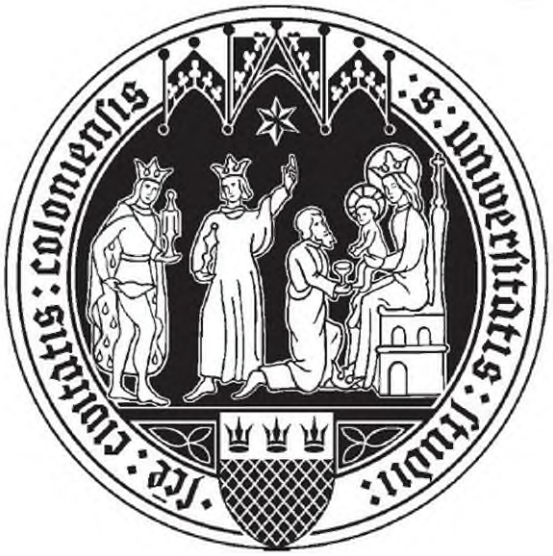

Local adaptation of *Daphnia magna* to protease inhibitors in cyanobacteria –

The characterization of the serine protease CT448

Inaugural-Dissertation

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vorgelegt von

Jacqueline Lange

aus Hagen

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Gutachter: Prof. Dr. Eric von Elert

Prof. Dr. Michael Bonkowski

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Abstract

Cyanobacteria are aquatic photoautotrophic organisms that proliferate in masses, so called blooms, under favorable conditions. Cyanobacterial blooms have increased in freshwater ecosystems due to a combination of eutrophication of waterbodies, rising temperatures and high levels of CO_2 with predicted increases in frequency along with a prevalence of potentially toxin-producing cyanobacteria. Many cyanobacterial genera produce toxic secondary metabolites and have been documented as harmful for humans and livestock when they are exposed to water with high concentrations of cyanobacteria. Moreover, cyanobacteria are known to negatively impact the fitness and somatic growth and increase the mortality of the freshwater Cladocera *Daphnia* sp., an important linker between primary producers and higher trophic levels. During blooms cyanobacteria represent a large proportion of the phytoplankton and thus provide the primary food source for the non-selective filter-feeding *Daphnia*. The negative effects of toxic canobacteria can be partially attributed to protease inhibitors (PIs), secondary metabolites that disrupt digestive proteases when ingested and are frequently found in surface blooms of cyanobacteria.

Daphnia's tolerance has been demonstrated to increase to toxic cyanobacteria through coexistence over time and space, which is referred to as local adaptation. A recent study demonstrated that positive selection at specific gene loci encoding for digestive proteases drove the evolutionary adaptation of a Swedish *Daphnia magna* population to cyanobacteria. The persistence of cyanobacterial blooms is a major challenge for lake management and requires multifactorial approaches. One potential approach would be the identification of *Daphnia* with increased tolerance to protease inhibitors produced by cyanobacteria, as these adapted *Daphnia* could be used to suppress and control toxic cyanobacterial blooms. Understanding the molecular basis of such local adaptation could offer insights into how proteases are mechanistically inhibited by secondary metabolites of cyanobacteria.

The aim of this dissertation is to elucidate changes on the protein level that explain tolerance of a *Daphnia magna* population to cyanobacterial protease inhibitors of a co-occurring *Microcystis* strain. Although some progress has been made in understanding the adaptation of *Daphnia* to cyanobacterial protease inhibitors, further data are required to gain a more comprehensive understanding.

In order to examine the mechanisms that contribute to the increased tolerance of a Swedish *D. magna* population to cyanobacterial protease inhibitors, a susceptible Polish population was chosen for comparison. I expressed the digestive serine protease CT448 from *D. magna*, which has been previously demonstrated to have undergone positive selection, in *Sf*21 insect cells. Subsequently, the purified precursor of this protease could be activated through tryptic

digestion. This work represents the first successful expression of an active serine protease from crustaceans that function in the gut of *Daphnia*. Proteomic analysis identified the substrate specificity and revealed that CT448 is a chymotrypsin-like elastase, contrary to previous publications. This study also demonstrated that CT448 is a target of cyanobacterial inhibitors. The studies of the chymotrypsin-like elastase CT448 were focused on investigating the structural properties using the machine learning tool AlphaFold 3. A primary objective was to gain insight into the structural characteristics of CT448, which exhibits a distinctive amino acid substitution at position 34 between the two *D. magna* populations that were the subject of this study. The predicted structure of CT448 indicates that the pro-peptide could play a role in the functioning of the protease and that the population-specific amino acid of the Swedish population at position 34 may contribute to the stability of CT448 through a salt bridge. Further comprehension of CT448's properties could be achieved in future studies via a crystal structure and inhibitor co-crystallization.

A comparative analysis of single Swedish and Polish clones of *D. magna* revealed that the Swedish clones exhibited a higher fitness compared to the Polish clones when exposed to the co-occurring *Microcystis* sp. BM25 and are capable to dominate a multiclonal experimental population in the presence of this toxic cyanobacterium. The study suggests that protease inhibitors (PI) coexisting with a tolerant Swedish *Daphnia* population may be involved in increased fitness. Hence, the isolation of those PIs can provide insights into the adaptive mechanisms of *Daphnia*. I modified the methodology of elastase activity assays in *D. magna* homogenates for the identification of cyanobacterial PIs and presented results on the inhibitory effect of methanol on elastase activity. Following the methodological adaptation, elastase activity assays were conducted using homogenates of *D. magna* clones from both populations, with fractions of *Microcystis* sp. BM25 cell extract. A population effect concerning elastase activity between the Swedish and Polish *D. magna* homogenates was not observed.

This dissertation provided new characteristics and insights into a serine protease that has been subjected to positive selection driven by toxic cyanobacteria. A combination of experimental techniques, advanced biochemical methodologies and artificial intelligence technology were employed to investigate the molecular mechanisms of adaptation in *Daphnia*. The results demonstrate novel properties and characteristics of elastases in *D. magna*, offering a novel perspective on their function in tolerance to toxic cyanobacteria.

General introduction

The term "anthropogenic climate change" describes the alteration of the global climate system by human activities, such as increased atmospheric concentration of greenhouse gases. Climate change is characterized by an increase in global average temperatures, or global warming, and impacts weather patterns, sea levels and ecosystems. In association with eutrophication, caused by extensive fertilization of agricultural lands, which leaches nutrients into aquatic ecosystems, human actions have extensive impacts on freshwater ecosystems, posing significant challenges to their biodiversity and functioning (Kernan et al. 2010; Moss et al. 2011). Freshwater bodies, including rivers, lakes, wetlands and small waterbodies, contain only around 0.01% of the world's water and cover only about 0.8% of the Earth's surface (Gleick 1996) yet play a crucial role in supporting biodiversity, providing ecosystem services, and sustaining human life (reviewed by Dudgeon et al. 2006).

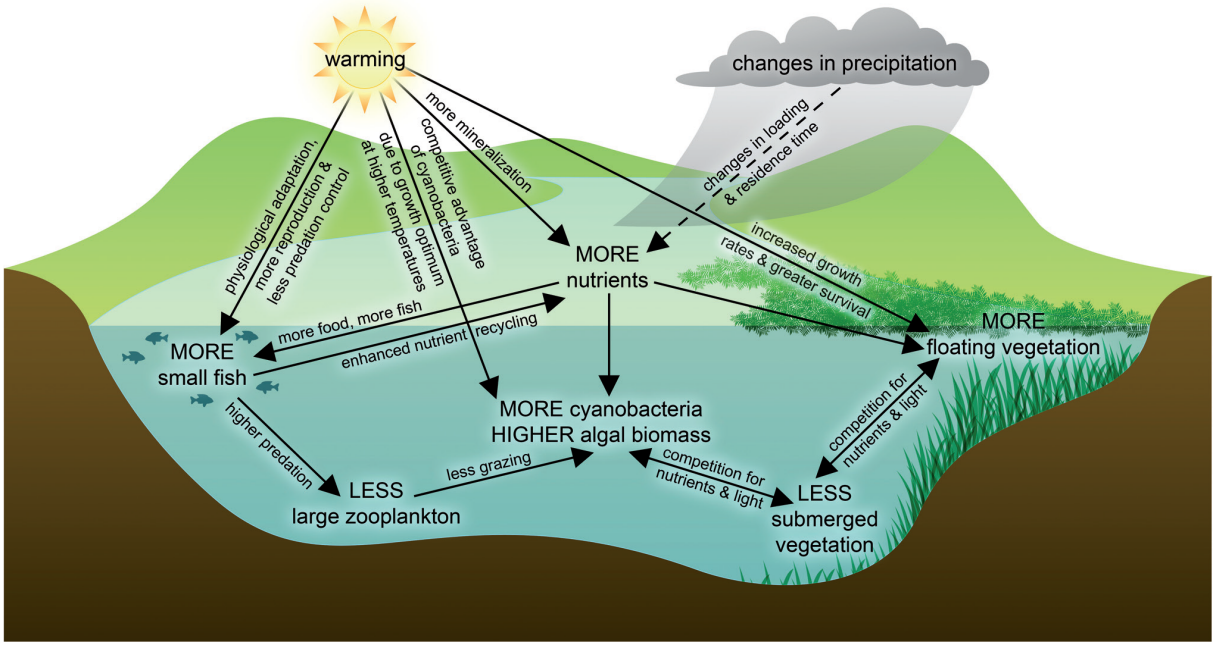


Figure 1: Established relationships of linked climate change and eutrophication symptoms (from Moss et al. 2011).

Increasing nutrient inputs and global warming are predicted to intensify the symptoms of eutrophication (Moss et al. 2011, Figure 1). One symptom of increased nutrient load and rising temperatures, along with high levels of CO_2, is the prediction of high frequencies of cyanobacterial blooms (Lampert and Sommer 1999; Downing et al. 2001; Paerl and Huisman 2008; Taranu et al. 2015) with a prevalence of potentially toxin-producing cyanobacteria (Trolle et al. 2015). It is generally accepted that cyanobacteria can cause major disruptions of the aquatic ecosystem (Christoffersen et al. 1993; Huisman et al. 2018; Zepernick et al. 2023).

This impacts trophic energy transfer of carbon from primary producers to consumers, e.g. higher trophic levels, where herbivore–plant-interactions play a key role.

The pelagic zooplankton of the genus *Daphnia* represents the most important consumer of phytoplankton. They inhabit most types of standing freshwater, where *Daphnia* species found in lakes with planktivorous fish are smaller (e.g. *D. galeata, D. cucullata, and D. hyalina*.) and larger species (e.g. *D. magna* and *D. pulex*) are usually found in freshwaters without intensive fish predation. Therewith, it serves as a food source for planktivorous fish (Luecke et al. 1990) and invertebrate predators (Pastorok 1981; Brandl 2005). Thus, *Daphnia* provide an important link in the transfer of energy from primary producers to higher trophic levels.

Daphnia is not a predator, as it differs from zooplanktonic copepods (Kerfoot 1978; Brandl 2005) in that it passively consumes food particles. Their filtering apparatus enables the unselective ingestion of small, suspended algae, (cyano)bacteria and protozoa. The size of ingestible food particles ranges from 0.1 µm to 55 µm which corresponds to the average mesh size of the filter combs and the size of the carapace gape (Geller and Müller 1981; Gophen and Geller 1984). Consequently, they are classified as suspension or filter feeders.

Their feeding mode highlights the significance of the food composition of phytoplankton for *Daphnia*. Ongoing eutrophication and climate change with high levels of CO_2 and rising temperatures have increased the frequency of cyanobacterial mass developments (Lampert and Sommer 1999; Paerl and Huisman 2008), especially of the genus *Microcystis* (reviewed by Harke et al. 2016). In addition to *Microcystis*, the cyanobacterial genera *Planktothrix* and *Anabaena* are able to form blooms under favorable conditions with severe ecological (Elser and Goldman 1991; Wilson et al. 2006) and economic consequences, particularly with regard to drinking water production (Steffensen 2008; Paerl and Otten 2013) or massive fish mortality due to depletion of oxygen after blooms (reviewed by Sevrin-Reyssac and Pletikosic 1990). Cyanobacteria possess effective mechanisms for the uptake of CO_2 and nutrients, are well-protected from light and UV radiation, and demonstrate high adaptability, enabling them to effectively utilize changing environmental conditions in aquatic ecosystems (reviewed by Paerl and Paul 2012).

The co-existence of zooplankton with toxic cyanobacteria is common in freshwater ecosystems, but functionality of plankton ecosystems is dependent upon the biological characteristics and abundance of zooplankton (reviewed by Ger et al. 2016). Hence, generalist grazers (i.e., *Daphnia*) may regulate blooms in the absence of planktivorous fish where they can reach high population densities (Sarnelle 2007). Still, phytoplankton of eutrophic lakes is dominated by cyanobacteria (Scheffer et al. 1997; Gkelis et al. 2014; Parulekar et al. 2017). Thus, the major food source for *Daphnia* is often constrained by bloom-forming and/or filamentous cyanobacteria. These cyanobacteria clog the filtering apparatus of especially

larger *Daphnia* species (e.g. *D. magna* or *D. pulex*) and increase rejection rates when feeding on filamentous cyanobacteria (DeMott et al. 2001). Furthermore, despite the inedibility, cyanobacteria are considered to be of low-quality food source due to their nutritional value and secondary metabolites, e.g. cyanotoxins. *Daphnia* grows optimal with phytoplankton rich in sterols, ω-3 fatty acids, protein, and amino acids (Peltomaa et al. 2017). Their study demonstrated that zooplankton is constantly limited by sterols in lakes dominated by cyanobacteria with more than 40% biomass contribution of the total phytoplankton. Cyanobacteria exhibit low contents of polyunsaturated fatty acids and sterols (Ahlgren et al. 1992; Müller-Navarra et al. 2000; von Elert and Wolffrom 2001; von Elert et al. 2003). Furthermore, they lack the amino acids arginine and histidine which affect the reproductive mode of *Daphnia* (Koch et al. 2011; Fink et al. 2011) as well as the cladocera *Moina* (Bouchnak and Steinberg 2014). Cyanobacteria produce various secondary metabolites, many of which have strong biological activity (reviewed by Gademann and Portmann 2008). This was observed in several field studies where the biomass of *Daphnia* declined in the presence of cyanobacteria (Threlkeld 1979; Ghadouani et al. 2003; Hansson et al. 2007; Baumann and Jüttner 2008) and the toxicity, e.g. the anti-grazing properties, was linked to properties of secondary metabolites (DeMott et al. 1991; Agrawal et al. 2001). Besides the ecological impacts of cyanobacterial metabolites, the ingestion of cyanotoxins has been linked to liver, digestive and skin diseases, neurological disorders and even death in humans (Carmichael et al. 2001, reviewed by Pearson et al. 2010) and poses a major threat to freshwater ecosystems and reservoirs for drinking water.

Cyanobacteria produce a broad spectrum of toxic secondary metabolites, with the best-studied group being the cyanopeptides, particularly the heptapeptide microcystin (Janssen 2019). Besides the hepatotoxic effect on mammals (including humans) (Carmichael 1994), microcystin-variants impact food uptake, somatic and population growth rate, mortality and reproduction of *Daphnia* (Rohrlack et al. 1999; Rohrlack et al. 2001; Lürling 2003a; Ghadouani et al. 2004; Semyalo et al. 2009). The ongoing global processes will impact cyanobacterial biomass and microcystin concentrations (Lürling et al. 2017) and the spatial distribution of cyanotoxins, such as microcystins and its concentration, is primarily influenced by water temperature (Mantzouki et al. 2018). Further it was stated that temperature-related mechanisms result in the selective development of well-adapted cyanobacterial strains, selecting for the prevalence of a few highly toxic strains.

Nevertheless, several studies have demonstrated toxic effects by cyanobacterial extracts that cannot be attributed solely to the presence of microcystins alone (Banker and Carmeli 1999; Baumann and Jüttner 2008; Keil et al. 2002; Lürling 2003b; Smutná et al. 2014). This suggests that the risk assessment should also consider the potential impact of other bioactive metabolites. Lürling (2003b) was able to link growth reduction of *Daphnia* to the *Microcystis*

aeruginosa strain NIVA Cya 43 which lacks microcystins. Subsequently, the negative effect could be attributed to the identified protease inhibitors (PIs) nostopeptin BN920 and cyanopeptolin 954 (CP954) (von Elert et al. 2005). Cyanobacterial protease inhibitors are of interest due to their widespread distribution, as they have been found in many cyanobacterial blooms (Carmichael 1994; Agrawal et al. 2001, reviewed by Janssen 2019 and Schwarzenberger 2022) and could be linked to a mass mortality of *Daphnia* during a bloom of *Planktothrix* (Baumann and Jüttner 2008).

Protease inhibitors with significant inhibitory potential belong to the Ahp-cyclodepsipeptides, a class of non-ribosomal peptides (reviewed by Köcher et al. 2020). All Ahp-cyclodepsipeptides harbor a distinctive (3S)-amino-(6R)-hydroxy piperidone (Ahp)-moiety at a fixed position which is crucial for their bioactivity. The binding mode is highly similar among the co-crystallized Ahp-cyclodepsipeptides (Köcher et al. 2020). Cyanobacterial protease inhibitors were crystallized with serine proteases (Lee et al. 1994; Nakanishi et al. 2000; Matern et al. 2003; Salvador et al. 2013), mainly (porcine) pancreatic elastases. It revealed that the protease inhibitors bind to the proteases in a substrate-like manner, where the N-terminus of the Ahp-moiety bonds to the substrate pocket, while the Ahp-residue occupies the S1' site and the neighboring two c-terminal amino acids the S2' and S3' sites (Figure 2) (Köcher et al. 2020). The S1 site is of special interest as serine proteases display strong substrate specificity at this site (Hedstrom 2002; Matern et al. 2003). No enzymatic hydrolysis occurs between the amide bond S1 and S1' therewith inhibiting the bound serine protease (Köcher et al. 2020).

