.7). Recently it has been found that enclosed developing pitchers are CO_2-enriched cavities up to 5000 ppm of CO_2, and CO_2 forming carbonic acid in the pitcher fluid is one of the factors making it acidic before opening (Baby et al. 2017).

Higashi et al. (1993) tested several ammonium salts and also found that proton excretion from the pitcher tissue and low pH was caused by NH_4^+. Ammonium is probably taken up by an ammonium transporter which is expressed in the head cells of the digestive glands of the lower part of pitcher (Schulze et al. 1999). The uptake of prey-derived NH_4^+ by the ammonium transporter might induce H^+ release (Scherzer et al. 2013). Together with the plasma membrane H^+-ATPase characterized by An et al. (2001), they are the best candidates responsible for acidification of the digestive fluid. The possible mode of NH_4^+ action includes rather depolarization of the membrane potentials than a specific receptor. Our extracellular recording of membrane potentials of the digestive zone showed the depolarization of membrane potentials in response to NH_4Cl application. Although changes in Donnan potential due to binding of ions to cell walls cannot be ruled out, such a depolarization in response to NH_4Cl was documented also in sundew tentacles (Williams and Pickard 1972) and digestive glands of the Venus flytrap (Scherzer et al. 2013) which triggered the action potentials (not observed in *Nepenthes*). Depolarization of membrane potentials belongs to the first cellular events after herbivore attack inducing the accumulation of jasmonates which trigger the expression of defence proteins (Maffei et al. 2007). Recent studies have shown that carnivorous plants have employed the same signaling pathway, and the electrical activity is translated into biosynthesis of the biological active isoleucine conjugate of jasmonic acid (JA-Ile), which triggers the expression of digestive enzymes (Libiaková et al. 2014; Böhm et al. 2016; Bemm et al. 2016; Krausko et al. 2017; Pavlovič et al. 2017). In accordance with this, the accumulation of jasmonates after prey (or chitin) capture was recently documented also in *N. alata* (Yilamujiang et al. 2016). Ammonium induced the accumulation of jasmonates and secretion of cysteine endopeptidase dionain in the digestive fluid of the Venus flytrap (Libiaková et al. 2014). The study of the carnivorous pitcher plant *Sarracenia purpurea*, which also traps insects into the pitcher traps, showed that BSA and NH_4Cl were powerful inducers of proteolytic, phosphatase and RNAase activities (Gallie and Chang 1997). Although it has been long believed that *Sarracenia* relies more on microbial digestion, recent data documented the presence of AP similar to nepenthesin in the digestive fluid (Fukushima et al. 2017). All these data and our results demonstrate that protein and probably its breakdown product (ammonia) are inducers of proteolytic activities.

In comparison to ammonium, chitin upregulated the expression of *NepI* and *NepII* only slightly (Fig. 4a, b) and could not built up an optimal pH for the proteolytic enzymes; thus the enzymes worked under suboptimal conditions (Fig. 2). In accordance with this, the increase of proteolytic activity of NEPI and NEPII in the digestive fluid after chitin treatment was almost negligible (Fig. 1b). Yilamujiang et al. (2016) found an upregulation of proteolytic activity in the digestive fluid after chitin addition in *N. alata* after 24 and 48 h, and concluded that chitin induced only a fast and transient stimulation of proteolytic activity. As mentioned above, for a sustained production of the enzyme, proteinaceous stimuli are necessary. This is also strongly supported by the fact that addition of chitin-free plant materials into the pitcher of *N. alata* can induce a comparable proteolytic activity in comparison to chitin-rich prey (Buch et al. 2015).

Chitin strongly and transiently upregulated *ChitIII* expression after 18 h. This indicates a second, separate signaling

pathway in prey sensing. In plants, chitin is usually sensed by the LysM receptor (Miya et al. 2007). However, for significant accumulation of CHITIII (and CHITIV) and significant endochitinolytic activity of digestive fluids, the presence of foreign proteins (BSA in our case) is again important. Yilamujiang et al. (2016) found that live prey upregulated the expression of *ChitIII* stronger than chitin alone, indicating another regulatory factor. Indeed the proteins and NH_4 can also upregulate the expression of *ChitIII* (Fig. 4c, d). This result is consistent with the findings of Matušíková et al. (2005) and Jopčík et al. (2017), who found an increased expression of chitinase and an increased chitinolytic activity in the digestive fluid not only after chitin application but also after addition of proteins in sundew plant *D. rotundifolia*. Our unpublished results showed that Venus flytrap also increased transcription and secretion of type I chitinase after BSA addition more strongly than after chitin addition. Proteins and their breakdown product ammonium may induce chitinase expression probably through the depolarization of membrane potentials. Depolarization of membrane potentials in plants is an important element in signal transduction pathways. Wounding which triggers the depolarization in the form of variation potentials can induce transcription of chitinase in plants without chitin sensing (Rawat et al. 2017). Wounding also induced secretion of type I chitinase in Venus flytrap (Pavlovič et al. 2017). The absence of the chitinases and low endochitinolytic activity in the digestive fluid after treatment with ammonium can be caused by very acidic conditions triggered by ammonium and the stability of chitinolytic enzymes at acidic pH (Fig. S4), and thus represent posttranslational regulation. The optimal pH for activity of recombinant CHITIII and CHITIV from *N. alata* was 3.9 and 5.5, respectively (Ishisaki et al. 2012a, b), pH values reached after addition of chitin but not ammonium (Fig. 1a). The activity of recombinant CHITIII was close to zero at pH 2 (Ishisaki et al. 2012a).

