Friedrich-Schiller-University Jena
Faculty of Biology and Pharmacy

Max Planck Institute for Chemical Ecology
Department of Bioorganic Chemistry
Research Group Defense Physiology

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Study on enzyme activity of nepenthesins in carnivorous *Nepenthes alata*

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Submitted by
Marietheres Jaeger
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Evaluated by:

1. Priv. Doz. Dr. Axel Mithöfer
Max Planck Institute for Chemical Ecology
Department of Bioorganic Chemistry, Research Group Plant Defense

2. Prof. Dr. Nicole van Dam
Friedrich Schiller University Jena
Institute of Ecology, Chair Molecular Interaction Ecology

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III Abbreviations

BSA	Bovine serum albumin
H_2O	distilled water
PFU-093	FITC(Ahx) – Val – Val – LysDbc, Kaman *et al.* (2011 and 2013)
FITC	fluorescein isothiocyanate
LysDbc	Lysin-Dabcyl
Ctrl	control
Std Dev SD	Standard deviation
M	Mean

1 Introduction

"It is a plant […] which carries at the end of its leaves […] a hollow flower or fruit resembling a small vase, with its own lid, a wonderful sight." (from French, Phillips A and Lamb A 1996) – like this Étienne de Flacourt described the pitcher plant in his work *Histoire de la Grande Isle de Madagascar* from 1658. While the *Nepenthes* plants are without a doubt a wonderful sight, over the centuries more was learned about this species. The small vase Flacourt described is neither a flower nor a fruit. These are instead specialized trap leaves, which are typical for carnivorous plants (Figure 1). While *Drosera* plants possess stalked sticky glands and *Dionaea,* the well-known snap trap, *Nepenthes* plants are more subtle. The pitchers of *Nepenthes* are not obviously traps. Due to that, in the botanic history many explanations of their use were made. A most interesting theory of the pitchers and their fluid was written down by the English naturalist John Ray in 1686. He quoted Grim, who described 1683 a "*planta mirabilis destillatoria*": "The root draws up moisture from the earth which with the help of the sun's rays rises up into the plant itself and then flows down through the stems and nerves of the leaves into the natural utensil to be stored there until used for human needs." (from Latin, Phillips A and Lamb A 1996). While the *Nepenthes* pitchers make good water cups apparently, the plant itself has a different reason for its special leaves.

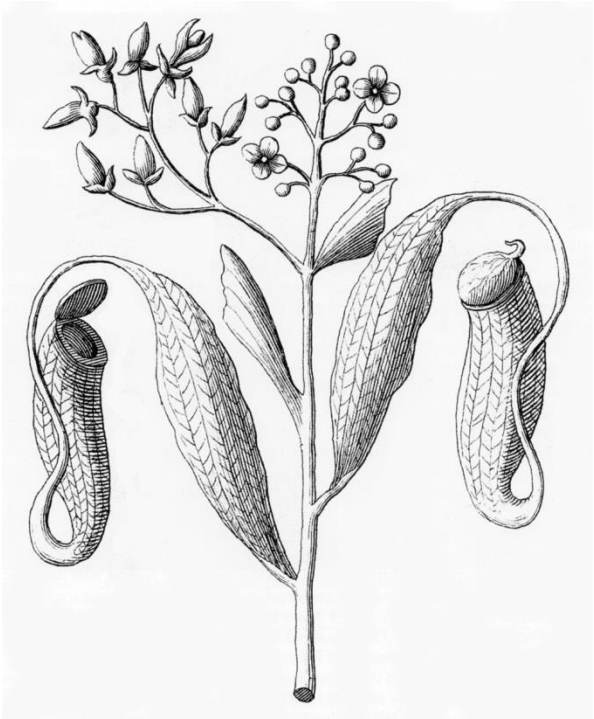

Figure 1: ***One of the earliest published illustrations of a pitcher plant.*** Drawing of *"Utricaria vegetabilis zeylanensium" (N. distillatoria)* from Plukenet, Leonard (1696) *Almagestum Botanicum*.

The goal of *Nepenthes* is to trap and digest prey in order to absorb the resulting products. These nutrients are the main source of nitrogen for *Nepenthes* plants. Like other carnivorous plants, *Nepenthes* lacks the necessary nitrogen sources in its environment (Darwin C 1875, Lloyd FE 1942). Since *Nepenthes* is unable to move from this nitrogen-lacking area, a different way to gather nutrients had to be found. If the plant cannot come to the nitrogen, the nitrogen must come to the plant. And that it does – in various forms. Most of the *Nepenthes* species trap small insects to feed on, while some are content with falling leaves, such as *N. ampullaria*. Bigger *Nepenthes* pitchers can trap different prey, such as small animals. This occasionally makes the news in articles like "Killer plant 'eats' great tit at Somerset nursery" (BBC News. 5 August 2011). Another way to gain nutrients is to let them be delivered by other organisms. This is the case for *Nepenthes lowii*, *N. rajah* and *N. macrophylla*, as described in *Trap geometry in three giant montane pitcher plant species from Borneo is a function of tree shrew body size* (Chin L, Moran JA, Clarke C 2010). These big *Nepenthes* plants provide a secrete at its lid for tree shrews (*Tupaia montana*), which meanwhile relive themselves into the pitcher. The plants prefer to be a toilet rather than consuming the small animals, which is more effective over a prolonged period. It does not stop unfortunate accidents, due to which the *N. rajah* plants still consume rats on occasion (Phillipps A 1988).

Figure 2: *Nepenthes alata* pitchers. Photographed in the greenhouse of the Max Planck Institute for Chemical Ecology by Marietheres Jaeger, 2015.

The pitcher develops at the end of the leaves and grows in size (Figure 2). It is connected to the leaves with a tendril. This tendril curls up to warp around nearby plants as support. During this growth period the pitcher remains closed with a lid but already contains the pitcher fluid. When the pitcher opens the trap leaf is finished in its construction. From the top the pitcher consists of the lid, which protects from falling water and debris. Some species have nectar glands at the inside of the lid to attract prey. The next part of the trap leaf is the opening with its ribbed structure. This part is slippery when wet and causes the prey to fall into the pitcher. Most *Nepenthes* plants prefer high humidity, which helps this part of the trap. The upper inside of the pitcher cup is slippery too, due to a waxy coating. This is to keep the prey in the cup. The base of the pitcher cup is filled with digestive fluid and covered with glands. The fluid inside the pitcher contains enzymes to digest the prey and the glands then absorb the nutrients (Figure 3).

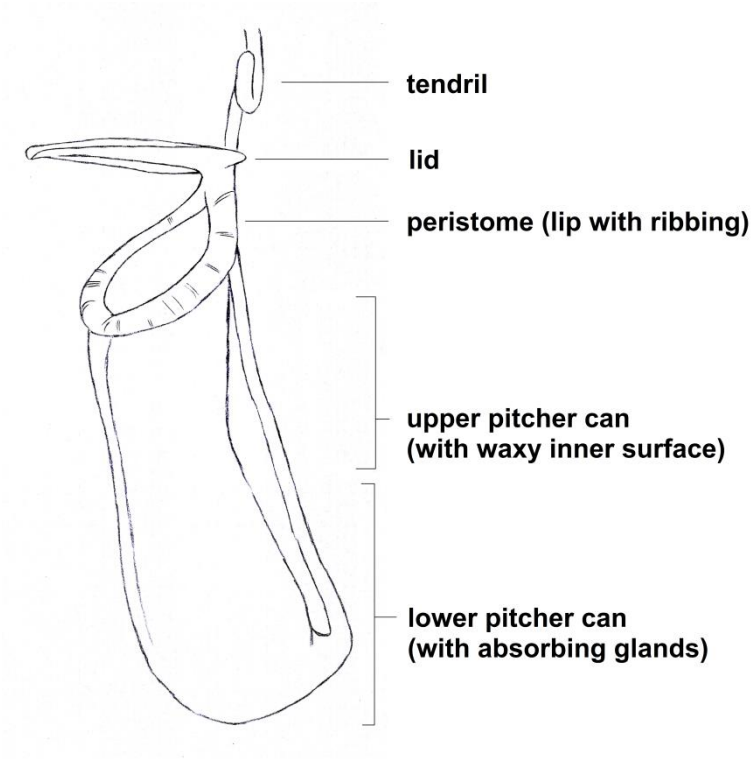

Figure 3: Sketch of hanging *Nepenthes alata* pitcher with description. Drawn by Marietheres Jaeger, 2015.