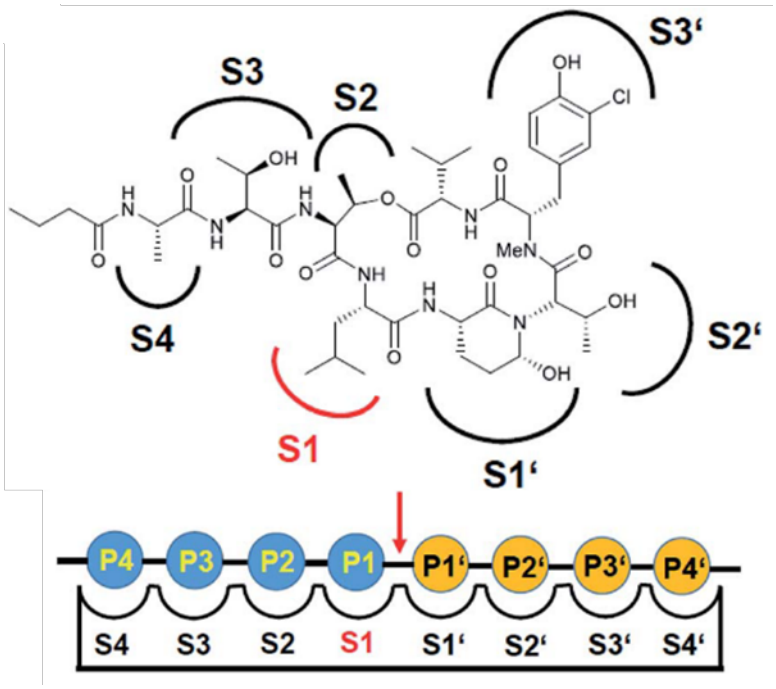


Figure 2: overview of the Ahp-cyclodepsipeptides scyptolin A (Matern et al. 2003) with the Schechter and Berger nomenclature for the description of protease-substrate or protease-inhibitor complexes (adapted from Köcher et al. 2020). The peptide side chain P binds to the protease subsites S and is annotated with 1-4 for the N-terminal scissile bond while the C-terminal is annotated with 1'-4' residues. The red arrow indicates the proteolytic attack of the protease on a substrate.

The inhibition of proteolytic serine proteases of *Daphnia* results in impaired digestion (Agrawal et al. 2005a). von Elert et al. (2004) could identify that the digestive proteolytic activity in

D. magna mainly derives from chymotrypsins and trypsins, which are serine proteases with a serine in the catalytic triad in addition to histidine and asparagine (reviewed by Hedstrom 2002). Both endopeptidases possess over 250 genes in *D. pulex* (Schwerin et al. 2009) suggesting that *Daphnia* are able to modulate the expression of digestive enzymes depending on the available food (Schwarzenberger 2022). In line with that, it has been shown that *D. magna* can express different protease isoforms when PI-producing cyanobacteria where present in their diet (Schwarzenberger et al. 2010).

This adaptability might be beneficial given that the production of PIs is dependent on the availability of nutrients, especially phosphorus (P) and nitrogen (N). It has been demonstrated that the PI content of *Microcystis* strains increases when P is limited but decreases when N is lacking in the growth medium (Schwarzenberger et al. 2013b; Burberg et al. 2019; Burberg et al. 2020). It is hypothesized that the PIs serve as a temporary storage for N, thereby making more resources available for cell growth (Burberg et al. 2019). A higher PI content under P limitation resulted in a stronger inhibition of the digestive proteases of *Daphnia*, while a lower PI content during N depletion led to a lower inhibition of the proteases, thereby promoting higher somatic growth rates of *Daphnia* (Schwarzenberger et al. 2013b).

PIs have not only been shown to impair the development (Rohrlack et al. 2004) or population growth (Lürling 2003b) of *Daphnia*, but also to inhibit their digestive serine proteases *in vitro* (Agrawal et al. 2005a; Czarnecki et al. 2006; Schwarzenberger et al. 2012; Schwarzenberger et al. 2013a; Schwarzenberger et al. 2020b) and *in situ* (Schwarzenberger et al. 2010; von Elert et al. 2012). Given the seasonal variation of protease inhibitor levels in the seston, with higher levels observed in summer (Küster et al. 2013), it is reasonable to hypothesize that selection pressure on *Daphnia* may occur during cyanobacterial blooms. This study did not demonstrate seasonal differences in tolerance between *D. magna* clones. Nevertheless, extended exposure to cyanobacteria has been demonstrated to alter zooplankton communities towards more adapted species, to select for genotypes within a species that are more tolerant (reviewed by Ger et al. 2016).

Local adaptation refers to the process by which populations of organisms have evolved traits that enhance their survival and reproduction in their specific local environments (Blanquart et al. 2013). In *Daphnia*, the local adaptation of *Daphnia galeata* in lake sediments was first demonstrated by the increased mean resistance of *Daphnia* genotypes to cyanobacteria during the eutrophication of Lake Constance (Hairston et al. 1999) and the intraspecific tolerance of *D. pulicaria* towards cyanobacteria with respect to their origins, which were characterized by low and high concentrations of bloom-forming cyanobacteria during the summer (Sarnelle and Wilson 2005). The underlying mechanism for “tolerance” was quantified without the physiological and genetic basis. Blom et al. (2006) isolated a potent PI and

described local adaptation to this toxic compound when the sensitivity of *Daphnia* sp., collected from a lake with annual mass developments of the *P. rubescens* and a lake almost free of this cyanobacterium, were compared with each other. This study has demonstrated that local adaptation does not only occur via rapid microevolution of *Daphnia* to microcystin (Hairston et al. 1999) but also with respect to other secondary metabolites, including PIs.

Two populations of *D. magna* got into focus: a Swedish population that was co-existing with a PI-producing *Microcystis* strain, e.g. *Microcystis* sp. BM25, and a Polish population that was naïve to this cyanobacterium. Schwarzenberger et al. (2017) demonstrated higher susceptibility of the naïve population to the PI-producing *Microcystis* strain compared to the local, Swedish population. Previously, three trypsin and three chymotrypsin genes (i.e., *ct383*, *ct448*, and *ct802*) have been identified encoding proteases which are active in the digestive tract of *D. magna* (Schwarzenberger et al. 2010). Of particular interest is the *ct448* gene, which has been demonstrated to exhibit copy number variation among populations, with fewer copies observed in a more tolerant population (Schwarzenberger et al. 2017). Population genetic tests on the tolerant population demonstrated that positive selection is most likely acting on the serine proteases *ct448* and *ct802* gene loci (Schwarzenberger et al. 2020a). It is likely that CT448 is the target of selection, and thus may represent the genetic basis for local adaptation of *Daphnia* populations to cyanobacterial protease inhibitors. The analysis of polymorphism frequencies provides evidence in support of the hypothesis that positive selection is acting on the *ct448* gene due to a recent selective sweep (Schwarzenberger et al. 2020a). The hypothesis of a selective sweep in CT448 is also supported by the observation of an N-terminal amino acid substitution at position 34, which has occurred between glutamic acid (Glu34) in the Swedish population and glutamine (Gln34) in the Polish *D. magna* population (Schwarzenberger et al. 2020a). This amino acid substitution may have resulted in the formation of a more stable isoform of CT448 in the Swedish population. However, the biochemical mechanisms underlying this process have not yet been investigated. The role of CT448 in the adaptation to toxic cyanobacteria is supported by the observation that the expression of protease-encoding genes is upregulated when cyanobacteria are present in the diet of *Daphnia* (Schwarzenberger et al. 2010; Schwarzenberger et al. 2012; Drugă et al. 2016; Schwarzenberger and Fink 2018).

Understanding the drivers and mechanisms of local adaptations could prove essential in elucidating the co-evolutionary dynamics between cyanobacteria and their grazers, e. g. *Daphnia*, and may prove crucial in regulating toxic blooms. The interaction between cyanobacterial defenses and zooplankton tolerance appears to be an underlying mechanism regulating the ecology and management of eutrophic waters, including the stability, toxicity, and control of cyanobacterial dominance (reviewed by Ger et al. 2016). It was demonstrated that the biomass of *Daphnia* is a crucial factor in effectively suppressing cyanobacterial

biomass (Wright and Shapiro 1984; Chislock et al. 2013), and for successful food chain manipulation (Leibold 1989). Therefore, adapted populations could facilitate the control of cyanobacteria by *Daphnia*.

Thesis aim

This dissertation aims at gaining an understanding of the evolutionary driven tolerance of *Daphnia magna* in co-existence to cyanobacterial protease inhibitors. Despite advancements in the comprehension of the molecular basis of local adaptation of *Daphnia* to protease inhibitors in cyanobacteria, further data are required. In order to identify the mechanisms that led to the locally increased tolerance of a Swedish *D. magna* population to co-occurring cyanobacterial PIs, a susceptible Polish population has been selected for comparison. This includes functional comparison of heterologously expressed digestive serine proteases CT448 of Swedish and polish *D. magna* in respect to their enzymatic properties.

Chapter 1

Heterologous expression and characterization of a novel serine protease from *Daphnia magna*: A possible role in susceptibility to toxic cyanobacteria

corresponds to the publication:

Lange, J., Demir, F., Huesgen, P. F., Baumann, U., von Elert, E., & Pichlo, C. (2018). Heterologous expression and characterization of a novel serine protease from D*aphnia magna*: A possible role in susceptibility to toxic cyanobacteria. Aquatic toxicology, 205, 140-147

The study presented in chapter 1 demonstrates the successful expression of crustacean CT448 in *Sf*21 insect cells. The purified and inactive precursor (zymogen) could be activated by tryptic digestion. I accomplished the first example of a recombinant expression of an active crustacean serine protease, which functions in the gut of *Daphnia*. Proteomic identification of protease cleavage sites (PICS) and hydrolyzation of various synthetic substrates showed that CT448 is a chymotrypsin-like elastase. In contrast to previous studies, this study classified CT448 as an elastase and confirmed it as a target of cyanobacterial PIs.

Authors contribution

The experiments were performed by me, Jacqueline Lange, and Fatih Demir. I expressed and purified the protein and analyzed the data together with Christian Pichlo, Fatih Demir and Pitter F. Huesgen. The study was designed by me, Christian Pichlo and Eric von Elert. Eric von Elert, Ulrich Baumann, Pitter F. Huesgen supervised the work. Jacqueline Lange wrote the paper with input from all authors.

Data availability statement

The mass spectrometry proteomics data for the PICS experiment have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al, 2015) partner repository with the dataset identifier PXD010978.

All data generated or analyzed during this study are included in the manuscript or supplements. Raw data generated during this study are available from the corresponding author upon request.

1.1 Abstract

Mass developments of toxin-producing cyanobacteria are frequently observed in freshwater ecosystems due to eutrophication and global warming. These mass developments can partly be attributed to cyanobacterial toxins, such as protease inhibitors (PIs), which inhibit digestive serine proteases of *Daphnia,* the major herbivore of phytoplankton and cyanobacteria. To date, mechanisms of this inhibition in the gut of the crustacean *Daphnia magna* are not known. Here, we characterize a single serine protease, chymotrypsin 448 (CT448), which is present in the gut of the crustacean *D. magna*. Sequence alignments with human serine proteases revealed that CT448 has a putative N-terminal pro-peptide which is extended compared to the mammalian homologs and within this pro-peptide two N-linked glycosylation motifs were found. CT448 was heterologously expressed in *Sf*21 insect cells using a baculovirus expression system for optimized protein production and secretion into the medium. The protein was purified via a one-step affinity chromatography, which resulted in a protein yield of 3.45 mg/L medium. The inactive precursor (zymogen) could be activated by tryptic digestion. This is the first example of a recombinant expression of an active crustacean serine protease, which functions in the gut of *Daphnia*. Proteomic identification of protease cleavage sites (PICS) and hydrolysation of various synthetic substrates showed that CT448 is a chymotrypsin-like elastase. In this study, we confirm that CT448 is a target of cyanobacterial protease inhibitors. Local evolutionary modifications of CT448 might render this proteolytic enzyme less susceptible against cyanobacterial secondary metabolites and might improve the fitness of Daphnia during cyanobacterial blooms.

1.2 Introduction

Cyanobacterial blooms have increased in lakes and ponds due to nutrient input and rising temperatures and are predicted to further increase in frequency (Downing et al., 2001; Paerl and Huisman, 2008). Cyanobacteria produce secondary metabolites that are harmful to humans and, in some cases, cause death of livestock when exposed to water with high cyanobacterial concentrations (Cheung et al., 2013). Therefore, cyanobacterial mass developments are a major issue of concern in lake management (Carmichael et al., 2001). Multiple strategies have been conducted to control harmful cyanobacteria blooms, *e.g.* the reduction of nutrient input or phytoplankton by herbicides, but also manipulation of the food chains has been proposed (Shapiro et al., 1975; Hansson et al., 1998). This manipulation addresses the concept of increased size and abundances of herbivorous zooplankton by the manipulation of higher trophic levels (Shapiro et al., 1975; Wright and Shapiro, 1984) in order to reduce cyanobacterial biomass.

During these blooms, cyanobacteria represent a major part of the phytoplankton and thus of the diet of the non-selective filter feeder *Daphnia*. Cyanobacteria are of particularly low food quality for *Daphnia* (von Elert and Wolffrom, 2001; von Elert et al., 2003; Martin-Creuzburg et al., 2008). And more importantly cyanobacterial filaments interfere with the filtering apparatus of *Daphnia* (Gliwicz and Lampert, 1990; DeMott et al., 2001) resulting in a decreased fitness of the grazer. The biomass of *Daphnia* is important for the effective suppression of cyanobacterial biomass (Wright and Shapiro, 1984; Chislock et al., 2013) and crucial for successful food chain manipulation (Leibold, 1989). The prevalence of cyanobacteria in lakes and ponds leads to the well-known low abundances of *Daphnia* in summer (Threlkeld, 1979; Sommer et al., 1986; Ghadouani et al., 2003), which, to a large degree, is caused by toxic and inhibitory cyanobacterial metabolites (Wilson et al., 2006). Many cyanobacterial species produce these toxic secondary metabolites with negative effects on the fitness of *Daphnia* (Sivonen and Jones, 1999; Rohrlack et al., 2001; Lürling, 2003; Gademann and Portmann, 2008) and several studies tried to understand the toxicological impact of the well-studied secondary metabolite microcystin on *Daphnia* and to elucidate molecular mechanisms behind the inhibition of *Daphnia* (Dittmann and Wiegand, 2006; Sadler and Elert, 2014a, 2014b). Among the huge variety of biologically active secondary metabolites (Gademann and Portmann, 2008), we focus here on protease inhibitors (PIs), as they are more frequently found in surface blooms of cyanobacteria (Agrawal et al., 2001) than the well-studied protein phosphatase inhibitor microcystin.

These cyanobacterial PIs specifically inhibit digestive proteases in the gut of *Daphnia* and thus reduce growth of this herbivore (Lürling, 2003; Schwarzenberger et al., 2010). von Elert et al. (2004) demonstrated that two classes of serine proteases, chymotrypsin and trypsin, account

for 80% of the total digestive protease activity in the gut of *Daphnia* and a strain of *Microcystis aeruginosa* was shown to produce secondary metabolites, which specifically inhibit chymotrypsins in the gut homogenate of *Daphnia magna* (von Elert et al., 2012). Moreover, two chymotrypsin inhibitors, nostopeptin BN920 and cyanopeptolin 954 (CP954) were identified in this cyanobacterium (von Elert et al., 2005). To date there is no detailed understanding of the interaction of cyanobacterial PIs with digestive proteases in the gut of *Daphnia.* Thus, studies on the protein-inhibitor-interaction are crucial with respect to the control of cyanobacterial blooms.

In nature, *Daphnia* clones differ in their tolerance to PIs depending on their coexistence with cyanobacteria in their habitat, which points at local adaptation of *Daphnia* to cyanobacterial serine PIs (Schwarzenberger et al., 2017). In response to exposure to dietary cyanobacterial protease inhibitors, *D. magna* has been shown to respond by the expression of compensatory isoforms, and by upregulated expression of dietary trypsins and chymotrypsins (Schwarzenberger et al., 2010; Schwarzenberger et al., 2012). These adaptations are further, epigenetically transferred to the offspring (Schwarzenberger and Elert, 2013). Using LC-MS/MS based proteomics, three chymotrypsin-like and trypsin-like peptidases were identified in the gut of *D. magna*, as well as the equivalent genes (Schwarzenberger et al., 2010). Furthermore, the copy number of the most relevant chymotrypsin-like gene, i.e. *ct448,* varied among different *Daphnia* clones. Nevertheless, these variations in gene copy numbers were not related to tolerance against PIs (Schwarzenberger et al., 2017), which strongly suggests that adaptation to cyanobacterial PIs in *Daphnia* is not due to adjusted gene expression levels of *ct448* but rather due to changes of the protein CT448 itself.

Chymotrypsin and trypsin belong to the S1 family of serine proteases that are of major importance for the digestion of proteins in humans and *D. magna*. These proteases are synthesized as inactive zymogens that require activation by proteolytic cleavage. Chymotrypsin and trypsin mainly recognize their substrates by the nature of the residue N-terminal to the scissile bond (S1 site). While chymotrypsin mainly cleaves behind large hydrophobic residues, trypsin cleaves behind positively charged amino acid residues. Elastases which are also chymotrypsin-like have specificity for small hydrophobic amino acids residues. Genes encoding for digestive proteases in *D. magna* have been identified. However, these digestive proteases have only been characterized in gut homogenates of *Daphnia* and not in isolation.