The conventional protein secretion refers to endoplasmic reticulum-Golgi-mediated secretory routes that co-translationally direct them to the endoplasmic reticulum. Subsequently, secretory proteins are packaged into vesicles and transported to the Golgi apparatus, where they undergo posttranslational modification. In postGolgi trafficking, secretory vesicles budding off the trans-Golgi network fuse with the plasma membrane and release their contents into the extracellular space (Drakakaki and Dandekar 2013). In carnivorous plants, recent studies indicate that release of the digestive fluid with enzymes and uptake of nutrients are mediated by exo- and endocytosis, respectively (Adlassnig et al. 2012; Scherzer et al. 2017). Although it is difficult to distinguished which vesicles are released and which are taken up on Fig. 7, it is tempting to assume that both processes dominate in activated glands of *Nepenthes*. Although glands are responsible for enzyme secretion, the study of Rottloff et al. (2011) demonstrated that *ChitIII* was upregulated also in glands' surrounding tissue indicating that all pitcher tissue might be employed in the generation of enzymes, but only glands secrete them, a situation very similar to sundew leaves as in situ hybridization of type I chitinase mRNA indicates (Matušíková et al. 2005).

Not all species of *Nepenthes* are equally good hunters. Several species of *Nepenthes* have evolved adaptations to obtain nitrogen from different sources, and interspecific differences in ecology are mirrored by differences in nutritional physiology (Moran et al. 2010; Thorogood et al. 2018). We confirmed interspecific differences in the ability to acidify the digestive fluid. After prey addition, the majority of species built up the optimal pH for the activity of nepenthesins. However, in *N. ampullaria* and *N. bicalcarata,* the pH remains relatively high and these species actively manipulated H^+ fluxes to maintain less acid pitcher fluid (Moran et al. 2010). Higher value of pH in these two species was found also in their natural habitat (Clarke 1997) and was attributed to special nitrogen sequestration strategies (Moran et al. 2010). *N. ampullaria* is unique among the Nepenthaceae in that, although it catches also invertebrate prey, it derives a significant amount of its nitrogen from abscised leaves that have fallen into its pitchers from the forest canopy above (Moran et al. 2003; Pavlovič et al. 2011). Although *N. bicalcarata* pitchers catch only invertebrate prey, it has a mutualistic association with a species of swimming ant *Camponotus schmitzi.* The ants increase the nutrient retention in the pitchers by attacking newly caught insects. They prevent prey escape, also by preying infaunal flies which would otherwise eventually leave their hosts (Bonhomme et al. 2011; Scharmann et al. 2013). When feeding on prey, it appears to target large prey items only preventing putrefaction (Clarke 1997). The nutrients from consumed and digested prey are transferred to the host plant via *C. schmitzi* faeces and carcases. The ants have been observed to clean the peristome of the fungal hyphae and other contaminants, thereby maintaining high trapping efficiency over the long pitcher's lifespan (Thornham et al. 2012). They also protect pitchers against pitcher-destroying weevils of the genus *Alcidodes* (Merbach et al. 2007). Both species benefit from their adaptations (Pavlovič et al. 2011; Bazile et al. 2012). It has been hypothesized that nepenthesins might be either absent or inactive in *N. bicalcarata* and *N. ampullaria,* and that digestion is partly undertaken by bacteria and other organisms at higher pH (Moran et al. 2003; Bazile et al. 2012). However, immunoblotting revealed the presence of nepenthesins in the digestive fluids of both species, although the proteolytic activities are rather low in comparison to other species of pitcher plants and they work at suboptimal pH values (Fig. 3b, c). This may indicate a partial relaxation of functional constraints on the production and enzymatic activities of nepenthesins. Plants partially

rely on the digestive ability of animals and microbial organisms and thus safe the available resources. To generalize this hypothesis, however, more work is necessary on species like *N. lowii* and *N. hemsleyana* which have evolved mutualistic relationships with small mammals and obtain substantial amount of nitrogen from faeces (Clarke et al. 2009; Grafe et al. 2011; Yilamujiang et al. 2017).