The digestive fluid of *Nepenthes* contains various enzymes, such as chitinases (Eilenberg *et al.* 2006, Rottloff *et al*. 2011) to digest chitin shells of insects, and proteases, to digest proteins as nutritional source for nitrogen. Proteases in *Nepenthes* were described early on (Vines SH 1879). In this work the nepenthesins are of main interest. Nepenthesins are aspartic proteases and were purified and characterized in

various works, such as Amagase S, Nakayama S, Tsugita A (1969), Jentsch J (1972), Tökés ZA, Woon WC, Chambers SM (1974) and An CL, Fukusaki E, Kobayashi A (2002). Athauda *et al.* (2004) described two nepenthesins, nepenthesin I and nepenthesin II and these enzymes were identified as a new subspecies of aspartic proteases in this work, as well in Takahashi *et al*. (2005) and Takahashi K, Tanji M, Shibata C (2006). Due to the small amounts of expressed nepenthesin in *Nepenthes* plants, the regulation and induction of these aspartic proteases was difficult to analyse. However, with a innovative FRET (fluorescent resonance energy transfer)-based method described and established by Buch F, Kaman WE, Bikker FJ, Yilamujiang A, Mithöfer A (2015) it is possible to detect and monitor protease activity directly in the *Nepenthes* pitcher fluid.

2 Objective

The aim of this work was to investigate the enzymatic activity status of nepenthesins in the pitchers due to the presence of external chemical signals. Nepenthesins as aspartic proteases are part of the digestive fluid of *Nepenthes* plants. With a FRET-based method (Buch *et al.* 2015) it is possible to determine directly the presence of enzyme activity over long time periods.

In this work, the species *Nepenthes alata* was used and stimulated with either chitin or BSA, representing various nutritional sources. BSA was chosen as protein source because nepenthesins digest proteins in the pitcher fluid; chitin was chosen because it is the main component of the exoskeleton of insects, thus representing the main prey of *Nepenthes*. By taking and freezing samples over a chosen period it was possible to study the nepenthesin activity in various timeframes.

3 Materials and methods

3.1 Materials

Table 1: Plants used for the experiment.

plants /organism	
Nepenthes alata Blanco	pitcher plant, pitchers used

Table 2: Chemicals used for the experiment.

chemicals / substances	
H_2O	
PFU-093	80 µM
KCl	25 mM
Tris-HCl buffer	pH 8.5 , 100 mM
chitin	5 mg/ml
BSA	10 mg/ml

Table 3: Quantity of stimulants used.

stimulants	quantity used for the experiment
chitin	1 mg/ml per pitcher
BSA	1 mg per pitcher

Table 4: Tools used for the experiment.

tools	
Tecan infinite M200	microplate reader
i-control 1.8 (for infinite reader)	software for reader
black 96-well plates	

3.2 Method

3.2.1 FRET-based method

To determine the enzyme kinetic of nepenthesins in *Nepenthes alata* a FRET (fluorescent resonance energy transfer)-based technique was used. This method was described in Buch *et al.* (2015) as "[…] highly sensitive FRET […], for the direct, easy and rapid detection and characterisation of protease activity […]". The fluorescence substrate is PFU-093 (Figure 4), coded as such by Kaman *et al.* (20011 and 2013). It consists of a fluorophore connected to a quencher with an amino acid bridge. The fluorophore is fluorescein isothiocyanate and the quencher is Lysin-Dabcyl. The amino acid bridge consists of two valines. With the bridge intact no activity can be detected, due to the proximity of the LysDbc (Figure 4: 1). The nepenthesin cleaves the peptide bond between the amino acids and the quencher is removed from the vicinity of the fluorophore (Figure 4: 2). The fluorescence intensity of the active substrate can be measured using a microplate reader. The higher the resulting relative fluorescence, the more active nepenthesin is in the sample. Due to the influence of pH on the method, the recommended amount of buffer was used to stabilize the reaction after incubation (Buch *et al*. 2015).

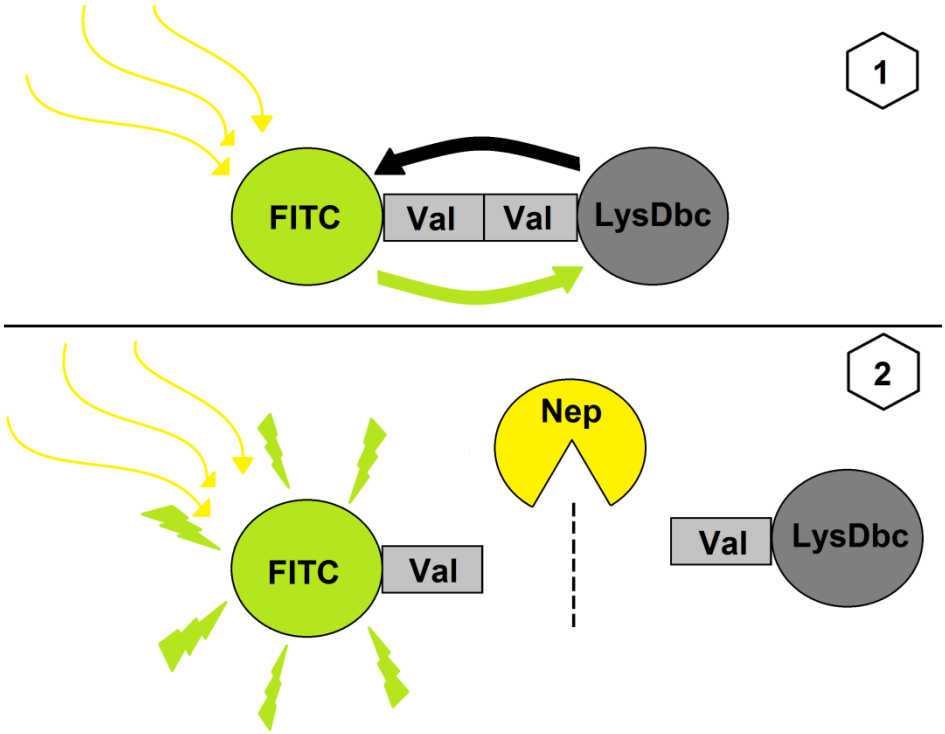

Figure 4: Simplified structure of PFU-093. 1: Due to the close vicinity to LysDbc the FITC fluorescence is quenched. 2: The nepenthesins (Nep) cut the bridge of two valines (Val). LysDbc is apart from FITC and cannot quench it. FITC is now fluorescent and the intensity of fluorescence can be measured.

3.2.2 Step by step

The pitcher plants were grown in the greenhouse of the Max Planck Institute for Chemical Ecology in Jena. The *Nepenthes alata* were kept under controlled conditions. The photoperiod was from 6.30 to 20.30 (14 h light/ 10 h dark). The day/ night temperature was 23 – 25 °C/ 19 – 21 °C and the humidity was between 50 % and 60 % full-time. The plants were watered every two days (Monday, Wednesday and Friday) and if necessary on the weekend.

Figure 5: Example of used pitcher (left) and the same covered in gauze (right). The pitcher is covered in gauze in order to keep semi-sterile conditions. It keeps contaminations, like water and small insects away. These might enter the pitcher when it opens up during the experiment. All used pitchers had the size of about 8 cm.