In the current work, we investigated a novel serine protease expressed in *D. magna*. For the first time, a functional serine protease of *Daphnia* was recombinantly expressed using a baculovirus-insect cell expression system and purified by Strep-tag affinity chromatography. The purified protein was proteolytically activated and its specificity was characterized by

proteomic identification of protease cleavage sites (PICS) revealing that the protein has a elastase-type specificity. Furthermore, we tested its ability to hydrolyze different synthetic substrates and the susceptibility to cyanobacterial inhibitors. Taken together, we provide a novel approach to study *Daphnia* gut proteases at the molecular level with the ultimate aim of providing new approaches for the management of cyanobacterial blooms.

1.3 Results

In silico analysis of CT448

Sequence similarity searches using BLAST reveal that CT448 shares around 33 % sequence identity to the catalytic domain of human elastase-2a, a pancreatic chymotrypsin-like elastase (Fig. 1). Further, multiple sequence alignments with human trypsin, chymotrypsin and elastase 1, 2 and 2A revealed that CT448 has a putative N-terminal pro-peptide with two predicted glycosylation site. The pro-peptide is extended compared to the mammalian homologs. Mammalian pancreatic trypsins and chymotrypsin are activated by a cleavage at the C-terminal site of the positively charged residue of the pro-peptide, leading to neo-N-termini starting with isoleucine or valine. In line with that, also in CT448 an arginine (R77) at the very C-terminus of the putative pro-peptide could be found, followed by two isoleucines, suggesting that CT448 is activated in an analog fashion as its homologs in mammals. An analysis of the residues involved in S1 pocket formation was conducted revealing that CT448 shares no strict conservation to any of the other protease at relevant sites (colored in red) and thus permitting to make accurate prediction about the specificity of this protease.

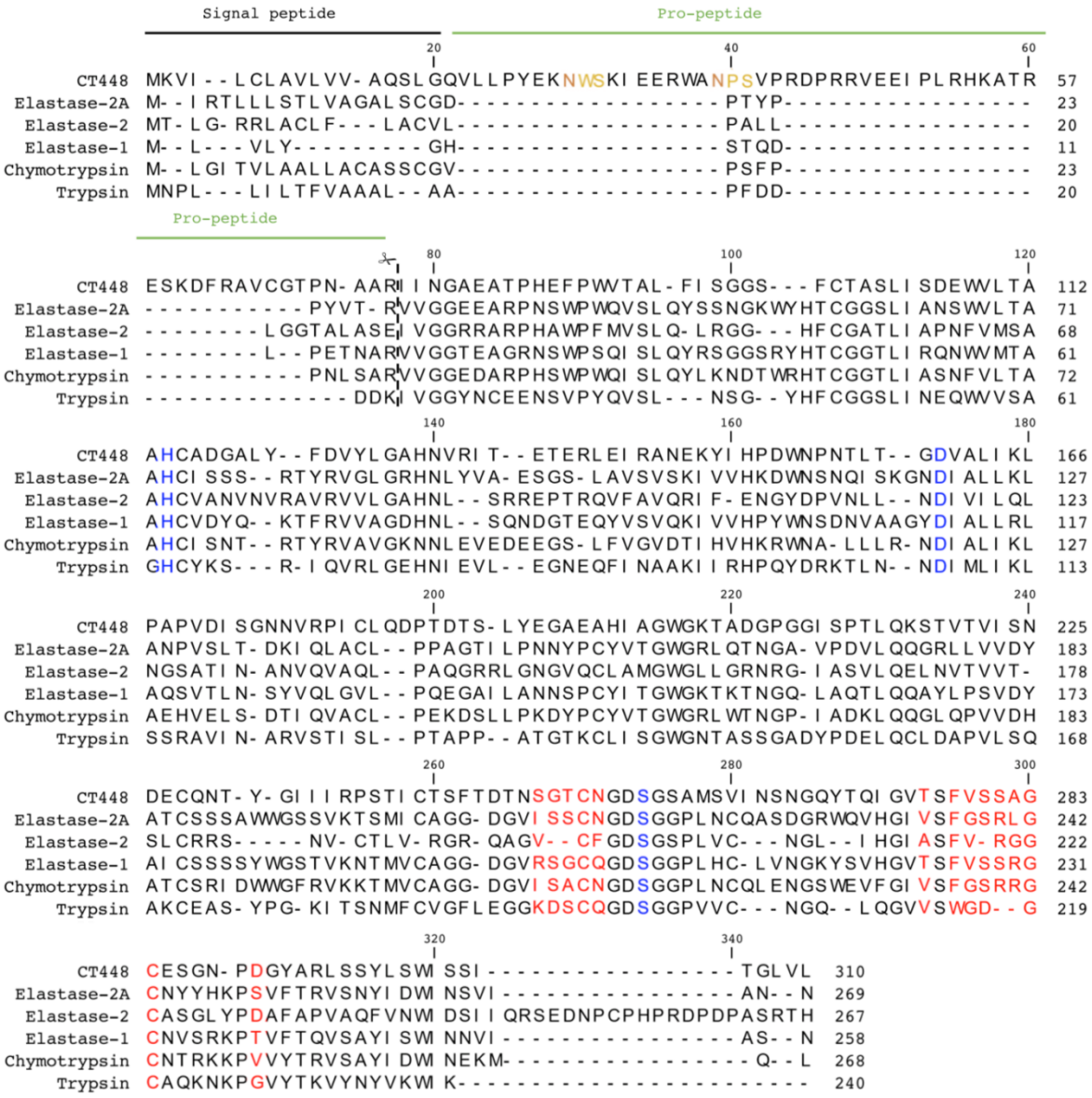


Fig. 1: Multiple alignment of crustacean CT448 with human serine proteases. Important regions are highlighted with different colors: The pro-peptide in green, amino acids of the catalytic triad (H122, D174 and S275) in blue and residues relevant for substrate binding in red. The putative glycosylated asparagines are in dark yellow, while residues of the N-glycosylation consensus sequence are in yellow. Glycosylation sites were predicted using the NetNGlyc 1.0 Server. Scissor icon indicates the position for trypsin cleavage and activation

Heterologous expression and purification of *D. magna* serine protease in *Sf*21 cells

We cloned the CT448 gene of *D. magna* into the pFL transfer vector of the Multibac system in order to generate afterwards a baculovirus for the heterologous expression of CT448 in in *Sf*21 cells. For an efficient secretion, the putative signal-peptide of CT448 was replaced by a melitin signal peptide. In addition, a Twin-Strep-tag was fused to the C-terminus for straightforward one-step purification. For the recombinant expression of CT448, *Sf*21 cells were infected with the baculovirus resulting in a proliferation arrest approximately 24 h after addition. This time

point was termed the day of proliferation arrest (dpa). At different time points after dpa, samples of the culture were taken for analysis. Cells were separated from the medium by centrifugation to obtain cell-free medium (M). Subsequently, the cells of the pellet were lysed in order to obtain supernatant and pellet (S+P) and, after centrifugation, supernatant of lysate (S). Western blot analysis of these samples revealed that CT448 became first detectable 48 h after dpa within cells lysates (Fig. 2A). After 72 h, a significant amount of the protein had been secreted into the medium. This time point was set as the optimal day for harvest of the culture medium, as 24 hours later, CT448 was no longer detectable in the medium.

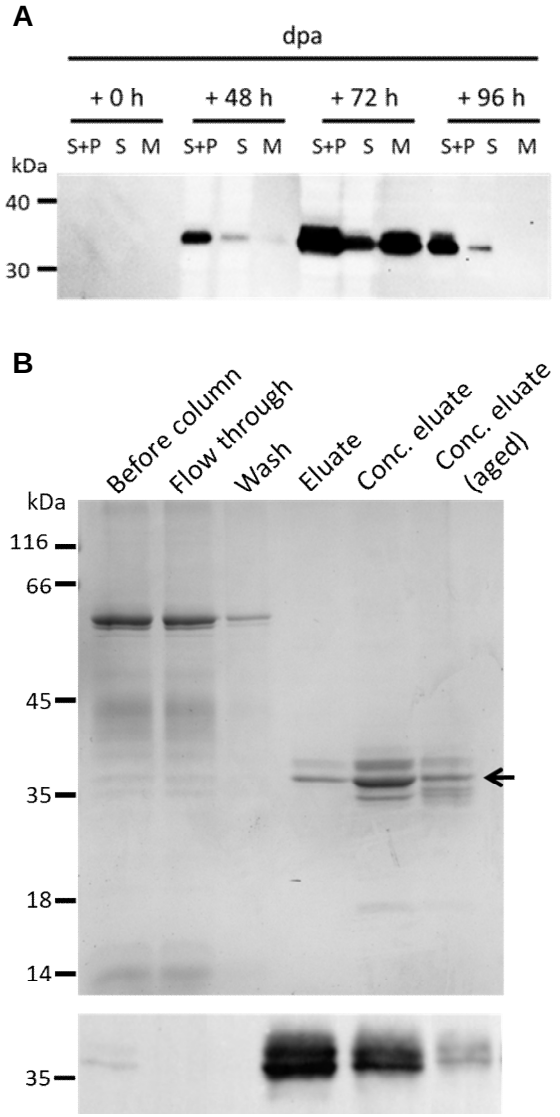


Fig. 2**:** Analysis of the expression and purification of C-terminally tagged CT448 by western blotting and SDS-PAGE. A) The protein was expressed in *Sf*21 cells after infection with a baculovirus that lead to an arrest of proliferation. CT448 was detected at the day of proliferation arrest (dpa) and at different time intervals, thereafter in supernatant and pellet (S+P), in supernatant of the lysate (S) and in the medium (M). Using Strep-Tactin-HRP conjugate, CT448 was first detected 48 h after dpa and CT448 levels increased until 72 h after dpa. These levels decreased 96 h after dpa and CT448 was not longer detectable in the medium. B) SDS-

PAGE analysis from samples taken during purification. A commassie-stained Polyacrylmide gel is shown in the upper panel while a western blot analysis is shown in the lower panel. CT448 was secreted into the medium and was, after separation from the *Sf*21 cells (before column), purified by affinity chromatography with thoroughly washing of the column (flow through and wash). The eluted protein (eluate) was concentrated in an ultrafiltration centrifugal device (conc. eluate) for subsequent enzymatic assays. Different species of pure CT448 (highlighted by an arrow) with a molecular weight of around 35 kDa were obtained. CT448 was detected using the Strep-Tactin-HRP conjugate (lower panel) and revealed CT448-specific bands at around 35 kDa which faded upon aging.

The cell-free supernatant, containing the protein of interest, was loaded onto a column for affinity chromatography. Figure 2B (upper panel) shows the individual steps of purification with a Strep-Tactin resin. The Coomassie-stained SDS-polyacrylamid gel displayed that washing of the column removed impurities, which were present in the medium. After the elution from the column (eluate) and after concentration, distinct bands with a molecular weight of around 35 kDa were detected and are consistent with the expected mass of 35.7 kDa for the pro-enzyme of CT448 (containing its pro-peptide). Western blot analysis (Fig. 2B, lower panel) and tryptic mass fingerprint confirmed that all species around 35 kDa were CT448. Further, MS-analysis of tryptic-digested protein revealed that all CT448 species had a complete Twin-Strep-tag (Fig. S1), suggesting that the distinct species have different N-termini due to an inaccurate cleavage of the signal peptide or N-terminal degradation. The mature enzyme (without pro-peptide (trypsin treated)) would have a calculated molecular mass of 29.1 kDa and the pro-peptide a mass of 6.6 kDa. For both, we could not observe a corresponding band during SDS-PAGE analysis. A stability control, incubation of the eluate at 4°C for 4 days (conc. eluate aged), showed a broader pattern of bands (35-37 kDa), which indicated protein degradation

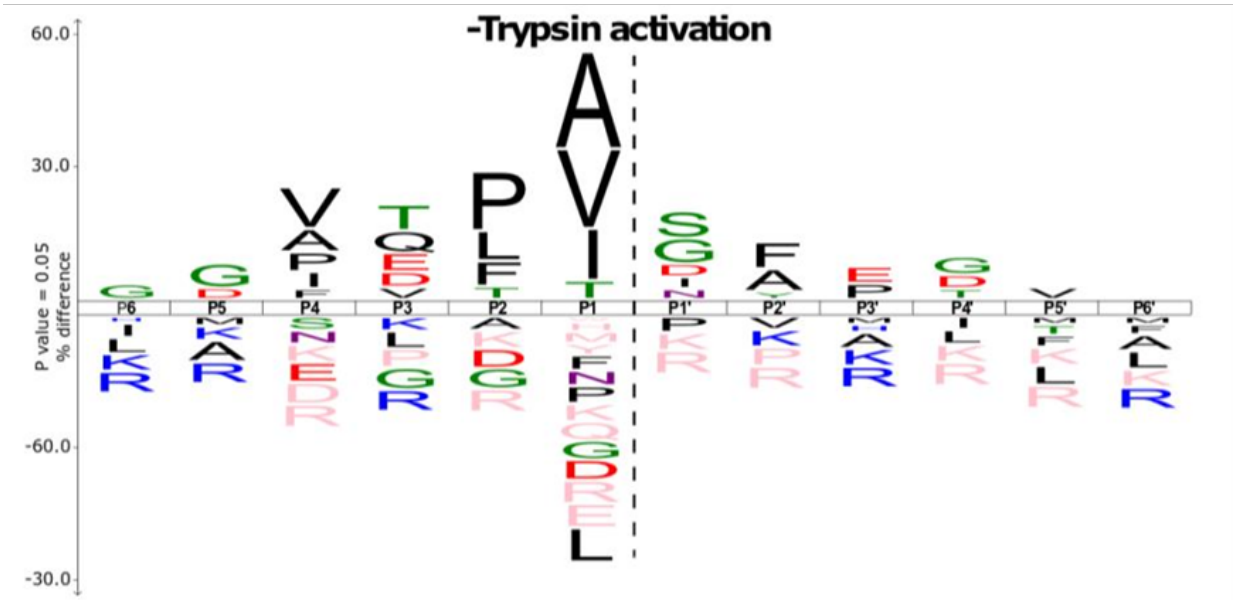
Overall, the purification via a Twin-Strep-tag and Strep-Tactin resin followed by concentration of the eluate seemed to be an adequate procedure to purify CT448 from *Sf21* medium for subsequent assays. The average yield from 1 liter medium was 3.45 mg of protein.

CT448 – a chymotrypsin-like elastase

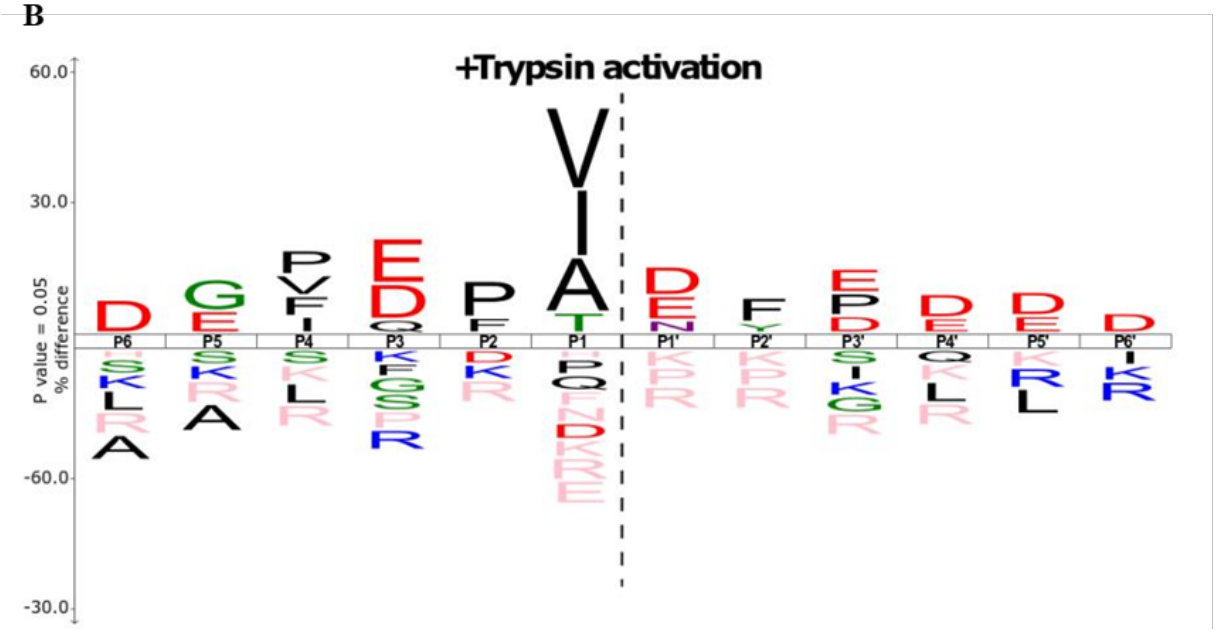
With purified recombinant CT448 protein in hands, we proceeded to determine its sequence specificity with proteome-derived peptide libraries using the Proteomic Identification of protease Cleavage Sites (PICS) approach (Schilling and Overall, 2008; Biniossek et al., 2016). In this approach a trypsin-digested E. coli proteome was used as peptide library, allowing to determine the specificity of non-tryptic proteases. The library was incubated with non-activated CT448 (-Tryp), trypsin-activated CT448 (+Tryp), or buffer control. Afterwards peptides which altered in abundance in the different samples were identified and annotated by a MS-based proteomic analysis from which a specificity profile was obtained. This profile showed that non-activated CT448 preferentially cleave at sites with Pro at P2 and Val/Ile at P1 for CT448 (Fig.