Our study showed only the potential of certain chemical agents in inducing digestive enzymes. The concentration of a given chemical stimulus under natural conditions strongly varies, and is dependent on species, hunting strategy, and environment. How much insects and thus chitin, protein, and ammonia (and their ratio) is captured within a certain period of time in a certain volume of pitcher fluid (volume range from few mL to 2 L in *N. rajah*) may affect the gene expression. Thus, we suggest that plants will react differently if only one fly is captured in a volume of 2 L digestive fluid in *Nepenthes rajah* during a day in a cool montane climate or a whole colony of termites is captured in a few milliliters of the digestive fluid of *Nepenthes albomarginata* during one night in a hot lowland (Merbach et al. 2002). In addition, the diet of different *Nepenthes* species differs ranging from ammonia rich bat guano and mammal faeces or nitrogen poor and chitin-free leaf litter (Moran et al. 2003; Clarke et al. 2009; Grafe et al. 2011). Therefore, we are still far to understand how the digestive process is regulated in different species under natural conditions.

In conclusion, our study demonstrated that pure chemically unmodified chitin upregulates enzyme activities in *Nepenthes* only very slightly. This is in accordance with the finding that the pure chitin is not such a good source of available nitrogen in comparison to proteins in carnivorous sundews (Pavlovič et al. 2016). Indeed, the hard chitin exoskeleton of a digested prey does not seem to be dissolved or significantly affected after digestion in carnivorous plants (Juniper et al. 1989). Chitinases may be more involved in the inhibition of fungal growth to avoid unwanted competition for the nutrients that are released from the digested prey (Mithöfer 2011; Buch et al. 2013). The weak stimulation of chitin of the proteolytic activity resembles the behavior of the Venus flytrap and sundew plants in response to wounding, and is probably a result of evolutionary history (Krausko et al. 2017; Pavlovič et al. 2017). Sharing the same signaling pathways for defence and carnivory, wounding mimicking mechanical stimulation of prey triggered a low enzyme activity in the digestive fluid in Venus flytrap and sundews without the presence of any prey (Krausko et al. 2017; Pavlovič et al. 2017). The same may hold for chitin which is an important signaling molecule for the initiation of defence response and serves as a microbe-associated molecular pattern (MAMP) for the detection of various potentials pathogens (Newmann et al. 2013). Thus, wounding as well

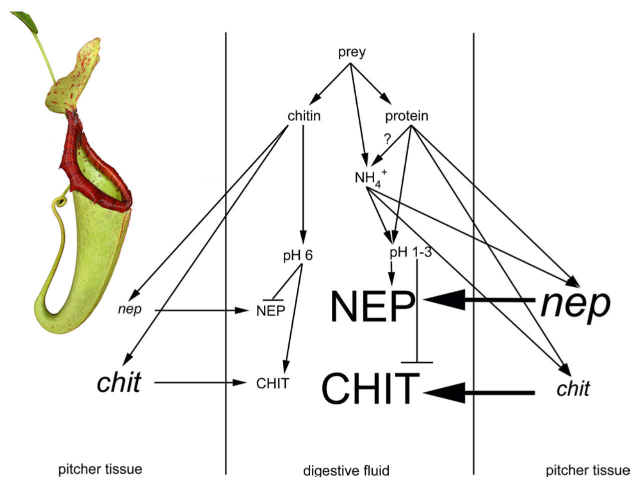


Fig. 8 Regulation of enzyme activities in pitchers of *Nepenthes* × *Mixta*. Three components of insect prey (chitin, protein, and ammonia) were studied separately. Protein and ammonia in particular strongly induce, probably through depolarization of membrane potentials, the transcription of AP nepenthesins which are subsequently released into the digestive fluid. They also induce the excretion of protons from pitcher tissue decreasing the pH of the digestive fluid (pH 1–3), thus creating the pH optima for catalytic activity of nepenthesins. Transcription of chitinases is also induced and they accumulate in the digestive fluid especially after addition of proteins. Low pH induced by ammonia can degradate the chitinolytic enzymes and the low pH is not optimal for chitinolytic activities. On the other hand, chitin lowered the pH and induced the transcription of nepenthesins only slightly; transcription of chitinase III is increased but not of chitinase IV. In comparison to protein addition, chitin induces only a small increase of enzymes in digestive fluid. The font size depicts the intensity of gene transcription in pitcher tissue (italic) or abundance of the enzyme in the digestive fluid (capital letters)

as chitin trigger low enzyme activity, and the significant production of digestive enzymes starts only after protein sensing, the important source of nitrogen for carnivorous plants (Pavlovič et al. 2016). We do not know if the protein affects gene transcription directly, or if it is mediated by the released ammonium; both ways are possible. The fact that proteins strongly increase the activity not only of proteases but also of phosphatases and chitinases indicates that they are universal and effective signals for enzyme induction (Fig. 8). This was also found in the pitcher plant *Sarracenia purpurea* (Gallie and Chang 1997). The plant interest in proteins is also supported by the fact that after prey capture many species of *Nepenthes* actively manipulate and keep pH values several days close to the optimal activity for proteases rather than chitinases, and the acidic pH can even destroy the chitinolytic enzymes. The participation of ammonium from deaminated proteins in acidification of the digestive fluid is highly probable.

Author contribution statement AP designed the study; MS, BB, and TS performed the experiments; AP, MS, and BB

analysed the data; MS and AP wrote the manuscript, AP provided materials and financial support.

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