For the experiment the pitcher fluid of closed *N. alata* pitchers was used. Pitchers with approximately the same size and volume of digestive fluid were chosen. If possible, the pitchers were on the same plant. The chosen pitchers were covered with gauze for semi-sterile conditions, in case the pitchers would open during the experiment (Figure 5). The samples of pitcher fluid were collected using sterile pipette tips. To do that the pitcher was punctured about 1 cm above the filling level and tilted to remove the fluid (Figure 6). The removed samples were kept frozen at – 20 °C until use.

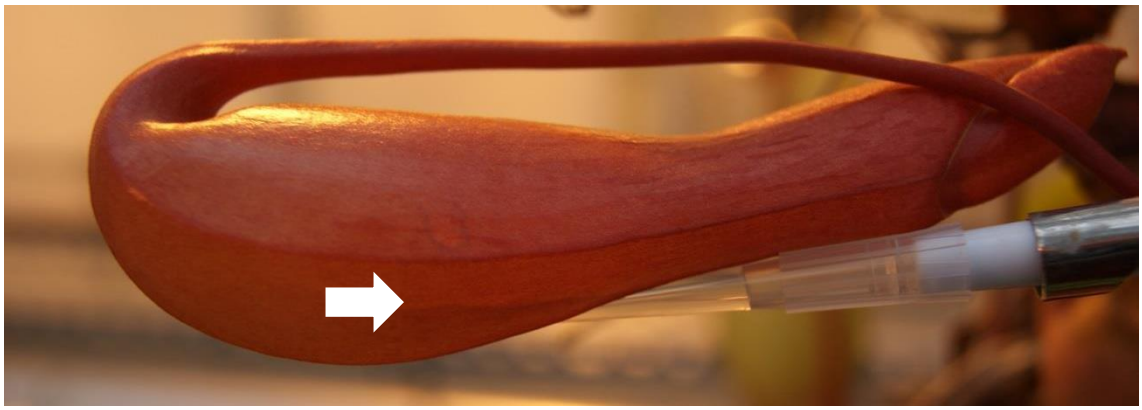

Figure 6: To remove the pitcher fluid, the pitcher was tilted. To keep the fluid semi-sterile, sterile pipet tips were used to take samples. The white arrow indicates the fluid level, while tilting.

Chitin or BSA was used to stimulate and follow up the proteolytic reaction of *N. alata*. The experiment was divided into two test series – one with chitin and the other one with BSA treatment. Each of the solid stimulants was dissolved in H_2O. The resulting concentration is listed in Table 3. Each time six pitchers were chosen. To three pitchers stimulant (chitin or BSA) was added. H_2O was added as control in the other three pitchers.

In the chitin test series 500 µl of the chitin solution were added to the pitchers, the same volume H_2O to the control pitchers. In case of the BSA run 100 µl of the BSA solution or H_2O were added into the pitchers.

After removing a sample with the volume of 200 µl, the taken volume was replaced with the same amount of 25 mM KCl according to Buch *et al.* (2013). The first sample was taken before the stimulation (referred to as 0 h). For the treatment of *N. alata* with chitin, the volume of pitcher fluid in each chosen pitcher was estimated. This was done with the help of similar sized, unused pitchers as volume reference. The final concentration was 1 mg/ml in each pitcher. In case of BSA a final concentration of 1 mg per pitcher was added to stimulate a reaction.

At different time points 200 µl samples of pitcher fluid were taken and replaced with 25 mM KCl. The samples of the chitin treatment were collected every 24 h until 144 h, then every 48 h until 288 h (12 d). The BSA samples were collected every 12 h until 96 h.

After all samples were taken (and kept frozen) the FRET-based technique described in Buch *et al.* (2015) was used to determine the nepenthesin activities. The samples were thawed on ice and the reagents prepared. PFU-093 is sensitive to light and should be treated as such. It is denser than water, so the substrate should be carefully homogenized before pipetting and during pipetting as needed. In triplicate 50 µl of the samples were pipetted in black 96-well plates. 39 µl of H_2O were added and 1 µl of PFU-093 in a concentration of 80 mM. This mixture was left in the dark for 5 h to incubate. After the incubation 10 µl of Tris-HCl buffer was added into each well. Each plate was placed into the microplate reader (Tecan infinite M200) and the fluorescence activity measured. With the resulting data the measurement tables were constructed, as well as diagrams visualizing the reaction of *N. alata* pitchers towards chitin and BSA.

4 Results and discussion

4.1 Stimulation with chitin

The used method works well to determine the presence of nepenthesin enzyme activity over time. The stimulation of *N. alata* with chitin worked well, as shown in Figure 7.

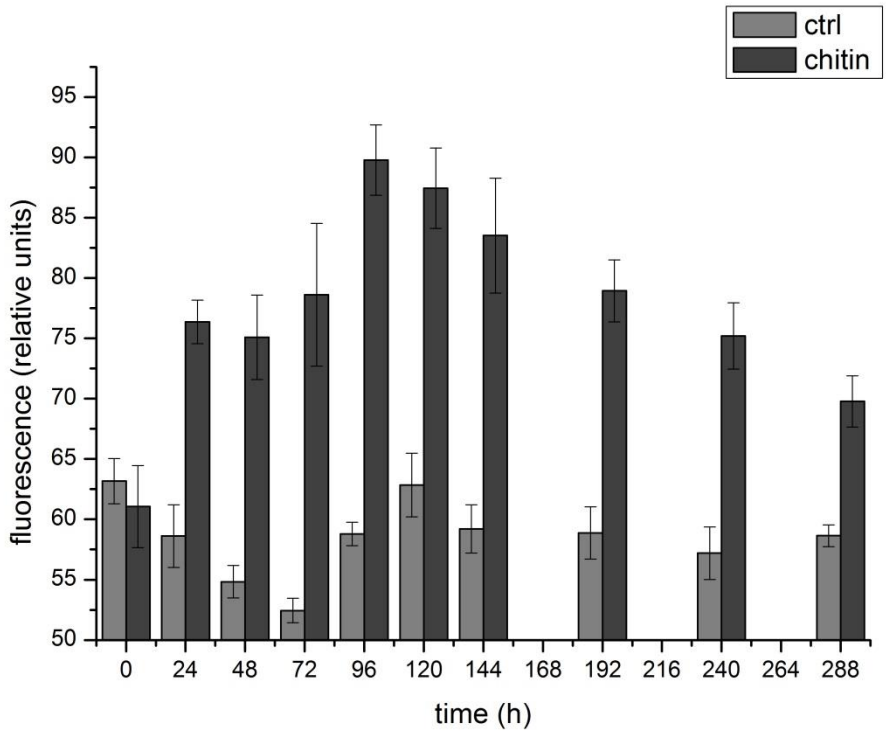

Figure 7: Nepenthesin activity in pitchers after treatment with 1 mg/ml chitin. The samples of pitcher fluid were taken every 24 hours at 2 pm until 144 h, followed by 48 hour intervals until 288 h. The sample at 0 h was taken directly before the stimulation. The amount of PFU-093 used for the measurement was 8 mM. The diagram shows the control and the chitin sample, each calculated with the data from three pitchers measured in triplicate.

The plant reacts to chitin indicated by higher nepenthesin activity in the digestive fluid over time. The enzymatic activity increases continuously and passes an optimum at 96 h whereas the control samples are rather constant over time and the here observed decrease and following ascend at 72 h and 120 h are most likely due to the setup of the experiment. An explanation can be that after removing the samples the volume is filled up again with 25 mM KCl, which dilutes the first samples. I think the plant gets used to the loss of enzymes. After all in nature similar things can happen. Either a dilution due to rain during the rainy season or a leak due to damage of the pitcher can

cause a regular loss of enzymes. To correct this from happening, the plant expresses more enzymes instead. Interestingly, the control plants need about the same time (96 h to 120 h) to correct the dilutions of pitcher fluid as the stimulated plants need to react to the chitin. The only difference is in the strength of that reaction. The *N. alata* is apparently capable to differentiate between KCl with no nutritional value, and chitin, which it can digest and use.