3A), resembling the specificity of neutrophil elastase (Biniossek et al., 2016). Activation with trypsin did not alter substrate specificity significantly (Fig. 3B).

A



B



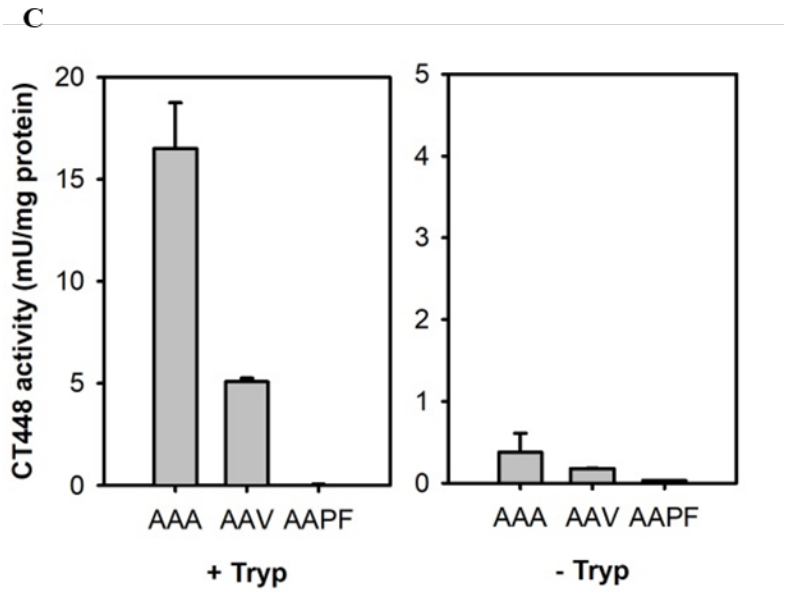


Fig. 3**:** Characterization of CT448 protease specificity by Proteomic Characterization of Cleavage Sites (PICS) and specific enzymatic activity on different synthetic substrates. A) For PICS analyses, an *E. coli* K12 total proteome-derived peptide library, prepared by tryptic-digest, was incubated with recombinant CT448 protease. IceLogos of 351 aligned cleavage sites of CT448 and B) 181 aligned CT448 cleavages sites of trypsin-activated CT448 display the favored (above horizontal line) and disfavored (below line) amino acids at each position, expressed as difference against the expected amino acid abundance in the *E. coli* K12 proteome (p-value 0.05). The dashed line indicates the cleavage site. Color coding of amino acids depicts the different physical-chemical properties: black for hydrophobic, green for hydrophilic, red for acidic and blue for alkaline amino acids. The height of one amino acid in a stack (P1-6; P1'-6') reflects its frequency. C) Further, synthetic substrates consisting of different peptides coupled to C-terminal *para*-nitroanilide were subjected to identical concentrations to CT448 (- Tryp) and with trypsin-activated CT448 (+ Tryp). N-Succinyl-alanine-alanine-proline-phenylalanine-para-nitroanilide (AAPFpNA), N-Succinyl-alanine-alanine-valine-para-nitroanilide (AAVpNA) and N-Succinyl-alanine-alanine-alanine-para-nitroanilide (AAApNA) were used as substrate with a concentration of 100 µM. Protease activity was monitored by detecting the release of p-nitroaniline by measuring the absorption at 380 nm. Only AAApNA and AAVpNA showed high specific activity of trypsin-activated CT448. Depicted is the mean specific enzymatic activity (n = 3, ± SD). Note different scaling of y-axes.

To validate the PICS results, we performed CT448 enzyme activity assays with synthetic peptide substrates, two specific for elastase and one specific for chymotrypsin. Trypsin-activated CT448 (+ Tryp) showed a significantly higher activity for the elastase substrates N-Succinyl-alanine-alanine-alanine-para-nitroanilide (AAApNA, 16.5 ± 2.24 mU/mg) and N-Succinyl-alanine-alanine-valine-para-nitroanilide (AAVpNA, 5.07 ± 0.17 mU/mg), than for the chymotrypsin-specific substrate N-Succinyl-alanine-alanine-proline-phenylalanine-para-nitroanilide (AAPFpNA, 0.045 ± 0.008 mU/mg) (one way ANOVA: $F=113.299$; $p < 0.001$; Tukey $p < 0.05$, Fig. 3C). CT448 without prior activation (- Tryp) showed a similar preference for the elastase substrates AAApNA (0.37 ± 0.23 mU/mg) and AAVpNA

(0.177 ± 0.002 mU/mg) although the activity was strongly reduced compared to the trypsin-activated CT448 (Fig. 3C). Only very little hydrolysis of the chymotrypsin substrate AAPFpNA (0.032 ± 0.002 mU/mg) close to background levels was detected. Trypsin, which was used for activation of CT448, did not hydrolyze any of these substrates (data not shown).

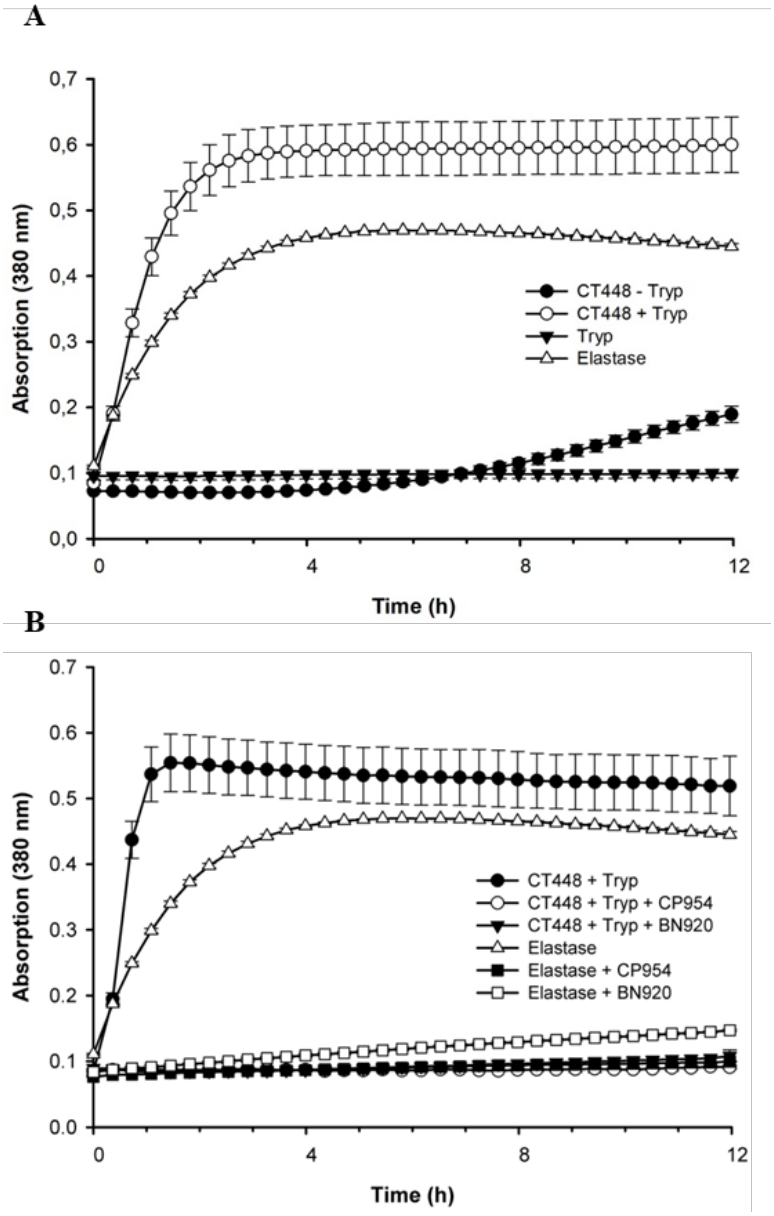


Fig. 4: Hydrolysis of a synthetic substrate in the absence/presence of cyanobacterial PIs by heterologously expressed CT448 of *D. magna* and of porcine elastase. A) Comparison of *D. magna* CT448 to porcine elastase on AAApNA. Non-activated (CT448 – Tryp) and trypsin-activated CT448 (CT448 + Tryp), porcine elastase and trypsin were tested, the latter did not hydrolyze the elastase specific substrate. B) Addition of 20 µM of the cyanobacterial serine protease inhibitors cyanopeptolin 954 (CP954) or nostopeptin 920 (BN920), which had been isolated from the cyanobacterium *Microcystis aeruginosa* NIVA Cya 43, to *D. magna* CT448 and porcine elastase. Both serine proteases were completely inhibited with CP954 or BN920. The measurements were carried out at 27°C for 12 hours. Depicted is the mean absorption$_{380nm}$ (n = 3, ± SD).

As the specificity of CT448 was similar to that of elastases, we compared the CT448 of *D. magna* to the well-studied porcine pancreatic elastase (Fig. 4A). When using AAApNA as substrate, similar activities of trypsin-activated CT448 (CT448 + Tryp) and porcine elastase were detected. Instead, CT448 - Tryp was only active after a lag phase of 6-7 hours, which suggested that auto-activation over several hours was required, before proteolytic activation became detectable. To exclude contaminating protease activity we used 1 mM and 10 mM of EDTA for metalloprotease inhibition, 1 mM and 10 mM of iodoacetamide for cysteine protease inhibition and 50 µM chymostatin as positive control. As expected, the activity of CT448 was only affected by chymostatin showing that the main activity was derived from a serine protease (Fig. S2).

Digestive proteases of *Daphnia* are often exposed to the well-studied cyanobacterial PIs cyanopeptolin 954 (CP954) and nostopeptin 920 (BN920). We therefore tested CT448 activity in the presence of these two PIs and observed complete inhibition of CT448 by both inhibitors (Fig. 4B). Interestingly, BN920 and CP954 also reduced the activity of porcine elastase to a minimum. Here, CP954 seemed to have a stronger impact than BN920. This demonstrates that these two cyanobacterial PIs, which have previously been reported as chymotrypsin inhibitors (von Elert et al., 2005; Schwarzenberger et al., 2010), act more broadly as inhibitors of mammalian and crustacean chymotrypsin-like elastases.

1.4 Discussion

Among the major digestive serine proteases in *Daphnia*, there is the chymotrypsin-like protease CT448, which has shown to be of major relevance for the interaction of *Daphnia* with cyanobacterial PIs. Evidence for the importance of CT448 is based on the upregulation of *ct448* gene expression and on an increase in proteolytic activity in *D. magna* clones when being fed with PI producing cyanobacteria (Schwarzenberger et al., 2012). Further, it has been shown that the tolerance of *D. magna* clones to cyanobacteria that contain chymotrypsin inhibitors is correlated to the tolerance of *D. magna's* gut proteases to in-vitro inhibition by these inhibitors (Gademann and Portmann, 2008). However, the molecular mechanism of the increased tolerance to PIs remained unclear. So far, proteases of *D. magna* could not be analyzed, so that no specific characteristics of individual digestive enzymes could be accomplished. Here, we were able to express a specific gut protease with the here described method in adequate amounts (3.45 mg/L) for subsequent analyses. Nevertheless, that we used the highly specific Twin-Strep-tag for purification, several protein bands with molecular masses ranging from 35-37 kDa in the purified fractions (eluate, conc. eluate and conc. eluate aged, Fig. 2B) were observed. Western blot and MS analysis confirmed that all species were CT448. As the Twin-Strep-tag was intact, the distinct species were most likely the result of N-

terminal truncations or post-translational modifications such as glycosylation. This, however, requires further investigations.

The digestive protease CT448 is annotated as a chymotrypsin (Schwarzenberger et al., 2010). Here, we showed by PICS analysis and by using synthetic substrates that CT448 preferentially cleaves behind small aliphatic residues Ala, Val and Ile meaning that the protease is an elastase, rather than a classic chymotrypsin. Further, will the elucidation of the CT448's specificity allows the development and use of more specific substrates and inhibitors to considerably enhance the sensitivity of assays. It remains to be determined if other digestive chymotrypsin-like proteases in *Daphnia* also have an elastase-like specificity, which now can be elucidated with our approach. Interestingly, the study demonstrated that CP954 and BN920, originally described as chymotrypsin inhibitors, also act as elastase inhibitors. For CP954 and BN920, IC_{50} values of 4.5 nM and 3.1 nM for the inhibition of bovine chymotrypsin have been reported (von Elert et al., 2005), which classified them as the most potent inhibitors containing 3-amino-6-hydroxy-2-piperidone (Ahp). Similar to CP954 and BN920, scyptolin A isolated from the cyanobacterium *Scytonema hofmanni* PCC7110 was demonstrated to inhibit elastase by occupying parts of the active center. Four N-terminal amino acid residues of scyptolin A bind at subsites S1 through S4 and prevent hydrolysis (Matern et al., 2001; Matern et al., 2003). Like scyptolin A, CP954 and BN920 belong to the class of cyanopeptolines and share similar structures, we suggest that these cyanopeptolines share similar specificities and binding properties with respect to target proteases. To test, whether inhibition of CP954 or BN920 is comparable to the binding of scyptolin A to elastase, we would suggest co-crystallization of CP954 or BN920 with elastase for structure analysis and ultimately with CT448. In several cases *Daphnia* have been shown to evolutionarily adapt to the presence of cyanobacteria (DeMott et al., 1991; Hairston Jr et al., 1999; Sarnelle and Wilson, 2005). The *D. magna* clone used in this study, was isolated from a lake where *D. magna* coexists with cyanobacteria that produce PIs (Schwarzenberger et al., 2013). We expect that CT448 has adapted to these natural cyanobacterial PIs in a way that rendered CT448 less susceptible to these natural inhibitors.

In the future, the approach presented here will be used to compare different *D. magna* clones with regard to their tolerance to cyanobacterial PIs: Local adaptation of *Daphnia* to toxic cyanobacteria has been demonstrated (Sarnelle and Wilson, 2005), and understanding the molecular basis of this adaptation might be helpful for the management of cyanobacterial blooms. In high densities *Daphnia* have the capability to suppress cyanobacteria before they become dominant (Christoffersen et al., 1993). Chislock et al. (2013) demonstrated that *Daphnia* suppressed a toxic cyanobacteria dominated bloom within a few weeks. In this enclosure experiment 96% of the phytoplankton was composed by cyanobacteria and grazing

of *Daphnia* reduced this cyanobacterial biomass by 76%. Further, it has been shown that *Daphnia* are able to suppress even an established bloom of cyanobacteria (Sarnelle, 2007).

As *Daphnia* is able to adapt to toxic secondary metabolites of cyanobacteria and is capable of suppressing cyanobacterial mass formation, we suggest that *Daphnia* clones with a high tolerance to serine protease inhibitors might facilitate the suppression of cyanobacterial blooms. Thus, adapted clones might be used as a tool for the management of cyanobacteria in lakes and ponds in the future.

Conclusion

In this study we have provided an approach for recombinant expression of *D. magna* proteases and have shown that CT448 of *D. magna* encodes a chymotrypsin-like elastase with specificity for alanine residues. This will facilitate comparison of the sensitivity of digestion proteases from different *Daphnia* clones to cyanobacterial PIs and thus help to clarify their contribution to adaptation.

1.5 Experimental procedures

Protease substrates and inhibitors

The synthetic substrates N-Succinyl-alanine-alanine-alanine-para-nitroanilide (AAApNA), N-Succinyl-alanine-alanine-valine-para-nitroanilide (AAVpNA), N-Succinyl-alanine-alanine-proline-phenylalanine-para-nitroanilide (AAPFpNA) and porcine pancreatic elastase were purchased from Sigma-Aldrich (St. Louis, USA). The cyanobacterial protease inhibitors, cyanopeptolin 954 (CP954) and nostopeptin 920 (BN920), were isolated from the *Microcystis aeruginosa* strain NIVA Cya 43 via HPLC according to von Elert et al. (2005). Stock solutions of the substrates were prepared in DMSO.

Generation of recombinant baculovirus

The full length sequence of *ct448* of *D. magna* clone Sweden May17, collected in May 2010 in Lake Bysjön (situated in Southern Scania, Sweden: N 55.675399 E 13.545805) was optimized for insect cell expression (GeneArt, Regensburg, Germany). Additional sequences were introduced so that the protein harbors an N-terminal melittin signal peptide and C-terminal Twin-Strep-tag. This gene construct was cloned into pFL MultiBac transfer plasmid (kind gift of Imre Berger) using BamHI and XhoI as restriction sites. Subsequently, chemical-competent *E. coli* DH10EMBacY cells (kind gift of Imre Berger) were transformed with the cloned transfer vector for *in vivo* Tn7 transposition generating a bacmid containing the expression cassette of CT448 (according to (Berger et al., 2004; Bieniossek et al., 2008; Fitzgerald et al., 2006). Putative recombinant pFLCT448 bacmids were selected by white/blue screening. Isolated

bacmid DNA was used to transfect *Spodoptera frugiperda Sf*21 cells (Vaughn et al., 1977) using X-tremeGENE HP (Roche, Basel, Switzerland) resulting in the recombinant virus (CT448-EmbacY).

Baculovirus expression and protein purification

*Sf*21 cells were grown in suspension in Insect-Xpress medium (Lonza, Basel, Switzerland) at 27°C. To analyze for recombinant protein production, cells were cultured in densities of 10^6 cells/ml and infected with CT448-EmbacY. Samples were taken to determine the time when cells stopped proliferating (day of proliferation arrest, dpa), usually 24 h post infection. Medium and cells were separated and analyzed for recombinant protein production. Cells were lysed in PBS and the cell extract was separated from insoluble material by centrifugation (11 000*g*, 5 min). Proteins were separated in a 15 % SDS polyacrylamide gel. The presence of recombinant protein in either medium (M), supernatant of the lysate (S) or supernatant and pellet (S+P) was detected using a horseradish peroxidase conjugated Strep-Tactin (Strep-Tactin-HRP, IBA, Goettingen, Germany) against the Twin-Strep-tag, in a standard Western-blot assay.