Table 5: T-tests between control and chitin stimulated pitchers at different time points. The group name consists of the data group (ctrl for control, c for chitin stimulated pitchers) and the time point (0 h to 288 h). The tests were done in the program SigmaPlot.

Group Name	N	Mean	Std Dev	SEM	
ctrl0	9	63,167	5,596	1,865	t(16) = 0.543
c0	9	61,056	10,238	3,413	P = 0.595
ctrl24	9	58,611	7,759	2,586	t(16)= -5.635
c24	9	76,361	5,394	1,798	P = <0.001
ctrl48	9	54,833	4,049	1,35	t(16) = -5.398
c48	9	75,083	10,501	3,5	P = <0.001
ctrl72	9	52,444	3,041	1,014	t(16) = -4.
c72	9	78,611	17,73	5,91	P = <0.001
ctrl96	9	58,778	2,906	0,969	t(16) = -10.119
c96	9	89,778	8,719	2,906	P = <0.001
ctrl120	9	62,833	7,925	2,642	t(16) = -5.786
c120	9	87,444	10,001	3,334	P = <0.001
ctrl144	9	59,194	5,994	1,998	t(16) = -4.708
c144	9	83,528	14,302	4,767	P = <0.001
ctrl192	9	58,861	6,501	2,167	t(16) = -5.974
c192	9	78,944	7,71	2,57	P = <0.001
ctrl240	9	57,194	6,508	2,169	t(16) = -5.140
c240	9	75,194	8,247	2,749	P = <0.001
ctrl288	9	58,639	2,707	0,902	t(16) = -4.820
c288	9	69,778	6,382	2,127	P = <0.001

Both the control pitchers (M = 63.167, SD = 5.596) and the stimulated ones (M = 61.056, SD = 10.230) were tested at 0 h. The resulting measurements showed no significant difference between the two groups (t(16) = 0.543, P = 0.595). That means the experiment starts with similar data.

At 24 h the stimulated group shows higher results (M = 76,361, SD = 5,394) than the control group (M = 58,611, SD = 7,759). There is a significant statistical difference between the two groups (t(16) = -5.635, P = <0.001). This means, that the chitin stimulation significantly increases the activity of nepenthesins in *N. alata* pitchers. The significant statistical difference exists also between the two groups at the following time points, a noted in Table 5.

Table 6: ANOVA-test of control measurements. The group name consists of "ctrl" for control and the numbers refer to the time points. The test was done in the program SigmaPlot.

Group	N	Missing	Median	25%	75%
ctrl0	9	0	64	57,875	67,625
ctrl24	9	0	56,5	51,875	66,375
ctrl48	9	0	52,5	52	59,25
ctrl72	9	0	51,75	50,5	55
ctrl96	9	0	59,25	55,875	61,125
ctrl120	9	0	59,75	56,875	67,75
ctrl144	9	0	58,75	53,125	65,75
ctrl192	9	0	54,5	53,5	65,75
ctrl240	9	0	56,75	53	59,25
ctrl288	9	0	59,75	57	60,75
H = 24.073 with 9 degrees of freedom. (P = 0.004)					

In Figure 7 a decrease in nepenthesin activity is visible for the control pitchers. The minimum is at 72 h and then the activity ascends again. To check if this is significant, an ANOVA test was done with the control data (Table 6). The different time points were treated as different data groups. The differences in the median values among the different time points are greater than would be expected by chance; there is a statistically significant difference (P = 0.004). That means that the decrease in activity is a reaction of the pitcher plants to the experimental procedures. It is to note, that the decrease would happen in the stimulated pitchers too. So the nepenthesin activity between 24 h and 96 h would be stronger in reality. Considering that, the maximum of nepenthesin activity in case of chitin stimulation is between 72 h and 96 h.

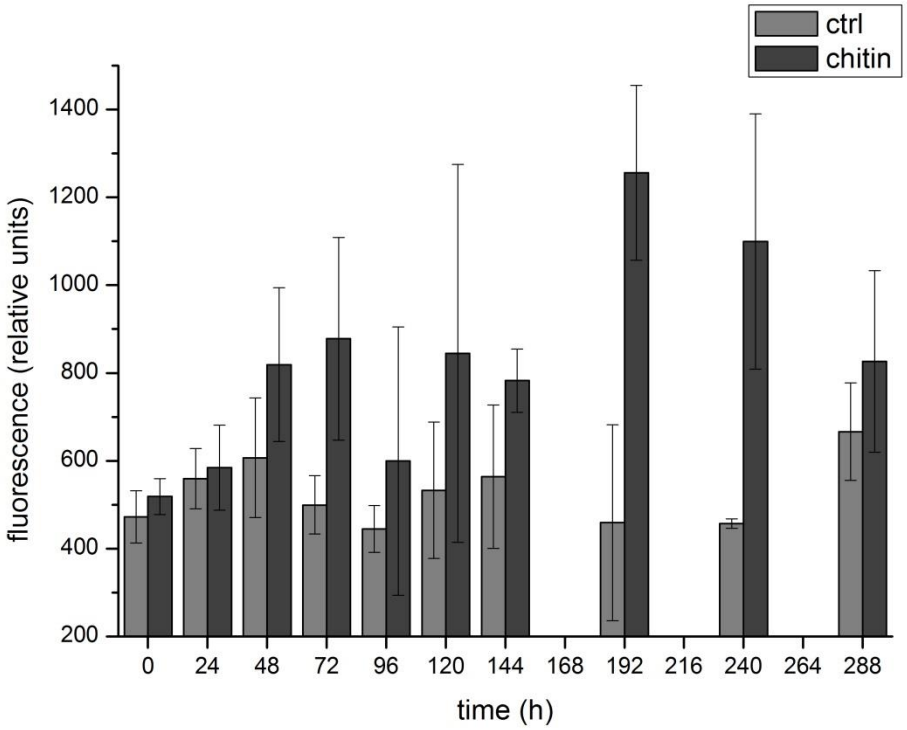

Figure 8: Nepenthesin activity in pitchers after treatment with 1 mg/ml chitin, 80 mM PFU-093. The samples of pitcher fluid were taken every 24 hours at 2 pm until the test point 144 h, then in 48 hour intervals until 288 h. The sample 0 h was taken directly before the stimulation. In contrast to the usage of 8 mM PFU-093 in the first measurement, here 80 mM of PFU-093 per sample was used. The different time points were measured with only single samples. The error bars represent technical replicates.

In the first run only 8 mM of PFU-093 were used instead of 80 mM. Due to that the analysis was repeated with the remaining samples, i.e. only one measurement per plant and time point. Therefore, a statistical analysis was not possible. However, it is still visible that the *N. alata* reacts strongly towards chitin.

The difference between the control (8 mM: 55 – 60 relative units) and maximum of stimulation (95 relative units) point to doubled nepenthesin activity after four days. The activity of nepenthesin returned to initial level afterwards and should reach the amount of before (0 h) at the end of the second week. The results of both measurements show that it is possible to carry out the experiment with using a low amount of PFU-093 (8 mM), in case an overview of the nepenthesin activity is needed. However, I recommend using about 10 mM for chitin stimulation, to avoid cutting of the maximum activity in the measurements.

4.2 Stimulation with BSA

The stimulation with BSA worked as well, as shown in Figure 9. In case of this experiment the pitchers were stimulated with the same amount of BSA, not the same concentration – as done with chitin. Pitcher C had about half of the volume than the pitchers A and B. Thus, it was possible to examine differences in the activity of nepenthesins by various concentrations of stimulant.

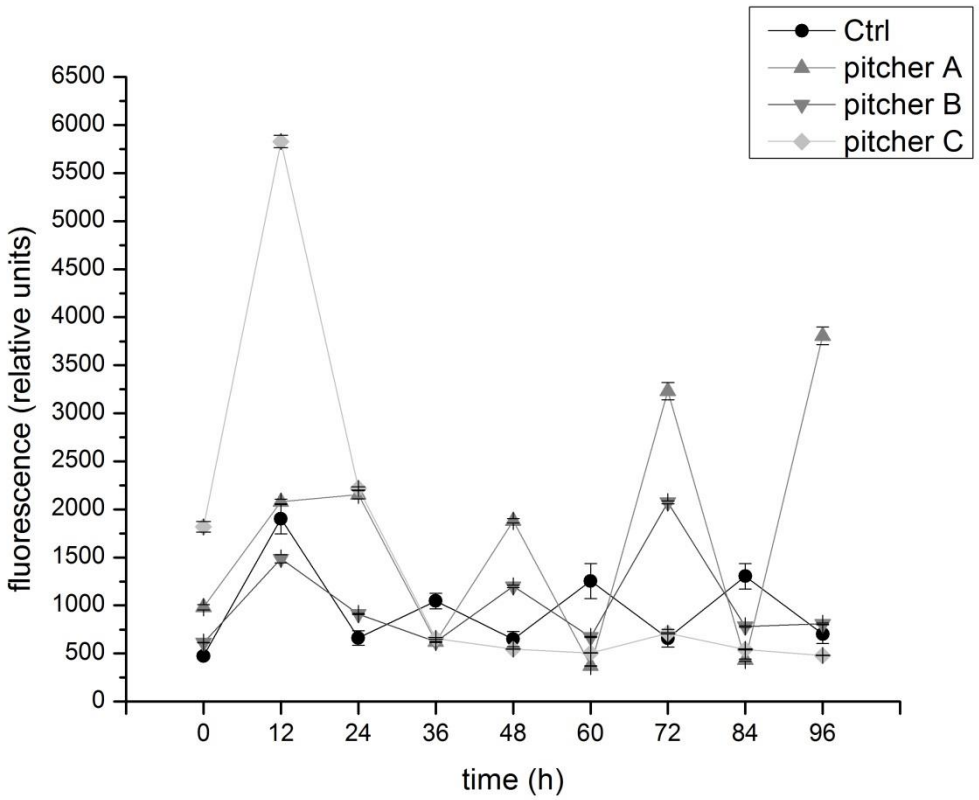

Figure 9: Nepenthesin activity in pitchers after BSA treatment. The samples of pitcher fluids were taken every 12 hours, at 10 am and 10 pm, until 96 h. The sample at 0 h was taken directly before stimulation with 1 mg BSA per pitcher. The amount of PFU-093 used for the measurement was 80 mM. The diagram shows the control, calculated with the data from three pitchers measured in triplicate, and the three different BSA samples, calculated from the measured technical triplicates each.

Figure 9 shows that the different pitchers react different in strength and time. The nepenthesin expression seems to happen faster compared with chitin treatment. The nepenthesin activity is higher when proteins are added to the pitcher fluid. Due to the fact that the same amount of BSA was used in different volumes of digestive fluid, it is not possible to align all (A-C) BSA-stimulated pitchers as demonstrated in Figure 9.

The final concentration in the pitchers is different and so the nepenthesin activity, too. For a better understanding, the following Figures 10 to 12 and Figure 15 show the control, as well as the stimulated pitchers, on their own. For the comparable pitchers A and B no clear nepenthesin activity optimum can be found (Figures 11 and 12) in contrast to pitcher C with a defined time optimum at 12 h after stimulation (Figure 13).In Figures 10 to 12 a zig-zag-pattern is visible suggesting an underlying circadian rhythm. This rhythm repeats in 24 h sequences.

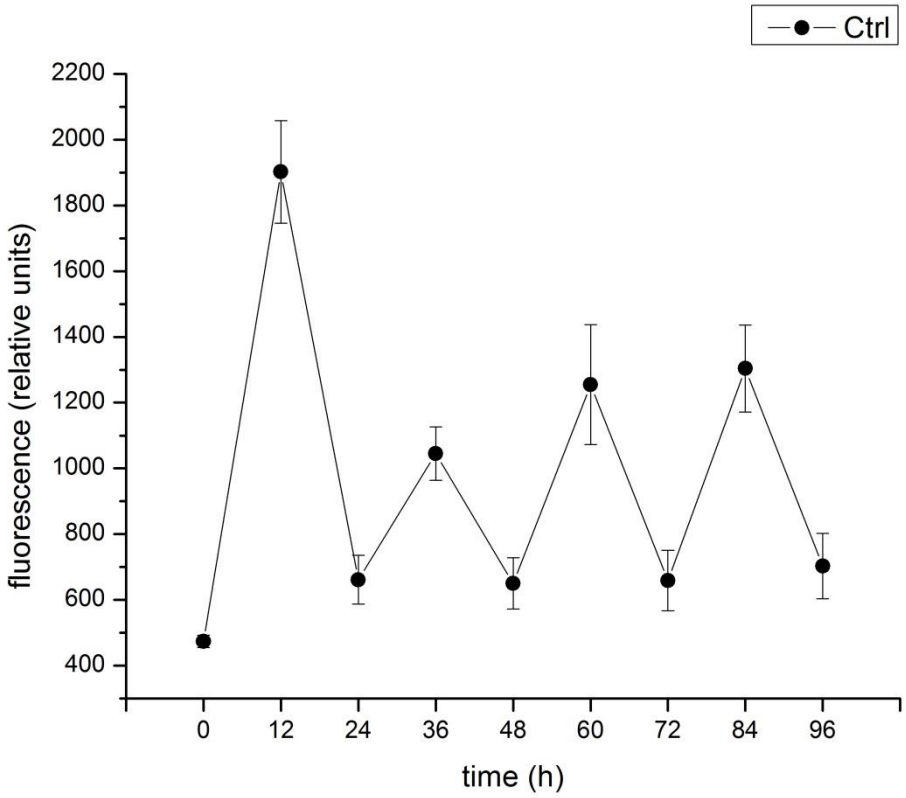

Figure 10: Nepenthesin activity in control pitchers. The samples of pitcher fluid were taken every 12 hours at 10 am and 10 pm until the test point 96 h, as well as a sample at 6 h. The sample 0 h was taken directly before the stimulation. The amount of PFU-093 used for the measurement was 80 mM. The diagram shows the control, calculated with the data from three pitchers measured in technical triplicates.

The control pitchers (Figure 10) react to the KCl at 12 h. After all the KCl replaced the taken volume and diluted the pitcher fluid. Due to that the concentration of enzymes in the digestive fluid differs. I suspect that *Nepenthes* expresses more nepenthesins and likely other enzymes than average for the plants to rectify the taken amount. After about a day the plants seem to get used to the varying concentrations and the control measurements are back at average levels.

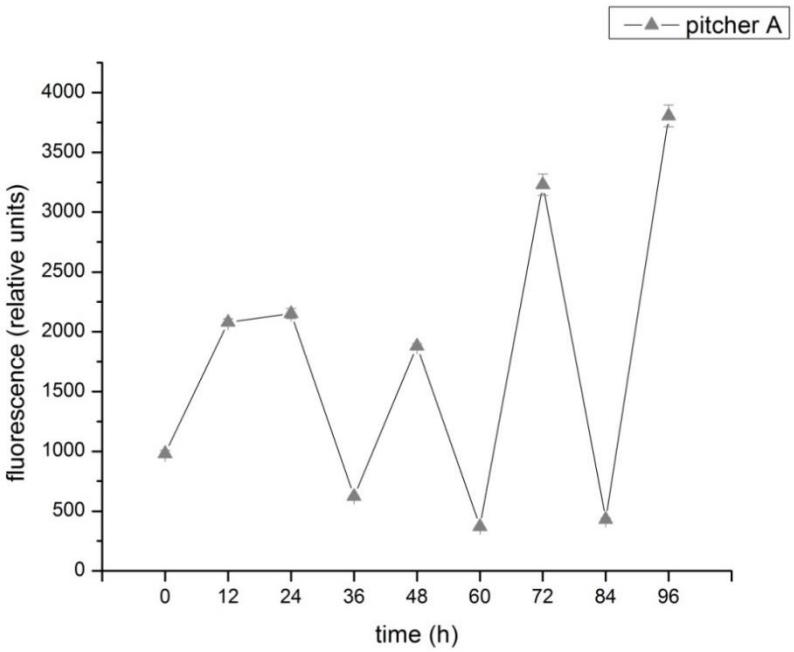

Figure 11: Nepenthesin activity in pitcher A after BSA (1 mg) treatment. The samples of pitcher fluid were taken every 12 hours at 10 am and 10 pm until the test point 96 h, as well as a sample at 6 h. The sample 0 h was taken directly before stimulation with 1 mg BSA per pitcher. The amount of PFU-093 used for the measurement was 80 mM. The diagram shows pitcher A, measured in technical triplicates.