For large-scale protein expression and purification, approximately 5 10^5 cells/ml were infected with CT448 EMbacY. Cells were harvested at the time point of highest protein secretion into the medium as the CT448 was secreted into the medium. Medium was collected, cells were removed by centrifugation (200*g*, 45 min) and prepared for affinity chromatography by adjusting the pH to 8.0 and by blocking of biotin with BioLock (IBA, Goettingen, Germany). After a second round of centrifugation (200*g*, 45 min), protein was purified by Strep-tag affinity chromatography using 1.5 ml Strep-Tactin XT Superflow resin (IBA, Goettingen, Germany) according to the user manual, except of exchanging manufacturer's Buffer B and BXT with a buffer containing 25 mM Tris pH 8.0 and 250 mM NaCl. For protein elution 50 mM biotin was supplemented into the washing buffer. Eluted protein was concentrated by centrifugal ultrafiltration. After the cells had been harvested, all steps were performed at 4°C.

Proteomic Identification of protease Cleavage Sites (PICS)

Proteome-derived peptide libraries were generated by tryptic digest of *E. coli* K12 lysates as described (Chen et al., 2017). The peptide library was split in three aliquots that were treated with recombinant CT448 with and without prior activation by trypsin or with a buffer control for 1.5 h at RT. After incubation, peptides were triplex stable isotope labelled by reductive dimethylation (Boersema et al., 2009). Isotopically light formaldehyde (CH_2O) and sodium cyanoborohydride ($NaBH_3CN$) was used to label control-treated library peptides, deuterated formaldehyde (CD_2O) and $NaBH_3CN$ were used to label the assay with recombinant CT448 without prior activation and heavy formaldehyde ($^{13}CD_2O$) and sodium cyanoborodeuteride

($NaBD_3CN$) were used to label samples after incubation with trypsin-activated CT448. Dimethylation was performed for 2 h at RT and quenched by addition of 100 mM Tris-HCl pH 7.5 for 1 h. Subsequently samples were mixed, desalted and purified by C18 StageTips (Rappsilber et al., 2007).

1 µg peptides were separated using a nano-HPLC system (Ultimate 3000 nano-RSLC, Thermo) operated in a two-column setup (Acclaim PepMap 100 C18, ID 75 µm, trap column length 2 cm, particle size 3 µm, analytical column length 50 cm, particle size 2 µm, Thermo) coupled online to a high resolution Q-TOF mass spectrometer (ImpactII, Bruker) as described (Rinschen et al., 2017). Peptides were eluted with a binary gradient from 5-35 % B for 90 min (A: H_2O + 0.1 % FA, B: ACN + 0.1 % FA), followed by washing and re-equilibration steps to a total runtime of 2 h per sample. The Bruker HyStar Software (v3.2) was used to acquire line-mode MS spectra in a mass range from 200-1750 m/z at an acquisition rate of 4 Hz. For each MS spectrum, the Top17 most intense ions were selected for fragmentation and an exclusion window of 40s was applied.

Peptides were identified and quantified from the acquired MS spectra using the MaxQuant software package, v1.6.0.16 (Tyanova et al., 2016) and a UniProt *E. coli* K12 proteome library (downloaded Nov 2015). Trypsin was set as digestion enzyme for semi-specific searches (e.g. only one side of the peptide was required to match the trypsin specificity). Label multiplicity was set to three, considering light dimethylation (+ 28.0313 Da), medium dimethylation (+ 32.0564 Da) and heavy dimethylation (+ 36.0757 Da) as peptide N-terminal and lysine labels. Carbamidomethylation of cysteine residues (+ 57.0215 Da) was set as fixed modification, methionine oxidation (+ 15.9949 Da) and protein N-terminal acetylation (+ 42.0106 Da) was considered as variable modifications. PSM false discovery rate was set to 0.01.

Identified peptides that showed at least a fourfold increase in intensity after protease treatment compared to the control treatment or were exclusively present in the protease-treated condition were considered as putative cleavage products. An in-house developed Perl script was used to remove putative library peptides (trypsin specificity on both sides of the identified peptide) and to reconstruct the full cleavage windows from the identified cleavage products as described (Schilling and Overall, 2008). Aligned validated cleavage windows were visualized as icelogos (Colaert et al., 2009), displaying site-specific differential amino acid abundance calculated as per cent difference compared to the *E. coli* K12 proteome as reference set (p-value 0.05).

In-gel digest of recombinant CT448

Coomassie-stained bands in preparations of recombinant CT448 were subjected to in-gel digestion with trypsin as described (Demir et al., 2013). Mass spectrometry analysis was performed as noted above and the sequence of recombinant CT448 was appended to contaminant database for peptide identification.

CT448 activity assays

Activity measurements of CT448 were performed with trypsin activation (+Tryp) and without (-Tryp) immediately after protein purification. CT448 was activated with recombinant trypsin (Sigma-Aldrich, St. Louis, USA) in a 1:20 mass ratio to CT448. For investigation of the substrate specificity, 100 µM of AAApNA, AAVpNA or AAPFpNA were incubated with 25 µg or 10 µg, of each activated (+Tryp) or non-activated (-Tryp), of purified CT448 in a final volume of 150 µl of 100 mM potassium phosphate buffer pH 8.0. Activity was measured as the increase in absorbance at 380 nm within the first 12 h at 27°C in a Biotek Synergy H4 plate reader (Biotek, Winooski, USA). Further, the specific proteolytic activity (mU/mg) of CT448 was calculated from the steepest parts of the curves.

In order to compare CT448 with a mammalian serine protease, we additionally tested the activity of 25 µg of porcine pancreatic elastase (CAS Number: 39445-21-1, Sigma-Aldrich, St. Louis, USA) on 100 µM AAApNA under the same conditions as described above. The effect of the isolated cyanobacterial protease inhibitors cyanopeptolin 954 (CP954) or nostopeptin 920 (BN920) on the activity of CT448 and of porcine elastase was measured. Thus, 20 µM of both inhibitors were separately added to trypsin-activated and non-activated CT448 or porcine elastase. All measurements were carried out in triplicates.

Statistical analyses

The statistics were conducted with the program Sigmaplot 11.0 (Systat Software GmbH, Erkrath, Germany). The data were analyzed via one-way ANOVA and a post-hoc analysis (Tukey HSD). The level of significance was $p < 0.05$.

Data availability

The mass spectrometry proteomics data for the PICS experiment have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al, 2015) partner repository with the dataset identifier PXD010978.

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1.7 Supplements

Specific against CT448 alone (165 aa identified/152 aa digestable: 100%; 49.3% seq. cov. in total)

10 20 30 40 50 60

ADRQVLLPYE KNWSKIEERW ANPSVPRDPR RVEEIPLRHK ATRESKDFRA VCGTPNAARI

70 80 90 100 110 120

INGAEATPHE FPWVTALFIS GGSFCTASLI SDEWVLTAAH CADGALYFDV YLGAHNVR**IT**

130 140 150 160 170 180

ETERLEIRAN EKYIHPDWNP NTLTGDVALI K**LPAPVDISG NNVRPICLQD PTDTSLYEGA**

190 200 210 220 230 240

EAHIAGWGKT ADGPGGISPT LQKSTVTVIS NDECQNTYGI IIRPSTICTS FTDTNSGTCN

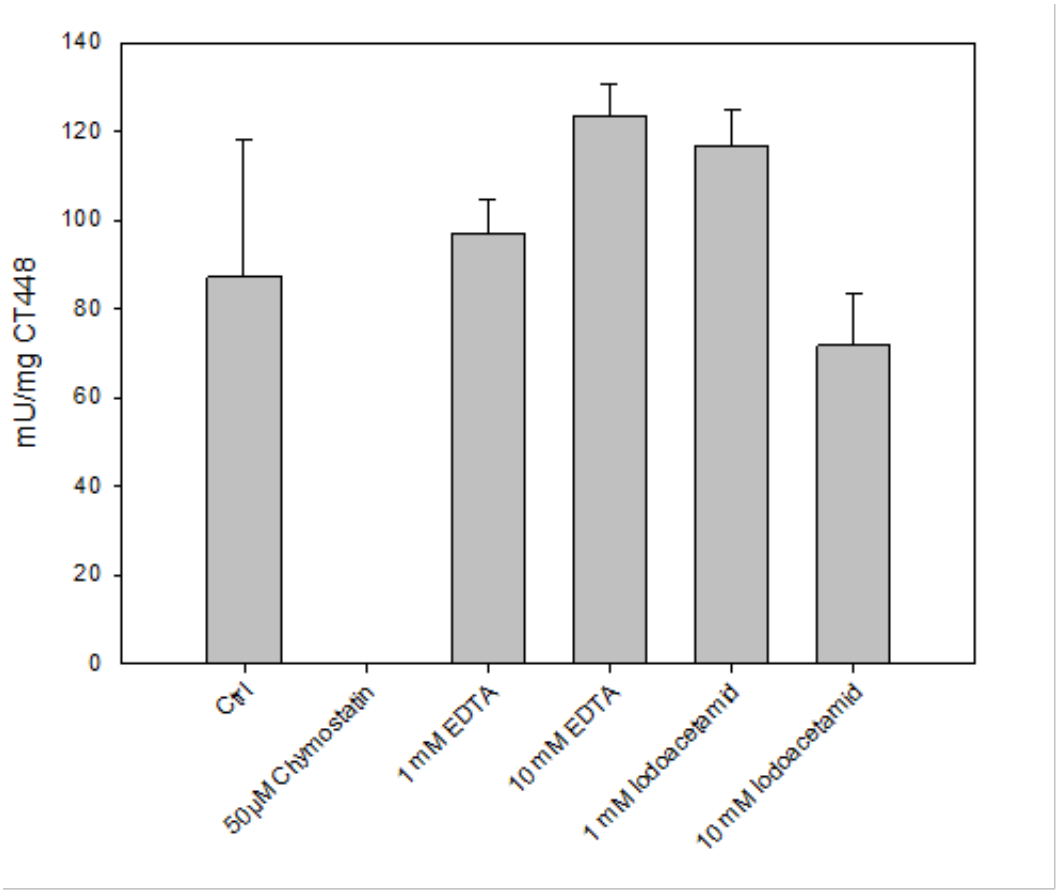
250 260 270 280 290 300

GDSGSAMSVI NSNGQYTQIG VTSFVSSAGC ESGNPDGYAR LSSYLSWISS ITGLVLENLY

310 320 330

FQGVDSAWSH PQFEK**GGGSG GGSGGSAWSH PQFEK**

Supplement 1: Peptide coverage of tryptic mass fingerprint of recombinant CT448. Bold red letters highlight identified peptides.



Supplement 2: Specific activity of trypsin-activated CT448 on N-Succinyl-alanine-alanine-proline-phenylalanine-para-nitroanilide (AAPFpNA, 100 µM) with different protease inhibitors. Protease activity was measured in presence of EDTA or iodoacetamide to exclude contaminating protease activity: EDTA was used to exclude metalloprotease activity and iodoacetamide for cysteine proteases. Chymostatin served as a positive control for chymotrypsins and diminished the activity. The control (Ctrl) was trypsin-activated CT448 without any inhibitor. Depicted is the mean specific enzymatic activity (n = 3, ± SD).

Chapter 2

Multi-clonal study of *Daphnia magna* with respect to adaptation to toxic cyanobacteria

corresponds to the publication:

Lange, J., Berges, A. C., & von Elert, E. (2023). Multiclonal study of *Daphnia magna* with respect to adaptation to toxic cyanobacteria. Limnology and Oceanography, 68(2), 514-524.

In order to demonstrate the adaptation of Swedish *D. magna* clones when compared to Polish clones, Chapter 2 presents a comparative analysis of *D. magna* clones from a Swedish lake, in which they coexist with the microcystin-free *Microcystis* sp. strain BM25, with clones from a Polish population naïve to cyanobacteria. Initially, I conducted the comparison of fitness in the presence of PI producing cyanobacteria in single-clone experiments, and subsequently, in a multiclonal experimental population. During this study I demonstrated, for the first time, that the Swedish clones are capable to dominate the mixed population in the presence of *Microcystis* sp. BM25, but not in its absence.

Authors contribution

The experiments were conducted and analyzed by me, Jacqueline Lange, and Amelie Berges. Jacqueline Lange and Eric von Elert designed the experiments and wrote the manuscript.

Data availability statement

All data generated or analyzed during this study are included in the manuscript or supplements. Raw data generated during this study are available from the corresponding author upon request.

2.1 Abstract

Mass developments of toxic cyanobacteria have increased in frequency due to global warming and eutrophication. Such cyanobacterial blooms impact whole freshwater ecosystems, especially reducing the abundance of herbivory species of the genus *Daphnia*. These negative effects on *Daphnia* have frequently been attributed to cyanobacterial secondary metabolites, among them hepatotoxic microcystins and protease inhibitors. Protease inhibitors inhibit major digestive proteases in the gut of *Daphnia* which results in reduced fitness, i. e. population growth. To date evidence for local adaptation of *Daphnia* to cyanobacteria is confined to microcystin-producing cyanobacteria and based on comparison of individual clones from different populations but lacks evidence from multi-clone microcosm experiments. In the present study, *D. magna* clones from a Swedish lake where they coexist with the microcystin-free *Microcystis* sp. strain BM25 were compared to clones from a Polish population without cyanobacteria, first in single-clone experiments and subsequently in a multi-clonal experimental population. The Swedish clones were assumed to be locally adapted to this protease inhibitor producing cyanobacterium and indeed showed higher population growth rates, a proxy for fitness, and dominated the population in the presence of dietary *Microcystis* sp. BM25, but not in the absence of this cyanobacterium. The results indicate an adaptive tolerance of the Swedish population and point at local adaptation to locally co-occurring protease inhibitor producing cyanobacteria.

2.2 Introduction

Cyanobacterial mass developments have increased in frequency over the last decades, due to eutrophication of waterbodies and climate change, which comes along with high levels of CO_2 and rising temperatures (Lampert and Sommer 1999; Paerl and Huisman 2008). Such cyanobacterial blooms are described as harmful for human and livestock (Falconer 1996), as many cyanobacterial strains produce toxins (Sivonen 1996). Furthermore, cyanobacterial blooms influence the aquatic ecosystem in terms of abiotic conditions, like light and turbidity (Paerl and Huisman 2008). Moreover, cyanobacteria are known to negatively impact the fitness of planktivorous zooplankton, like for example the freshwater cladoceran *Daphnia* sp.(reviewed by Ger et al. 2016), which is an important link between primary producers and higher trophic levels. Thus, cyanobacterial blooms reduce the flow of energy and material within the food web (Müller-Navarra et al. 2000). However, there are several main reasons why a cyanobacterial diet leads to reduced growth and reproduction rates of the non-selective filter-feeder *Daphnia* (Hansson et al. 2007; Ger et al. 2016). Besides low ingestibility of cyanobacterial colonies and filaments (Porter & McDonough 1984), deficiency of lipids in cyanobacteria that are essential for *Daphnia* (von Elert et al. 2003) and resultant changes in *Daphnia* behavior and anatomy (Bednarska & Dawidowicz 2007), the negative effects of cyanobacteria on *Daphnia* can at least partly be assigned to cyanobacterial secondary metabolites. Cyanobacteria produce a variety of biologically active secondary metabolites (Gademann and Portmann 2008; Janssen 2019) like microcystins and protease inhibitors , which have been shown to cause reduced growth and reproduction rates and increased mortality of *Daphnia* (Rohrlack et al. 2001; Lürling 2003; Gademann and Portmann 2008). Microcystins are a group of widely described cyanobacterial metabolites, but Lürling (2003) was the first who demonstrated that a microcystin-free cyanobacterial strain, *Microcystis aeruginosa* NIVA Cya 43, led to growth reduction and he suggested other secondary metabolites to impair the fitness of *Daphnia*. As a first case for negative effects of such other cyanobacterial secondary metabolites, Rohrlack et al. (2004) demonstrated molt inhibition in *Daphnia* by a cyanobacterial protease inhibitor. von Elert et al. (2005) identified two major protease inhibitors in *M. aeruginosa* NIVA Cya 43 and demonstrated that these two protease inhibitors reduced juvenile growth of *Daphnia*, when these protease inhibitors were fed via liposomes (von Elert et al. 2012). Hence, other unknown metabolites could be excluded as cause for the reduction of growth (von Elert et al. 2012). Similarly, Czarnecki et al. (2006) identified cyanobacterial inhibitors of *Daphnia* trypsins. Another microcystin-free strain is *Microcystis* sp. BM25 (Schwarzenberger et al. 2013b), which contains the three protease inhibitors micropeptins DR1056, DR1006 and MM978 (Schwarzenberger et al. 2010). These protease inhibitors are depsipeptides that inhibit digestive proteases in the gut of *Daphnia*, which is also known to result in reduced somatic growth and population growth rate, decreased

ingestion rates and increased mortality of *Daphnia* (Lürling 2003; Schwarzenberger et al. 2010).