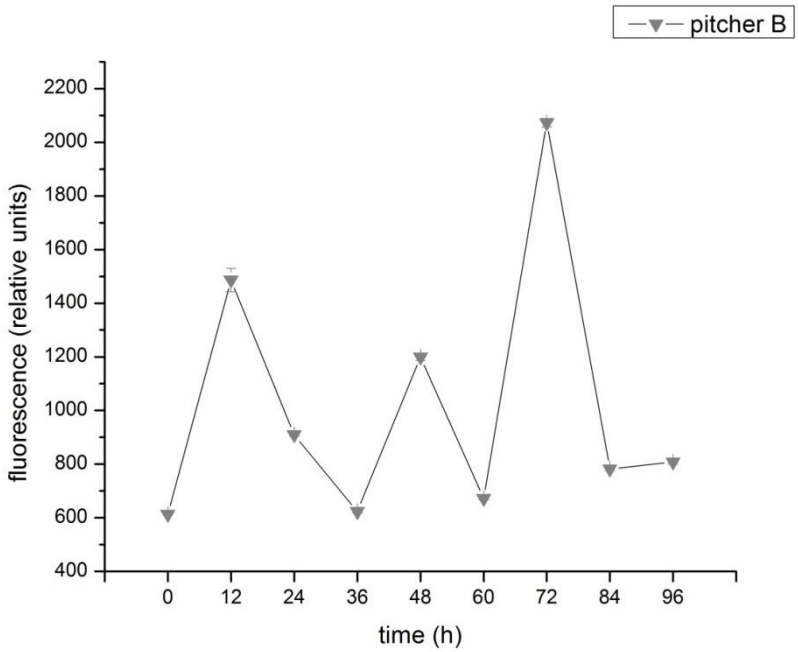

Figure 12: Nepenthesin activity in pitcher B after BSA (1 mg) treatment. The samples of pitcher fluid were taken every 12 hours at 10 am and 10 pm until the test point 96 h. The sample 0 h was taken directly before stimulation with 1 mg BSA per pitcher. The amount of PFU-093 used for the measurement was 80 mM. The diagram shows pitcher B, measured in technical triplicates.

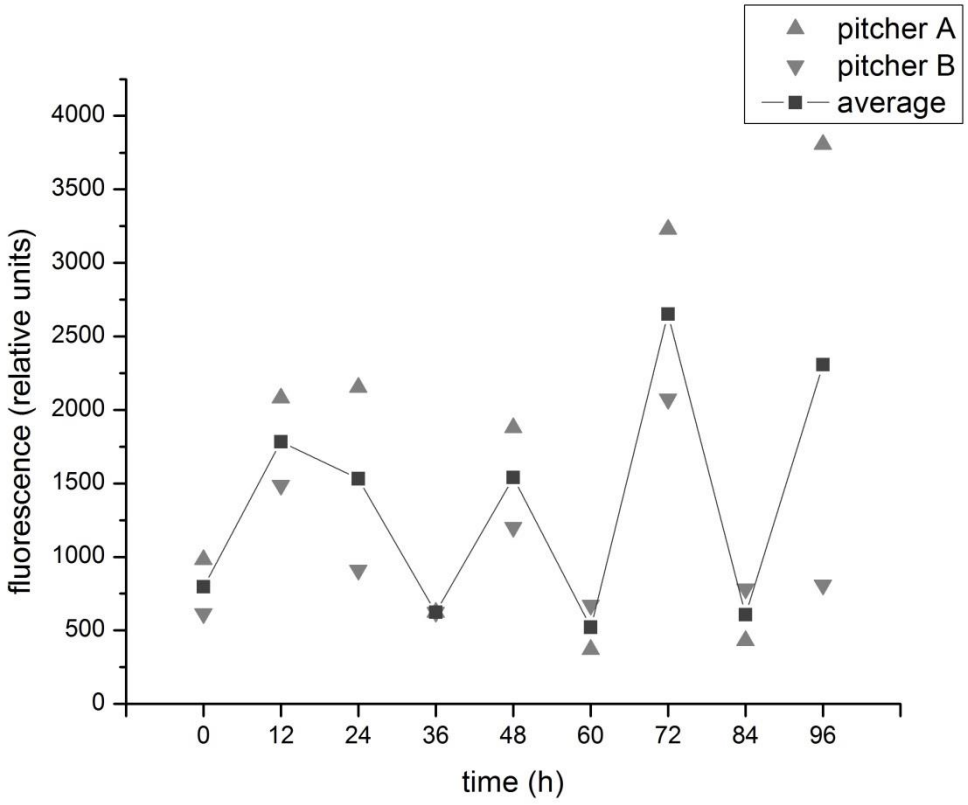

Figure 13: Averaged nepenthesin activity in pitchers A and B after BSA treatment. The samples of pitcher fluid were taken every 12 hours at 10 am and 10 pm until the test point 96 h. The sample 0 h was taken directly before the stimulation with 1 mg BSA per pitcher. The amount of PFU-093 used for the measurement was 80 mM. The diagram shows pitchers A and B, measured in technical triplicates, as well as the average of these two pitchers.

In pitcher A (Figure 11) and B (Figure 12) a kind of circadian rhythm is suggested. Due to their similarity in size and fluid volume an average in nepenthesin activity was calculated (Figure 13).

There is an ascend in nepenthesin activity at 12 h and a slow descend to 36 h. Afterwards the activity shows a zig-zag-pattern similar to the control (Figure 10). In Figure 14 the average of pitchers A and B is shown, directly compared to the control. A BSA-induced shift of 12 h is clearly visible. After the stimulation with BSA a peak is at 12 h detected for both control and BSA-treatment. The activity descends directly after the peak for the control data, a minimum reached is at 24 h. Meanwhile the descending of activity is slower for the stimulated pitchers; a minimum is at 36 h. The BSA shifts the activity of nepenthesin in *N. alata* for 12 h in case of stimulation with 1 mg per pitcher (estimated 0.20 mg/ml).

As the pitcher fluid samples were taken at 10 am and 10 pm, the later time point was 90 min after the light was turned off. Moreover, the BSA-induced shift is 12 h, so there is no connection between this circadian rhythm and the daylight cycle for *N. alata*. Would the cycle be connected to the daylight cycle, then there would be either no shift or a shift of 24 hours.

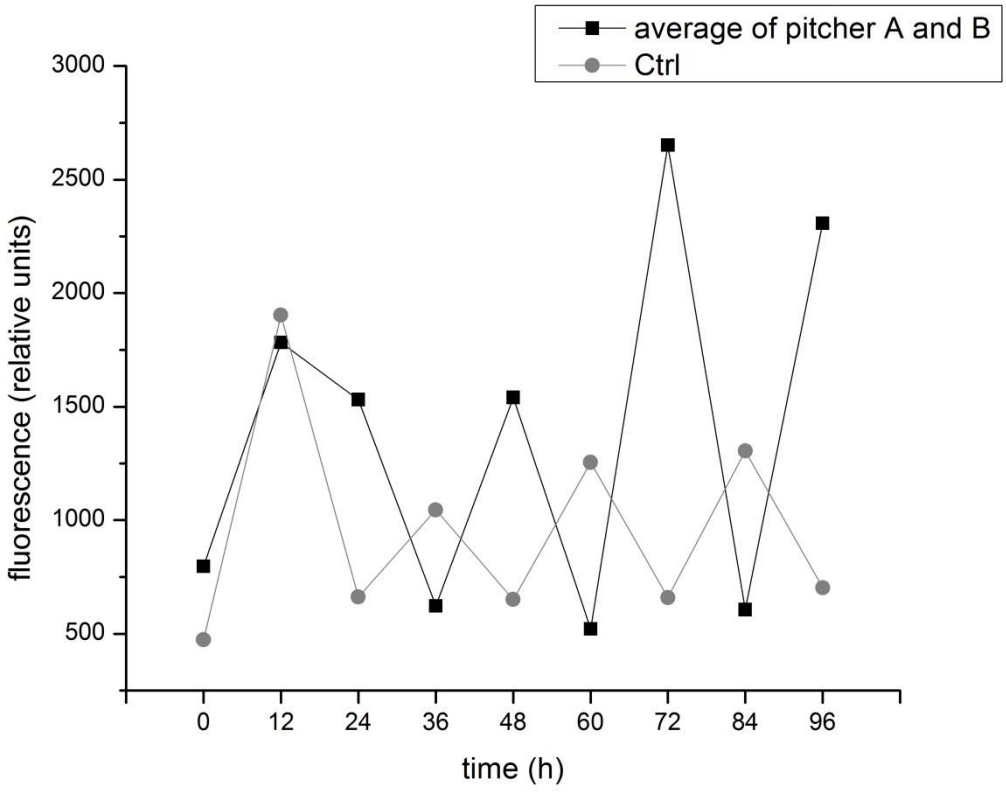

Figure 14: Averaged nepenthesin activity of BSA-stimulated pitcher A and B compared with control. The samples of pitcher fluid were taken every 12 hours at 10 am and 10 pm until the test point 96 h. The sample 0 h was taken directly before stimulation with 1 mg BSA per pitcher. The amount of PFU-093 used for the measurement was 80 mM. The diagram shows the average of pitchers A and B, compared to the control measurements.

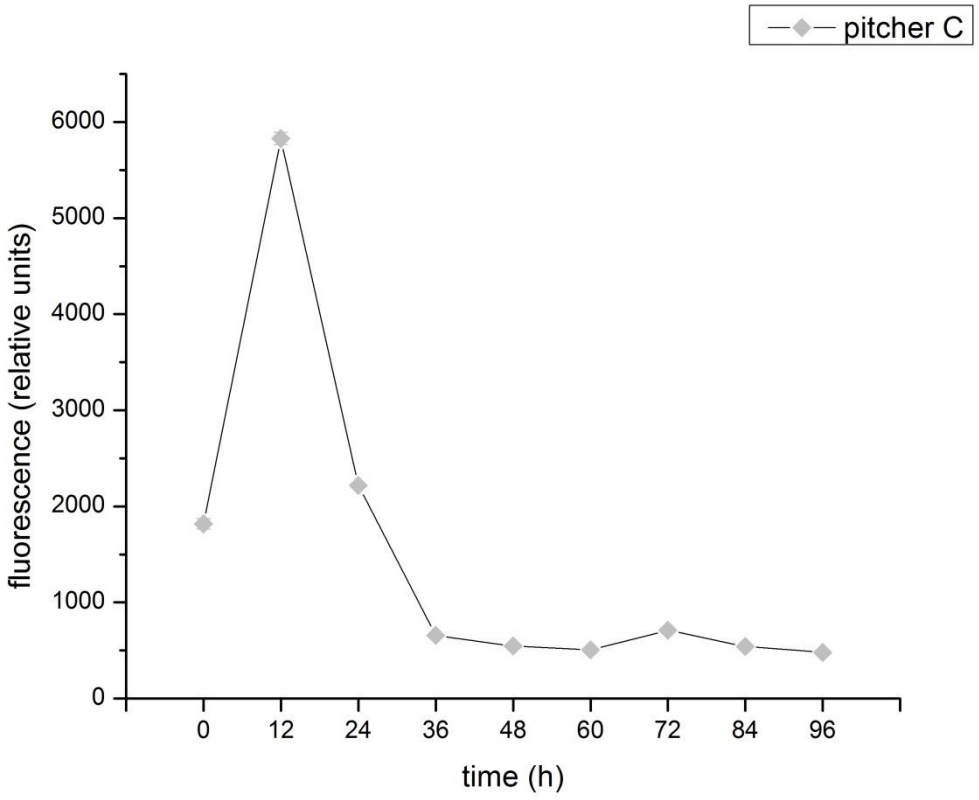

Figure 15: Nepenthesin activity in pitcher C after BSA (1 mg) treatment. The samples of pitcher fluid were taken every 12 hours at 10 am and 10 pm until the test point 96 h. The sample 0 h was taken directly before stimulation with 1 mg BSA per pitcher. The amount of PFU-093 used for the measurement was 80 mM. The diagram shows pitcher C, measured in technical triplicates.

In Figure 15 the results of pitcher C are shown. In this case the stimulation was done with a higher concentration of BSA (estimated 0.30 mg/ml). In this pitcher the strength of nepenthesin-dependent fluorescence at 12 h is 6000 relative units, six-times higher than the other data points (about 1000 relative units). The only difference is the reference (0 h) with 2000 relative units.

Perhaps due to the strong activity after only 12 h, the return to the former activity (0 h, 2000 relative units) takes some time and the following activity is lower than before. Unlike pitchers A and B, no rhythm is visible. There is only a small peak at 72 h, which could be a slow return to a rhythm. Maybe the higher concentration of BSA in pitcher C did not only shift the rhythm, but rather temporarily disabled it, similar to a refractory phase in in nerve, muscle and sensory cells.

While pitchers A and B (Figure 13 and 14) show that even after BSA stimulation the plants come back to a daily cycle in their activity of nepenthesin, pitcher C (Figure 15) shows a faster and stronger reaction. Whether there is a rhythm for other enzymes in the digestive fluid or not needs to be investigated. In addition, it should be studied whether this rhythmic activity changes of nepenthesin depends on different transcript level or amount of enzyme or on protein modifications. Due to the difference in the activity of nepenthesin and the rhythm between the different concentrations of BSA, it would be worth investigating at what concentration the rhythm is disabled (or perhaps shifted to a point of time I did not measure anymore).

Compared to the stimulation with chitin, the nepenthesin activity occurs faster and stronger. The difference in time can be explained by the nature of the plants. When confronted with chitin, they expect an insect in their pitcher. For this the first logical step is to remove the chitin shell with chitinases and later digest the proteins with proteases like nepenthesins. Due to that the nepenthesin activity is most likely delayed, compared to the stimulation with BSA.

4.3 Further research

Using PFU-093 to determine the kinetics of nepenthesin was successful and can be done for other stimulants. But it is recommended to toughly check the *N. alata* before. There are plenty differences even in the same growth conditions. The plants themselves have different sizes and numbers of open, closed and perishing pitchers which might influence the plants reaction. It is also not possible to check whether the plants have enough nutrients or not – and thus react faster due to the need for nitrates or not. While here I tried to find similar enough pitchers, there were always some differences. The volume of digestive fluid is different for every pitcher, even if they have the same size. This is shown in the results of the BSA stimulation. Due to the somewhat different volumes, the concentration of BSA was different. Thus, probably the expression of nepenthesin was different. It is recommended to use the same concentration of stimulant, when doing this experiment.