From the perspective of water management an increased biomass of *Daphnia* constitutes a means to suppress cyanobacterial blooms (Wright and Shapiro 1984; Leibold 1989). However, it is controversial in how far *Daphnia* are able to suppress cyanobacterial blooms. Several studies demonstrated that bloom forming cyanobacteria have a negative impact on the abundance of *Daphnia* (Threlkeld 1979; Hansson et al. 2007) and that they cannot be controlled by grazing zooplankton (Ghadouani et al. 2003). These results are in contrast to other studies that depict that in particular *Daphnia* plays an important role in controlling the development of bloom forming cyanobacteria: Chislock et al. (2013) demonstrated, that *D. pulicaria* was able to decimate a phytoplankton community consisting of 96 % microcystin producing *Microcystis* sp. and *Anabena* sp. by over 70 %. In line with this, Sarnelle (2007) depicted that *D. pulicaria* were able to suppress an already developed cyanobacterial bloom consisting of 90 % toxic *Microcystis aeruginosa*. These partly contradictory results may be explained by the finding that *Daphnia* co-existing with cyanobacteria can evolve increased tolerance to toxic cyanobacteria both in time (Hairston et al. 1999; Isanta-Navarro et al. 2021) and space (Sarnelle and Wilson 2005; Wojtal-Frankiewicz et al. 2013; Schwarzenberger et al. 2017) the latter resulting in local adaptation. From the perspective of lake management, the identification of *Daphnia* with elevated tolerance to protease inhibitor-producing cyanobacteria would be of high interest, as these *Daphnia* could possibly be used as a tool to suppress and control protease inhibitor containing cyanobacterial blooms. However, as even single cyanobacterial strains may contain more than one bioactive secondary metabolite, it is unclear which of these cyanobacterial metabolites has become more tolerated in locally adapted *Daphnia*. Recently evidences for locus-specific positive selection underlying evolutionary adaptation of a Swedish *Daphnia magna* population to cyanobacterial protease inhibitors have been presented (Schwarzenberger et al. 2020).

Despite these strong molecular evidences for positive selection due to cyanobacterial protease inhibitors in the Swedish *D. magna* population, evidence for local adaptation of the Swedish population is confined to two approaches: (i) effects of cyanobacterial extracts on proteases in body homogenates of *D. magna* clones (Schwarzenberger et al. 2017; Schwarzenberger et al. 2013a) and (ii) effects of cyanobacterial strains on somatic growth and clutch size of various single *D. magna* clones from this experienced population (Schwarzenberger et al. 2021). However, it still remains to be tested if this local adaptation can be demonstrated on the level of fitness of various single *D. magna* clones and if these evidences for local adaptation can be confirmed in multi-clonal experiments.

It has been demonstrated in recent studies that the outcome of single clone experiments does not necessarily predict the outcome of multi-clonal experiments (Weider et al. 2008; Drugă et al. 2016). In this study, we performed multi-clonal microcosm experiments with an experienced Swedish and with a naïve Polish experimental *Daphnia* population in the presence and absence of a protease inhibitor producing cyanobacterium that had been isolated from the environment of the experienced *Daphnia* population to determine the fitness of the experienced *Daphnia* population, which has undergone positive selection. We used microsatellite analyses to track changes in genotype frequencies over a time-period of 51 days in order to allow for several rounds of reproduction thereby assessing effects of the dietary cyanobacterium on *Daphnia* fitness.

2.3 Methods

Organisms and cultivation

The green alga *Chlamydomonas klinobasis* (strain 56, culture collection of the Limnological Institute, University of Konstanz, Konstanz, Germany) was cultivated semi-continuously in cyanophycean medium (Von Elert and Jüttner 1997) at 20°C at 130 µE m^{-2} s^{-1}, with 20 % of the medium exchanged daily. The cyanobacterium *Microcystis* sp. strain BM25 (kindly provided by Ineke van Gremberghe, Ghent University, Ghent, Belgium) originates from Lake Bysjön in Southern Skania, Sweden. Strain BM25 has been shown to inhibit *D. magna* chymotrypsins, but not trypsins, *in vitro* (Schwarzenberger et al. 2013a) and does not contain microcystins (Schwarzenberger et al. 2013b). The cyanobacterium *Microcystis aeruginosa* NIVA Cya 43 (culture collection of the Norwegian Institute for Water Research) does not produce microcystins, but the two protease inhibitors cyanopeptolin 954 and nostopeptin 920 (von Elert et al. 2005). Both cyanobacteria were cultivated in chemostats on cyanophycean medium (von Elert and Jüttner 1997) with a light intensity of 50 µE m^{-2} s^{-1}, at a temperature of 20°C and constant air supply and a dilution rate of 0.1 d^{-1}. The concentration of particular organic carbon (POC) in the food suspensions was estimated regularly by measuring the extinction of the cultures at 470 nm. Carbon concentrations were calculated by a previously determined food-specific calibration curve to estimate the required volume of the food suspension for *Daphnia* growth experiments.

The *D. magna* clones used in this study (Table 1) were isolated from Lake Bysjön, Sweden from May to July 2010 (Schwarzenberger et al. 2013a) and in August 2010 from a Polish pond near Warsaw and cultivated in the lab for nine years prior to the experiments. The Swedish population experienced frequently cyanobacterial blooms whereas the Polish population did not (Schwarzenberger et al. 2017). For regular cultivation, 15 *Daphnia* originating from one clutch of a single mother were cultured in 800 ml membrane-filtered aged tap water, at 19°C,

at low light conditions with a day and night rhythm of 16:8 h. Every second day all animals were transferred into fresh water and fed with 2 mg POC/L^{-1} of *C. klinobasis* as food alga. Third clutch neonates of one mother were kept for constant culture extension.

Table 1: List of *D. magna* clones used for the growth experiments according to Schwarzenberger et al. 2017.

***D. magna* clones**	**Sampling year**	**Origin; geographical coordinates**
Mai7/M7, Mai17/M17, Mai20/M20, Mai24/M24, Jun6/J6, Jun17/J17, Jul8/J8	2010	Lake Bysjön, Sweden; LAT 55.675448, LON 13.545070
P2, P4, P12, P13, P21, P27, P31	2010	Pond near Warsaw; LAT 52.322722, LON 20.730515

Growth experiments with single Swedish and Polish *D. magna* clones

Each *D. magna* clone (Table 1) of either population was exposed to either *Microcystis* strain NIVA or strain BM25 in separate single clone growth experiments. Each clone was grown in 0 % (control), 10 % or 20 % of POC derived from NIVA or BM25 and 100 %, 90 % or 80 % POC from *C. klinobasis*, respectively. All experiments were performed at low light, 19°C,16:8 D:N rhythm and a total food concentration was 2 mg POC*L^{-1}. For the exposure, newborns from the 3rd and 4th clutch from a cohort of synchronized *D. magna* mothers were used. They were randomly distributed at 5 ind /glass into 250 mL aged tap water containing the five food treatments with each food treatment being run in triplicate. The experimental animals were transferred every second day into fresh water and food until the first clutch of the test animals hatched., The number of hatched neonates and the number of females which had released them were counted to calculate the population growth rate r (Eq. 1).

$$r = \sum l_x \times mx \times e^{-rx}$$

Equation 1: Euler-Lotka-equation for the calculation of the population growth rate r (d^{-1}). lx: Age-specific survivorship, mx: Number of neonates at day x, x: Age in days.

Mean and standard deviation of the population growth rates were calculated for each clone. In order to illustrate the effect of 20 % BM25 on the population growth rate *r* of each clone, tolerance was calculated as the difference between *r* on the BM25 treatment and *r* on control food normalized to *r* on control food (Eq. 2).

$$tolerance\ (\%) = \left(\frac{r_{mean(treat)} - r_{mean(ctrl)}}{r_{mean(ctrl)}}\right) \times 100$$

Equation 2: Tolerance (%). rmean (treat): Average population growth rate of the clone in the BM25 treatment. rmean (ctrl): Average population growth rate of the clone in the control treatment (Brzeziński and von Elert 2007).

The single clone growth experiments could not all be performed at the same time but were performed in four different blocks. To account for possible changes in conditions across experiments, the clone M17 was included in all four blocks of these single clone growth experiments as a reference clone. Populations growth rates of M17 neither differed under control conditions (100 % green alga, one-way ANOVA, F = 0.608, p >0.05) nor in food mixtures with 10 % of the cyanobacterium strain BM25 (one-way ANOVA, F = 3.263, p >0.05) nor with 20 % of BM25 (one-way ANOVA, F = 0.434, p >0.05) across the four experimental blocks (Supplements Table S2). The treatments 10 % and 20 % of the cyanobacterium NIVA were performed in block 3 and 4 only, and the respective population growth rates of M17 for these treatments were neither statistically distinguishable (Welch's t-test, for 10 % NIVA: t = -0.235, p >0.05; for 20 % NIVA: t = -0.676, p >0.05). We conclude that for neither food treatment block effects were detectable, and we hence assumed the absence of block effects with respect to food quality. This assumption is corroborated by a principal component analysis of population growth rates of all clones across all treatments in which no separation of data according to experimental blocks was visible (Figure S1).

Hence, data from all experimental blocks were pooled. To compare the effect of both cyanobacterial strains on the growth rates of the two *Daphnia* populations, for each clone a mean population growth rate r on the control, the 10 % and 20 % BM25 treatment was calculated. These mean values (n = 7) from either population were used to test for statistical differences among the Swedish and the Polish *Daphnia* population. Statistical differences were calculated with an analysis of variance (ANOVA) and post hoc test (Tukey's HSD) after testing for normal distribution (Shapiro-Wilk test) and homogeneity of the data (Levene's test).

Multiclonal microcosm experiment

A microcosm experiment with Swedish and Polish clones was conducted in the presence and absence of 20 % *Microcystis* sp. BM25 in the diet. In the control treatment the feeding suspension consisted of 100 % *C. klinobasis*, while the BM25 treatment consisted of 80 % *C. klinobasis* and 20 % BM25 with a total food concentration of 2 mg POC*L^{-1}. These two treatments were conducted with five replicates each. The experiment was performed in 10 L buckets filled with 5 L water and feeding suspension. Within each replicate three Swedish (M7, M17, M24) and three Polish (P4, P27, P31) *D. magna* clones were inoculated together. In order to prevent a clone-depended bias of the results, M7 and P4 were chosen as low performing clones, M17 and P31 as moderately performing clones and M24 and P27 as good performing clones in the presence of 20 % BM25, based on the single clone experiments. All replicates

were inoculated with four neonates per clone (initial relative abundance of each clone 16.7 % in each replicate). Once a week, the water of each replicate was replaced by new aged tap water. The replicates were fed with 2 mg POC*L^{-1} every other day and it was assured that the food concentration never fell below 0.4 mg POC*L^{-1} within a replicate in order to exclude quantitative food limitation. The experiment took place at 19°C and low light conditions with a day and night rhythm of 16:8. Based on developmental times observed in the single clone experiments and to make sure that at least a first generation of each clone had hatched, the first sampling was performed on day 16. The population within a replicate was thoroughly mixed before sampling and 10 % of the total volume, i.e. 500 mL were removed from the bucket and the *Daphnia* within these 500 mL were counted and collected in PCR tubes (two animals per tube). If the overall population within a bucket exceeded a number of 250 animals in total, the population was reduced to 250 animals, to avoid crowding effects. The experiment was stopped after 51 days. To analyze the abundances of the clones over the timespan of seven weeks, the relative abundances of the clones were determined at day 16, 37 and 51.

Microsatellite analysis

The animals in the PCR tubes were frozen and stored (-20°C) until DNA extraction. For DNA extraction the animals were squished with a pipette tip in 15 µL squish buffer, containing 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0 and 24 mM NaCl. The squished *Daphnia* were lysed with 5 µL proteinase K (20 mg*mL^{-1}, VWR International Radnor, PA USA) and incubated at 37 °C for 30 min with a termination step at 95°C for 3 min. Subsequently, the samples were centrifuged for 5 min at 13 000 rpm to clear the supernatant, which contained the DNA for the multiplex PCR. The QIAGEN Multiplex PCR Kit 1000 (Qiagen GmbH, Hilden, Germany) was used according to the manual and (Brede et al. 2006) with the following primers (Table 2) and PCR reaction: An initial temperature step of 95°C for 15 min was followed by 40 cycles of 30 sec at 94°C and 90 sec at 56°C (primer temperature) and extension for 90 sec at 72°C, and a final step at 95°C for 10 min. The multiplex PCR products were diluted 1:150 and analyzed at the Cologne Center of Genomics (Cologne, Germany) with a fluorescence-based-DNA-electrophorese-analysis (GeneScan 500 ROX dye Size Standard, Thermo Fisher Scientific, Waltham, MA USA).

Table 2: Multiplex primers used for the multiplex PCR. Tm: Annealing temperature (°C). AN: NCBI accession number. F: Forward primer. R: Reverse primer. Dye label was used to label the forward primers (Brede et al. 2006).

Locus	AN	Size range (bp)	Primers (5'-3')	Dye label	Tm (°C)
B045	HQ234168	118-126	F: GCTCATCATCCCTCTGCTTC R: ATAGTTTCAGCAACGCGTCA	FAM	56.0
B074	HQ234174	194-204	F: TCTTTCAGCGCACAATGAAT R: TGTGTTCCTTGTCAACTGTCG	FAM	56.0
B050	HQ234170	234-248	F: TTTCAAAAATCGCTCCCATC R: TATGGCGTGGAATGTTTCAG	HEX	56.0

The resulting electropherograms were analyzed with the software Geneious (Version 6.1.8, Biomatters Ltd, Auckland CBD, New Zealand). Multiplex primers were used as according to Brede et al. (2006), and within the loci (Supplements Table S1) the alleles of the six *D. magna* clones were determined by calling the peaks and setting bins, according to the user manual of the microsatellite plugin of Geneious. In prior experiments it had been possible to distinguish the six clones of the multiclonal microcosm samples via this analytical procedure. The resulting data were then analyzed with Microsoft Excel (Version 2016) and subsequently with R (Version 3.6.1). Firstly, relative abundances of each clone and each replicate were calculated. Secondly, means and standard deviations were determined. The data of at all three time points passed the normality test and the equal variance test ($p > 0.05$). Thereafter, a repeated measures three-way ANOVA was performed with the factors 'population', 'treatment' and the repeated factor 'day'. Pairwise comparisons were run between the two *Daphnia* populations for 'day' (days 16, 37, 51) and the two treatments (control food and 20 % BM25) with subsequent Bonferroni adjustment.

Statistical analyses

Statistical analyses were conducted in R (version 3.5.3, R Core Team 2019) and RStudio (version 1.2.5033, RStudio Team 2019). We tested for normal distribution of the data using a Shapiro-Wilk test and used a Levene's test to test for homogeneity of variance.

2.4 Results

For each of the seven clones from the Swedish or Polish *D. magnas* (Table 1) population growth rates were determined on pure *C. klinobasis* and on mixtures of *C. klinobasis* with 10 % or 20 % of *Microcystis* strain NIVA or strain BM25. The population growth rates *r* for the Swedish and the Polish population represent means calculated from the single clonal means (*n* = 7) (Figure 1). Interestingly, the presence of strain NIVA did not affect *r* of either the Swedish or the Polish population (Figure 1a), whereas a significant interaction of population and strain BM25, a cyanobacterium that originated from the same lake as the Swedish *D. magna* clones, was observed: The 20 % BM25 treatment negatively affected the Polish population growth rate compared to the control (Figure 1b), whereas the Swedish population was not affected by BM25. In the presence of 20 % BM25, the Polish population growth rate *r* was significantly lower than that of the Swedish population (Figure 1b, Tukey HSD after two-way ANOVA, $p < 0.05$; Supplement Table S3). In conclusion, effects of NIVA did not differ among the *Daphnia* populations, whereas effects of BM25 on the population growth rate were affected by the population origin such that 20 % BM25 had no effect on the Swedish population but significantly reduced *r* in the Polish population.

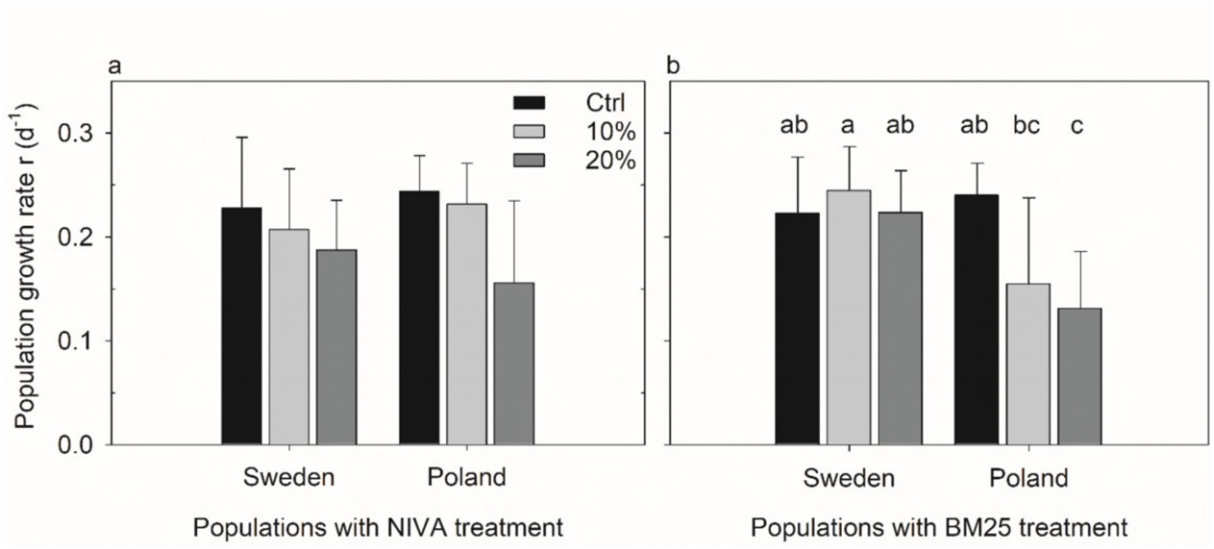


Figure 1: Population growth rates of Swedish and Polish *D. magna* clones exposed to different *Microcystis* strains. Population growth rates represent means of clonal means determined for each clone individually. a) Both populations were fed with the green alga *C. klinobasis* and either 0 % (Ctrl), 10 % or 20 % of *Microcystis aeruginosa* NIVA Cya 43 and b) Both populations were fed with the green alga *C. klinobasis* and either 0 % (Ctrl), 10 % or 20 % of *Microcystis* sp. BM25. Shown are means ± SD (*n* = 7) for each population. Tukey's HSD post hoc test after two-way ANOVA for A or B, $p < 0.05$. Letters indicate statistical differences between treatments within both populations.