In this work only the reaction to chitin and BSA were analysed. There are multiple stimulants yet to check, such as simple sugars (with and without nitrate), complex sugars like starch and chlorophyll or different structured proteins, as well as lipids. After all, there are different species of *Nepenthes*, which are specialized on various sources of nutrition such as falling leaves in case of *N. ampullaria* or feces in case of *N. lowii, N. rajah* and *N. macrophylla*. Due to these different specialized species it is possible that the plants reactions are different towards various stimulants.

As stated at the end of 4.2 there are also non-answered questions about the putative circadian rhythm for nepenthesin activity in the pitchers of *N. alata*. Are all digestive fluid enzymes controlled like this? Are the enzymes expressed at the same time or in a pattern? Do the rhythmic activity changes of nepenthesin depend on different transcript levels or amount of enzyme or on protein modifications? What is the reason for this cycle – might the plants be busy with attracting new prey half the day? Altogether there are plenty new questions to answer.

5 Summary

In this work the activity of nepenthesins was studied. Nepenthesins are aspartic proteases and found in the digestive fluid of carnivorous *Nepenthes* plants. To determine the enzyme activity a FRET (fluorescent resonance energy transfer)-based method (Buch *et al.* 2015) was used. It is possible to observe the nepenthesin activity over different periods of time. In this work, the species *Nepenthes alata* was used. These plants were stimulated with either chitin or BSA, respectively. By comparing the different stimulants for the expression of nepenthesin, it is possible to determine differences in the protease activity due to various nutritional sources. After taking and freezing samples over a chosen period the artificial nepenthesin substrate PFU-093 was added, the mixture incubated and used for microplate reading. The expressed nepenthesins cleaved the substrate and released an attached quencher. The higher the resulting relative fluorescence, the more nepenthesin is in the sample.

In case of the chitin stimulation, the *Nepenthes* pitchers were stimulated with 1 mg/ml chitin. The samples were taken every 24 h. The maximum nepenthesin activity was detected after about 96 h. When compared with the initial activity, the activity doubles in that time and reduces again afterwards.

The results after BSA stimulation were slightly different. The pitchers were stimulated with 1 mg per pitcher. This results in different concentrations per pitcher and divergent data. Due to that the pitchers were compared separately. The samples were taken every 12 h. The diagrams show a zig-zag-pattern, which fluctuates over 24 h, suggesting a circadian rhythm. When comparing the data, it is shown that the activity of nepenthesin is after 12 h at its maximum of 6000 relevant units for pitcher C, which was treated with a higher concentration of BSA. Afterward the activity descends in the next 12 h and stays constantly at 1000 relevant units until the end of the test run. For the other pitchers (A and B) no clear optimum can be found, due to the circadian rhythm. Compared to the reference of pitcher A and B (0 h) the activity of nepenthesin doubles at various points of time.

Compared to chitin, which was added in a bigger amount than BSA, the activity is not only faster but also stronger in case of BSA. This is most likely due to the fact, that the proteases digest proteins. When the Nepenthes plants are stimulated with chitin, the plants react like they do towards an insect. First the chitin shell needs to be removed by chitinases and only then the proteins can be digested. So the nepenthesin activity is delayed.

For further research other stimulants could be used. Since the *Nepenthes* need nitrogen, it would be worth knowing the protease activity when comparing stimulants with and without nitrogen. The day cycle of *Nepenthes alata* requires closer investigation and more information, too.

6 Zusammenfassung

In dieser Arbeit wurde die Aktivität von Nepenthesinen untersucht. Nepenthesine sind eine Subkategorie der Aspartatproteasen und kommen in *Nepenthes*-Pflanzen vor. Sie sind Bestandteil der Enzymmischung in der Kannenflüssigkeit. Bisher konnte die Aktivität der Nepenthesine nicht beobachtet werden, da sie nur in geringer Konzentration vorhanden sind. Mit der FRET (Fluoreszenz-Resonanzenergietransfer)-basierten Methode beschrieben in Buch *et al.* (2015) ist dies möglich. Hierfür wurden *Nepenthes alata* Pflanzen stimuliert. Die Stimulantien waren Chitin und BSA. Chitin wurde gewählt, da es der Hauptbestandteil von Insektenpanzern ist. So kann untersucht werden, ob Nepenthesin aktiviert wird, wenn die Pflanzen ein Insekt in der Kanne gefangen haben. BSA wurde genutzt um die Reaktion auf Proteine zu untersuchen, die von den Nepenthesinen verdaut werden sollen.

Die Nepenthesin-Aktivität nach der Stimulation mit Chitin steigt langsam an und erreicht ein Maximum bei circa 96 h. Zu diesem Zeitpunkt ist die Aktivität etwa doppelt so stark wie bei der Kontrolle. Danach reduziert die *Nepenthes*-Pflanze die Aktivität von Nepenthesin wieder auf das vorherige Niveau (0 h).

Bei BSA ist die induzierte Aktivität von Nepenthesin stärker, wahrscheinlich da es sich hier um Proteine handelt, die von Nepenthesinen verdaut werden können. In den Messungen zeigt sich ein Zick-Zack-Muster im 24 h-Rhythmus. Dies lässt auf einen Tageszyklus oder circadianen Rhythmus bezüglich der Nepenthesin Aktivität in *Nepenthes alata* schließen. Beobachtet man nur die Messwerte in 24 h-Abschnitten, beginnend mit 12 h (und 0 h als Referenz), dann ergibt sich ein sehr schneller Anstieg bei der Nepenthesin-Aktivität. Bereits nach 12 h ist die Aktivität maximal und reduziert sich danach langsam auf Normalniveau. Da in den Kannen unterschiedliche Konzentrationen vorlagen, wurden die Ergebnisse separat betrachtet. Bei zwei Kannen verdoppelt sich die Aktivität in 12 h, bei der dritten versechsfacht sie sich. Bei letzterer liegt die dreifache Konzentration an BSA im Kannensaft vor. Die Menge an eingesetzten BSA ist geringer, als an Chitin. So vermute ich, dass auch die Aktivität von Nepenthesin nicht nur schneller sondern auch stärker als bei der Stimulation mit Chitin ist. Dies ist logisch, da Nepenthesine Proteasen sind und Proteine verdauen sollen. Bei der Stimulation mit Chitin wird ein Insekt in der Kanne vorgetäuscht. Zuerst müsste der Panzer von Chitinasen zersetzt werden, bevor die Proteine im Inneren verdaut werden können. Entsprechend ist die Reaktion langsamer.

Ich erinnere daran, bei diesem oder einem ähnlichen Versuch Kannen zu stimulieren, die sich in Größe, Alter und Volumen möglichst ähnlich sind. Außerdem sollte darauf geachtet werden mit derselben Konzentration an Stimulans zu arbeiten, nicht nur derselben Menge. Obwohl es einen Grund gab, die unterschiedlichen Konzentrationen an BSA zu testen, ist es immer empfehlenswert in einzelnen Testreihen mit identischen Bedingungen zu arbeiten, um statistisch verwertbare Ergebnisse zu erhalten. Es wäre wissenswert, ob es Unterschiede in der Nepenthesin-Aktivität bei anderen Stimulantien gibt, besonders welchen mit und ohne Stickstoff. Immerhin ist das Ziel der Pflanze, Stickstoff zu erhalten – können *Nepenthes*-Pflanzen unterscheiden ob etwas für sie nahrhaft ist oder nicht? Außerdem sollte der Tageszyklus von *Nepenthes alata* genauer untersucht werden. Die Hauptaufgabe dieser Arbeit – ob man mit dem FRET-basierten Verfahren die Aktivität von Nepenthesinen bestimmen kann, und die Reaktion auf Chitin und BSA zu vergleichen – ist hier beantwortet worden.

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C Statement of authorship

I hereby certify that I wrote the present work independently and have used no other than the sources and aids stated. I declare that all elaboration and contemplation, which were taken verbatim or in spirit from other work, are indicated. This work was not jet part of a study, an academic performance or an examination performance in an identical or similar version.