In single clone experiments we determined the tolerance to either strain BM25 or strain NIVA. Within the Swedish and within the Polish *D. magna* population clones differed significantly in

tolerance to 20 % of strain NIVA (Figure S2 and Supplement Table S5), although tolerance was not distinguishable on the population level (Figure 1). With respect to 20 % strain BM25, significant clonal differences were apparent in both the Swedish and the Polish population (Figure 2) with an overall reduced tolerance of all Polish population (Supplement Table S8). Three clones of each population were chosen for high, medium and low tolerance to strain BM25 to ensure a balanced population for the subsequent multiclonal experiment with 20 % BM25. Clone Mai24 was significantly more tolerant to BM25 than the other tested clones (Figure 2a; Tukey HSD after one-way ANOVA, p <0.05; Supplement Table S4). Differences in tolerance were as well visible between the Polish clones. Here, P13, P12 and P27 were significantly more tolerant to BM25 and performed the best (Figure 2b; Tukey HSD after one-way ANOVA, p <0.05; Supplement Table S4). Therefore, for the Swedish population Mai24 was chosen as high performing, Mai17 as medium and Mai7 as low performing clone, and for the Polish population clone P27 represented a high performing clone, followed by P31 as medium and P4 as low performing clone. When considering population growth rates of these clones, the Swedish population, consisting of clones M7, M17 and M24, showed a significantly lower population growth rate r than the Polish population (clones P4, P27 and P31) under control conditions. This pattern was reversed when 20 % BM25 was present, thus the Polish population was significantly less tolerant against the cyanobacterium. (Supplement Figure S3).

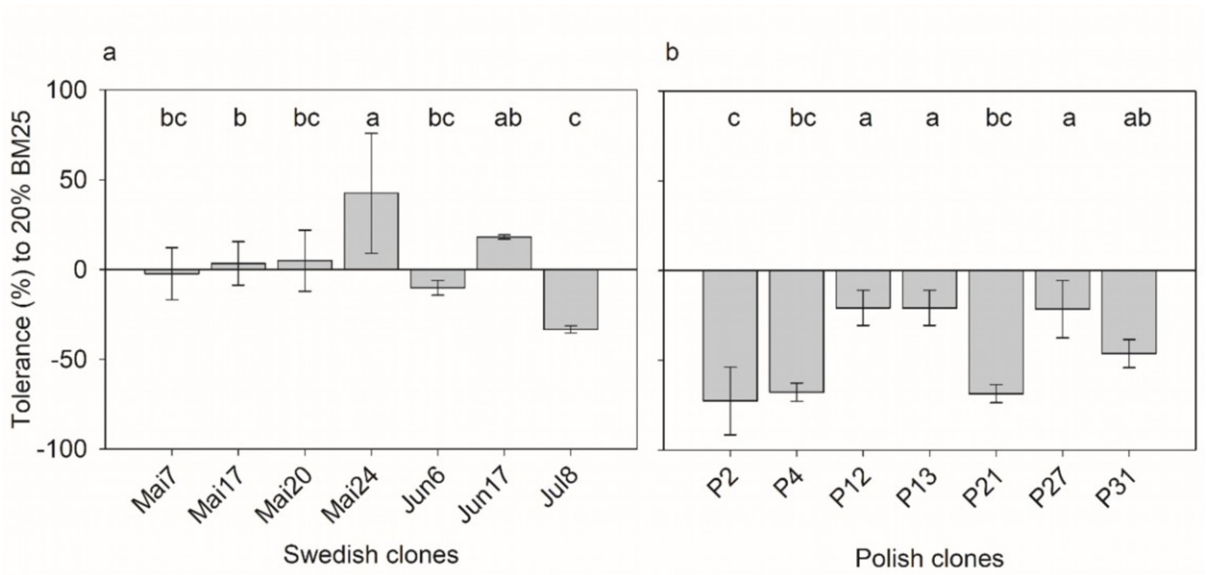


Figure 2: Tolerance of the Swedish and the Polish population of *D. magna* clones to *Microcystis* sp. BM25: a) Swedish clones Mai7, Mai17, Mai20, Mai24, Jun6, Jun17, Jul8 and b) Polish clones P2, P4, P12, P13, P21, P27, P31. Clones were grown on pure *C. klinobasis* ('control') and on a mixture of 20 % *Microcystis* sp. BM25 + 80 % *C. klinobasis* ('20 % BM25'). For each clone tolerance was calculated as the difference between population growth on 20 % BM25 and population growth on the control. Positive values indicate increased, negative values indicate decreased growth on 20 % BM25. Shown are means ± SD (n = 3) for each clone. Letters indicate differences between clones within the same population (Tukey's HSD post hoc test after one-way ANOVA $p < 0.05$).

In a subsequent microcosm experiment four synchronized neonates of each of these chosen Swedish and Polish clones were used to examine their performance within a mixed population with and without *Microcystis* sp. BM25 over a period of 51 days. Microsatellite analyses were conducted with samples from day 16, day 37 and day 51. Ten percent of the population was sampled and analysed to determine the abundance of the Swedish and Polish *D. magna* clones (Figure 3).

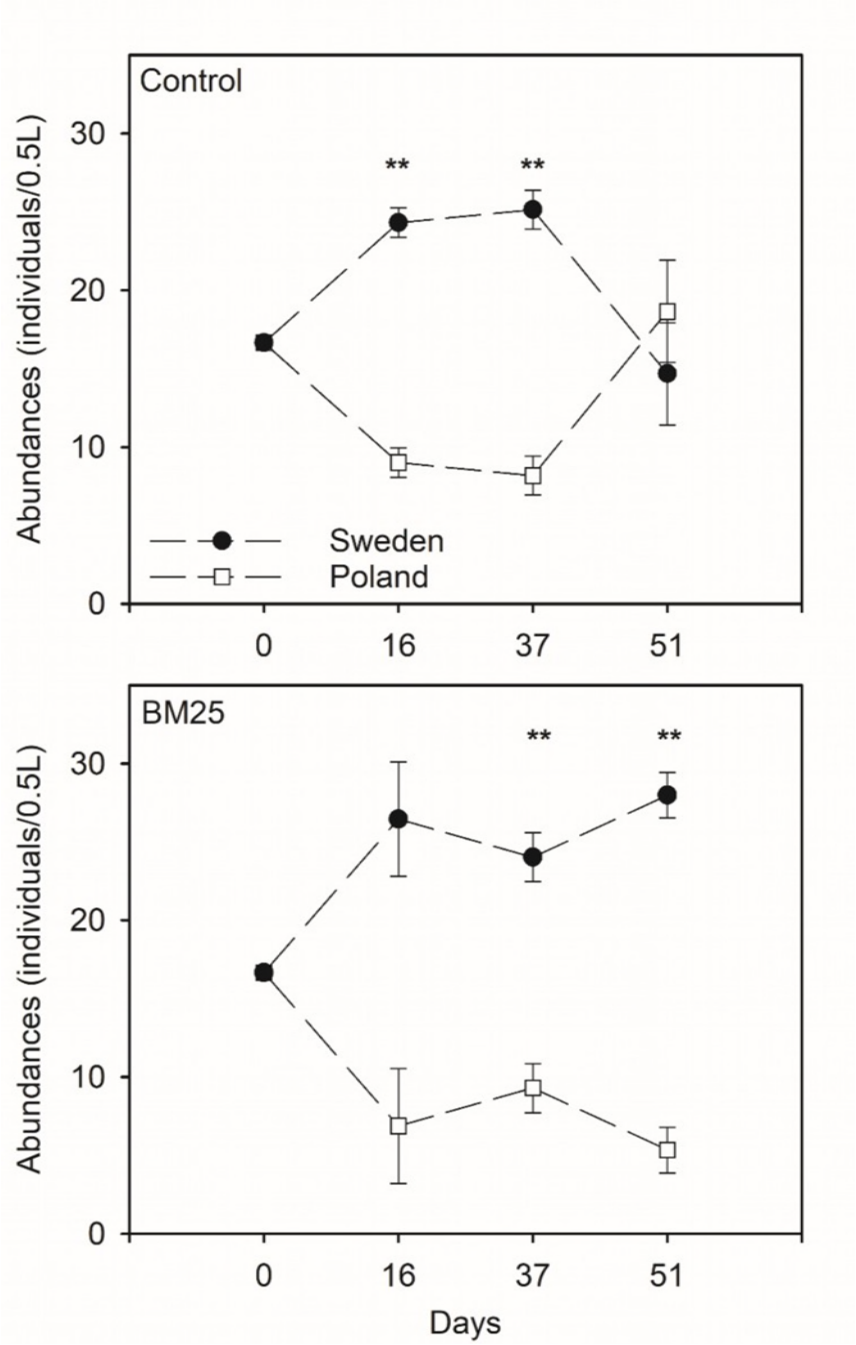


Figure3: Abundances over time of *D. magna* from the Swedish and Polish population during the microcosm experiment. Clones were distinguished by microsatellite analyses and pooled according to their population. Clones were tested in mixture with equal number of individuals in the beginning and exposed to two treatments: 'Control': 100 % *C. klinobasis* and '20 %; BM25': 20 % *Microcystis* sp. BM25 + 80 % *C. klinobasis*. Shown are mean ± SE (*n* = 5) for all

time points. Tukey's HSD post hoc test after repeated measures three-way ANOVA, p-values: *** <0.001; ** <0.01; * <0.05.

A repeated measures three-way ANOVA showed significant interaction of the factors population, day and treatment ($F(2,8) = 11.985$, $p = 0.004$) and of the factor population, $F(1,4) = 146.429$, $p = 0.000268$. Subsequently pairwise comparisons were run between the two *Daphnia* populations for "Day: 16, 37, 51" and the two treatments control and 20 % BM25 with subsequent Bonferroni adjustment. The abundances of the experimental Swedish and Polish population were significantly different in the control treatment on day 16 and 37 ($p < 0.05$) but not 51 ($p > 0.05$), whereas there was a significant difference between the two populations on day 37 and 51 ($p < 0.05$) in the 20 % BM25 treatment (Table 3).

Table 3: Results of pairwise comparison after repeated measures three-way ANOVA for the microcosm experiment. The data were grouped by 'day' and 'treatment', and pairwise comparisons were performed between the two *Daphnia* 'populations' ($n = 5$) with Bonferroni adjustment. The treatments consisted of the pure green alga *C. klinobasis* ('control') or a mixture of 80 % *C. klinobasis* with 20 % *Microcystis* sp. BM25 ('BM25').

Day	Treatment	Population	p-Value (adjusted)	Significance
16	BM25	Sweden - Poland	0.055	n.s.
16	Ctrl	Sweden - Poland	0.001	**
37	BM25	Sweden - Poland	0.009	**
37	Ctrl	Sweden - Poland	0.002	**
51	BM25	Sweden - Poland	0.001	**
51	Ctrl	Sweden - Poland	0.577	n.s.

In the control treatment the abundance of the Polish population increased towards the end of the experiment (day 51) while that of the Swedish population decreased, so that the abundance of both populations was not different. This pattern differed from that in the BM25 treatment where the Swedish population dominated the community in the latter part of the experiment (Figure 3, Table 3). Especially clone Mai24 was fairly abundant in both treatments (Figure 4, for further details see supplements Figure S4), corroborating its high tolerance to BM25 in the single clone experiments. In accordance with its relatively high tolerance to BM25 in the single clone experiments, the Polish clone P27 had a higher abundance (35 % of the population) than the two other Polish clones (P4: 11 %, P31: 10 %) in the BM25 treatment on day 51. The supposed disappearance of clones and their subsequent reappearance can be attributed to the low relative abundance of these clones which may lead to accidental absence in the subsamples taken for determination of clonal abundances.

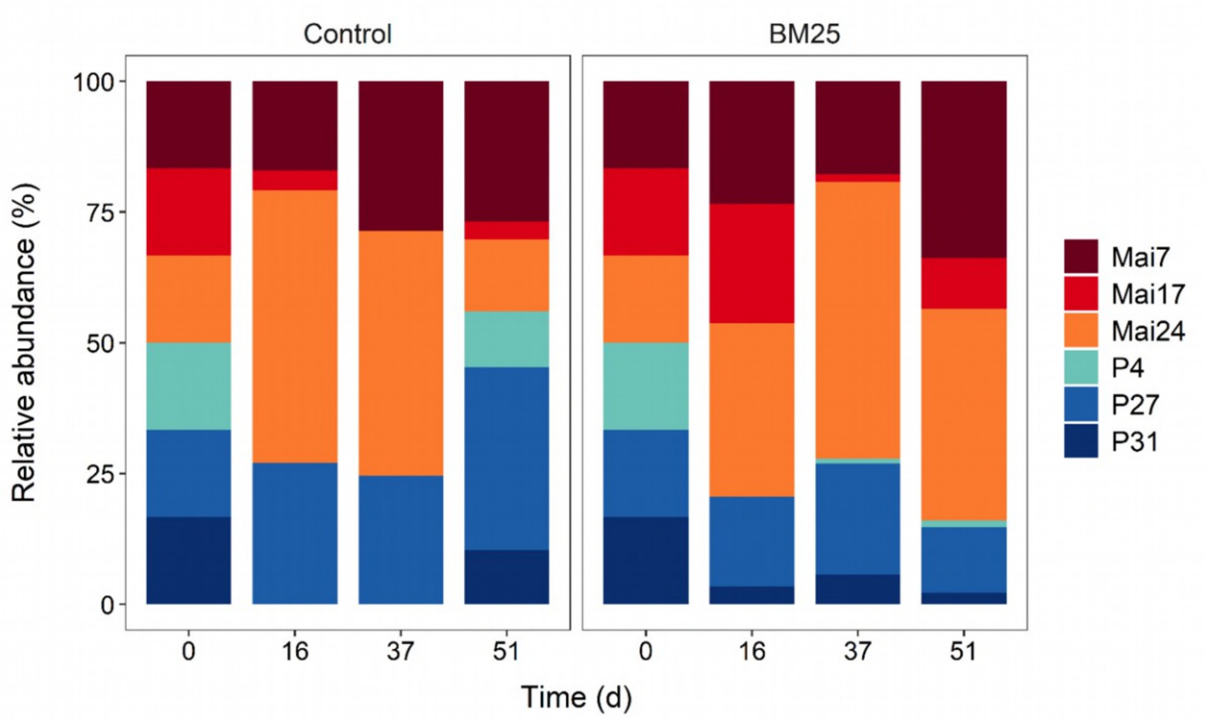


Figure 4: Community composition (relative abundances in %) of *D. magna* clones Mai7, Mai17, Mai24, P4, P27 and P31 in a microcosm experiment over a duration of 51 days. Clones were inoculated at identical relative abundances (16.4 %) and exposed to a Control (100 % *C. klinobasis*) and a BM25 treatment (20 % *Microcystis* sp. BM25 + 80 % *C. klinobasis*). Shown are mean values (n = 5) for all time points.

The strain *Microcystis* sp. BM25 originates from the same lake as the Swedish D. magna population. Hence, our results show that the experimental Swedish population had a higher fitness in the presence of Microcystis sp. BM25 but could not obtain dominance over the experimental Polish population under control conditions over time. The long-term dominance of the Swedish population in the presence of a cyanobacterium with the same origin indicates local adaptation to this cyanobacterial strain.

2.5 Discussion

Our results demonstrate local adaptation of a Swedish *D. magna* population to a *Microcystis* strain isolated from the same location and dominance over a naïve *Daphnia* population in the presence of this specific *Microcystis* strain. We showed that the fitness of the Swedish population was not negatively affected by *Microcystis* sp. BM25 whereas the Polish population was, when 20 % BM25 was present in the diet. This finding points at local adaptation of the Swedish population to a co-occurring cyanobacterium as it has been suggested in previous studies (Schwarzenberger et al. 2017; Schwarzenberger et al. 2020; Schwarzenberger et al. 2021). Such adaptations of *Daphnia* originating from lakes with annual exposure to toxic cyanobacteria are often assigned to microevolution (Hairston et al. 1999; Sarnelle 2007). Here

we used the two *M. aeruginosa* strains NIVA Cya 43 and BM25. Neither cyanobacterial strain produces microcystins, and both produce inhibitors of *Daphnia* digestive proteases, which have been shown to be inhibitors of digestive chymotrypsins. However, the strains synthesize chemically different inhibitors: The strain NIVA Cya 43 produces two inhibitory cyanopeptolines (Von Elert et al. 2005), whereas strain BM25, which was isolated from the Swedish lake, produces three inhibitory micropeptins (Schwarzenberger et al. 2013b). Secondary metabolites in *M. aeruginosa* strain BM25 and strain NIVA have only been characterized with respect to known protease inhibitors (von Elert et al. 2005; Schwarzenberger et al. 2013b). As single cyanobacterial strains may synthesize several classes of bioactive secondary metabolites (Gademann & Portmann 2008; Janssen 2019, Huang & Zimba 2019), we cannot rule out the possibility that the higher fitness of the Swedish *D. magna* population in the presence of BM25 is caused by substances other than protease inhibitors in BM25. However, due to the detailed investigation of *Daphnia*'s response to BM25 ranging from seasonal succession of *Daphnia* genotypes (Schwarzenberger et al. 2013a) over effects on growth and reproduction (Schwarzenberger et al. 2021) of varying inhibitor content (Schwarzenberger et al. 2013b) and sensitivity of gut proteases to protease gene copy numbers (Schwarzenberger et al. 2017) and evidence for site-specific selection on protease alleles (Schwarzenberger et al. 2020), we are confident that the observed higher fitness of the Swedish *D. magna* population may be attributed to the protease inhibitors present in strain BM25.

We as well observed an intra-population variance among of the *Daphnia* clones to the two cyanobacterial strains as Schwarzenberger (2021) reported previously. The idea that this variance within the Swedish *D. magna* population results from a strong punctual selection caused by the seasonal occurrence of cyanobacterial blooms could not be supported: Although a strong bloom of protease inhibitor-containing cyanobacteria occurred in May in the Swedish lake, no difference in tolerance, measured as IC_{50} values, to inhibition by natural lake seston from May was found between the clones from before and after the bloom (Schwarzenberger et al. 2013a), which corroborates findings obtained for a hypertrophic pond (Küster et al. 2013). However, in another case study, increasing tolerance to cyanobacteria over the season has been demonstrated in a *Daphnia* population (Schaffner et al. 2019).

It has been shown repeatedly that single clone experiments cannot predict the outcome of complex multiclonal competition experiments (Wieder et al. 2008; Drugă et al. 2016). Thus, it was necessary to test both populations in a microcosm experiment in order to verify the results of the single-clone experiments in a community context. Similar to other multi-clonal experiments, in which population dynamic effects were reported after 30-50 days (Jeyasingh et al. 2009; Engel and Tollrian 2009), our experiment lasted 51 days. The Swedish clones dominated the community in the 20 % BM25 treatment over the second half of the experimental time, but were not dominating in the control treatment after 51 days. This demonstrates that

under non-blooming conditions the Swedish clones do not show higher fitness over a long period.

Chymotrypsin and trypsin, which belong to the S1 family of serine proteases, are the most important digestive enzymes in the gut of *D. magna* as they account for 80 % of the whole proteolytic activity (von Elert et al. 2004). More than 20 protease inhibitors, which specifically inhibit chymotrypsins and trypsins, have been found in different marine and freshwater cyanobacteria (Gademann and Portmann 2008; Köcher et al. 2020). Protease inhibitor producing cyanobacteria have a negative impact on *Daphnia* and thus on the aquatic ecosystem (Baumann and Jüttner 2008). Schwarzenberger et al. (2020) showed that the digestive proteases in *D. magna* of the Swedish population have undergone positive selection on the loci of the serine proteases CT448 and CT802. Especially, CT448 harbors a nonsynonymous mutation in the Swedish clones, which might have altered the protein structure such that it resulted in higher tolerance to protease inhibitors, which in turn would require lower expression levels of CT448 to obtain the same protease activity in the presence of protease inhibitors. Given that this assumption is correct, it provides an explanation for the finding that increased tolerance to protease inhibitors in the Swedish population is associated with lower copy numbers of the CT448 that harbors this nonsynonymous mutation (Schwarzenberger et al. 2017). Although elevated tolerance to protease inhibitors still remains to be shown for CT448 itself, gut homogenate of the Swedish *Daphnia* population proved to be more tolerant to natural protease inhibitors (based on IC_{50} values) than the Polish *Daphnia* population (Schwarzenberger et al. 2017). The most abundant clones in our microcosm experiment (clones M24 and M7) have low copy numbers of CT448 (M24; two copies; M7: two copies; M17: three copies; P27: three copies; P4: three copies; P31: four copies) whereas the copy numbers of the other serine proteases CT383 and CT802 did not differ between the populations (Schwarzenberger et al. 2020). This suggests that a low copy number of CT448 might result in a reduced susceptibility against protease inhibitors likewise in the most dominant Swedish clones in our experiment.

Further studies have shown that CT448 is upregulated when cyanobacteria with protease inhibitors are present in the diet. Druga et al. (2016) conducted a competition experiment with *D. galeata* in which CT448 was by far the most up-regulated gene of all tested candidates when *M. aeruginosa* was present. Although Druga et al. (2016) could not correlate the tolerance of the single clones with an up-regulation of this gene, they suggested that CT448 strongly reacts to food quality changes. Still, we suspect that CT448 might play an important role in the tolerance to cyanobacteria and in local adaptation. Here, we have compared two *D. magna* populations in the presence of 20 % of a cyanobacterium that does not form colonies in a mixture with a high-quality food alga with the aim to limit dietary effects on *Daphnia* exclusively to the secondary metabolites of the *Microcystis* strains. This experimental setting

may not reflect the environmental scenario during cyanobacterial mass developments which may be characterized by the occurrence of filamentous and colonial cyanobacteria comprising considerably higher shares of phytoplankton, in particular during cyanobacterial mass developments. With respect to the control of cyanobacterial blooms, *Daphnia* biomass has been shown to be important for food chain manipulation in terms of lake management (Leibold 1989; Carmichael et al. 2001; Codd et al. 2005; Sarnelle 2007). However, the degree of suppression of cyanobacterial blooms is largely dependent on the initial conditions, i.e. the relative abundances of cyanobacteria and *Daphnia* in a complex way (Sarnelle 2007; for more details see reviews by Ger et al. 2016) and Moustaka-Gouini and Sommer (2020). Although progress has been made with respect to understanding the molecular basis of local adaptation of *Daphnia* to protease inhibitors in cyanobacteria (Schwarzenberger et al. 2020), the significance of these findings with respect to the control of cyanobacteria by *Daphnia* has yet to be determined. Here we have demonstrated superiority of the adapted Swedish *D. magna* population in the presence of 20 % of cyanobacteria, which rather mimics pre-bloom conditions.

In this context it has to be noted that the protease inhibitor content of a cyanobacterial strain may vary. As shown for strain NIVA Cya 54 (Burberg et al. 2019; Burberg et al. 2020), Schwarzenberger et al. (2013b) demonstrated that the protease inhibitor content of *M. aeruginosa* strain BM25 was strongly affected by available nutrient ratios. In accordance with the carbon-nutrient-balance hypothesis (van de Waal et al. 2014) the content of the N-containing protease inhibitors in BM25 decreased under N-limitation (C:N = 13) and increased under P-limitation (C:P = 234).

In the few cases in which adaptation of *Daphnia* populations to cyanobacteria has been demonstrated in time and space, evidence for adaptation is based on effects of dietary cyanobacteria on somatic growth rate (and partly on clutch size) in experiments with individual *Daphnia* genotypes (Hairston et al. 1999; Sarnelle and Wilson 2005; Schwarzenberger et al. 2017; Schwarzenberger et al. 2020). However, somatic growth rate is not a good predictor of fitness in case of xenobiotic stressor, which include toxic cyanobacteria (Trubetskova and Lampert 2002). Here, for the first time we used increased fitness as a proxy for adaptation of populations, here and show local adaptation to cyanobacteria of the Swedish *D. magna* population, which represents the only case in which the molecular basis of local adaptation of *Daphnia* to cyanobacteria has been understood (Schwarzenberger et al. 2017; Schwarzenberger et al. 2020). By calculating *F*ST values in neutral loci (microsatellites, exon sequences of selected single copy genes), Schwarzenberger et al. (2020) found evidences for genetic isolation of the Swedish and the Polish *D. magna* populations. Further, these two populations were genetically more distant in three protease loci (three chymotrypsin genes) than in the neutral loci, which suggested that the evolution of these protease genes has been

driven by adaptive selection by cyanobacterial protease inhibitors present in the Swedish lake. This suggestion was further confirmed by population genetic tests applied to the tolerant Swedish *D. magna* population (Schwarzenberger et al. 2020).

Here we determined population growth rates as a proxy for fitness of individual *Daphnia* genotypes and corroborate the evidence for local adaptation of this Swedish *D. magna* population to cyanobacteria. In a microcosm experiment with the absence/presence of a local Swedish cyanobacterium we demonstrate that the presence of cyanobacteria impacts the genetic composition of *Daphnia* and show that the Swedish *D. magna* population is capable of dominating a mixed population over a long period in the presence of cyanobacteria. Under control conditions this fitness advantage disappears, but there is no evidence that the Swedish *D. magna* population underperforms relative to the Polish population. Hence, we cannot provide evidences for a cost associated with higher tolerance to *Microcystis* sp. strain BM25, so that there is no evidence for local adaptation in the strict definition given by Kawecki & Ebert (2004); still the higher fitness of the Swedish *D. magna* population in the presence of the cyanobacterium and evidences for adaptive selection on the chymotrypsin loci in this *D. magna* population (Schwarzenberger et al. 2020) point at local adaptation to co-occurring cyanobacterial protease inhibitors.

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2.7 Supplements

Table S1: Alleles of microsatellites of the Swedish clones M7, M17 and M24 and the Polish clones P4, P27 and P31. The alleles were determined in prior experiments, where each clone was analyzed separately.

***D. magna* clone**	**Loci**					
	B045-1	**B045-2**	**B074-1**	**B074-2**	**B050-1**	**B050-2**
M7	120-121		197	200-202	238	243
M17	120-121			200-202	238	
M24	120-121			200-202		244
P4	120-121	123	197		238	243
P27	120-121	123	197			241
P31	120-121	123	197		238	

For the analysis of the electropherograms with the software Geneious (Version 6.1.8, Biomatters Ltd, Auckland CBD, New Zealand) the following rules were determined beforehand to ensure a consistent analysis for all replicates of all time points:

1. Allele 200-202 indicates presence of a Swedish clone, allele 123 indicates presence of a Polish clone.

2. Allele 244 always indicates the presence of clone M24, allele 241 always indicates presence of clone P27.

3. Is allele 200-202 or 123 missing, but allele 244 or allele 241 is present, rule no. 2 is still valid.

4. Is allele 200-202 or 123 missing, it is generally decided against a Polish or a Swedish clone.

Table S2: One-way ANOVA on population growth rate of the reference clone M17 (n = 3) within the four experiment blocks. The reference clone was either fed with control food (green algae), or a mixture of this green alga with 10 % or 20 % *Microcystis* sp. BM25.

	Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
10 % Bm25 control	**Block**	3	0.00192	0.000639	0.608	0.628
	Residuals	8	0.00841	0.00105		
20 % Bm25 control	**Block**	3	0.00663	0.00221	3.263	0.080
	Residuals	8	0.00542	0.000678		
	Block	3	0.00115	0.000382	0.434	0.734
	Residuals	8	0.00704	0.00704		

Table S3: Two-way ANOVA on the effect on population growth rates of Swedish and Polish *D. magna* clones exposed to either pure green alga ('control') or mixtures of this green alga with 10 % or 20 % of *Microcystis* strains BM25 or NIVA. The factors were 'population' (Sweden, Poland) and 'treatment' (Control, 10 %, 20 %) of *M. aeruginosa* NIVA or *Microcystis* sp. BM25 diet. For each *Microcystis* strain a two-way ANOVA was calculated.

	Source of Variation	**DF**	**SS**	**MS**	**F**	**P**	
BM25	Population	1	0.01864	0.018641	6.103	0.015679	*
	Treatment	2	0.01807	0.009033	2.957	0.057818	
	Population x Treatment	2	0.04909	0.024544	8.036	0.000671	***
	Residual	78	0.23825	0.003054			
NIVA	Population	1	0.01864	0.018641	5.272	0.0244	*
	Treatment	2	0.01877	0.009384	2.654	0.0767	
	Population x Treatment	2	0.01084	0.005418	1.532	0.2224	
	Residuals	78	0.27580	0.003536			

Table S4: One-way ANOVA on the tolerance of *D. magna* clones (n = 3) to a mixture of 80 % of the green alga with 20 % *Microcystis* sp. BM25 compared to growth on the green alga only. Shown are the results for the Swedish clones Mai7, Mai17, Mai20, Mai24, Jun6, Jun17, Jul8 and for the Polish clones P2, P4, P12, P13, P21, P27, P31. A one-way ANOVA was calculated for each population.

	Source of Variation	**DF**	**SS**	**MS**	**F**	**P**	
Sweden	Clone	6	0.9957	0.16596	5.737	0.0034	**
	Residual	14	0.4050	0.02893			
Poland	Clone	6	1.3156	0.21927	22.23	2.11e-06	***
	Residuals	14	0.1381	0.00986			

Table S5: One-way ANOVA on the tolerance of *D. magna* clones (n = 3) to a mixture of 80 % of the green alga with 20 % *Microcystis aeruginosa* NIVA compared to growth on the green alga only (see Figure S2). Shown are the results for the Swedish population represented by clones Mai7, Mai17, Mai20, Mai24, Jun6, Jun17, Jul8 and for the Polish population represented by clones P2, P4, P12, P13, P21, P27, P31. A one-way ANOVA was calculated for each population.

	Source of Variation	DF	SS	MS	F	P	
Sweden	Clone	6	5.502	0.917	83	3.92e-10	***
	Residual	14	0.155	0.011			
Poland	Clone	6	1.8591	0.30985	8.566	0.00049	***
	Residuals	14	0.5064	0.03617			

Table S6: Two-way ANOVA on the effect of a mixture of 80 % green with 20 % of *Microcystis* sp. strain BM25 on the population growth rate r compared to growth on the pure green alga. the factors 'population' and 'treatment' were used as factors. The Swedish population was represented by clones M7, M17, and M24; and the Polish population by clones P4, P27, and P31 (see Figure S3), n = 3 for each clone.

Source of Variation	DF	SS	MS	F	P	
Population	1	0.01177	0.01177	7.21	0.00846	**
Treatment	1	0.05981	0.05981	36.65	2.37e-08	***
Population x Treatment	1	0.10248	0.10248	62.80	2.97e-12	***
Residuals	102	0.16645	0.00163			

Table S7: Repeated measures three-way ANOVA for the microcosm experiment on the factors 'day', 'population' and 'treatment' in a ANOVA table (type III). The populations (Sweden and Poland) consisted of three different clones which have been fed with a green alga (control) or a mixture of 80 % green alga with 20 % *Microcystis* sp. strain BM25. Clone abundances were determined on day 16, 37 and 51 (n = 5).

Effect	DFn	DFd	F	P		ges
Day	2	8	-0.002	1.0		8.29e-21
Treatment	1	4	-0.004	1.0		7.73e-21
Population	1	4	146.429	0.000268	***	7.09e-01
Day x Treatment	2	8	-0.001	1.0		3.94e-21
Day x Population	2	8	1.590	0.262000		1.29e-01
Treatment x Population	1	4	2.711	0.175000		2.16e-01
Day x Treatment x Population	2	8	11.985	0.004000	**	3.15e-01

Table S8: Nested ANOVA on the tolerance to *Microcystis* sp. strain BM25 of the Swedish and the Polish population. The Factor "clone" is nested within the factor "population". Each population consisted of seven different clones (n = 3).

Source of Variation	DF	SS	MS	F	P	
Population	1	2.2856	2.2856	117.846	1.52e-11	***
Population (Clone)	12	2.3114	0.1926	9.931	3.57e-07	***
Residuals	28	0.5431	0.0194			

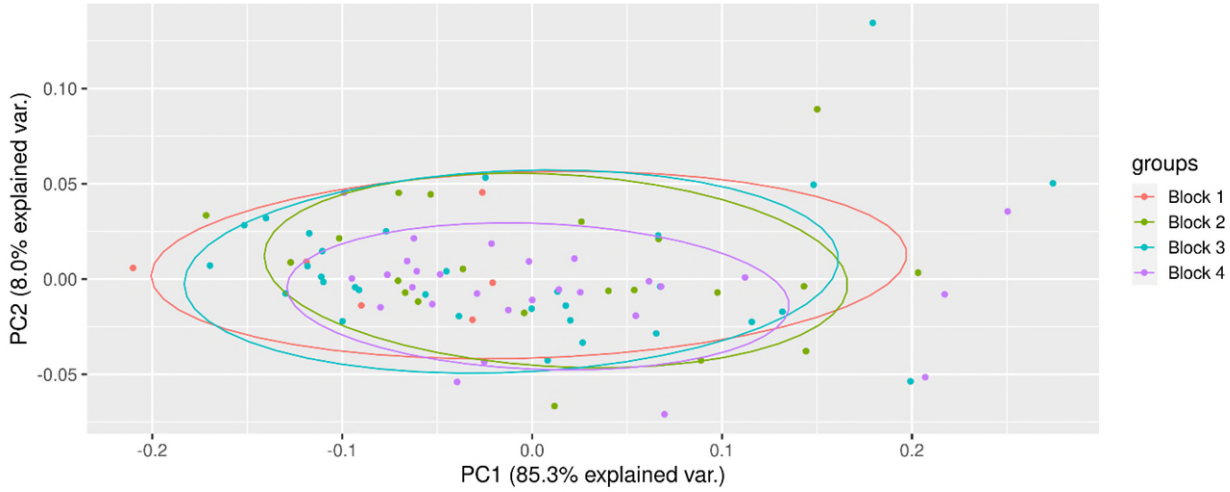

Figure S1: Principal Component Analysis of population growth rates of all *D. magna* clones on all five food treatments (100 % green alga, mixture of green alga with 10 % or with 20 % of the cyanobacterium *Microcystis* sp. strain BM25 or with 10 % or with 20 % of the cyanobacterium *M. aeruginosa* strain NIVA across all four experimental blocks. Ellipses indicate the 0.69 confidence intervals.

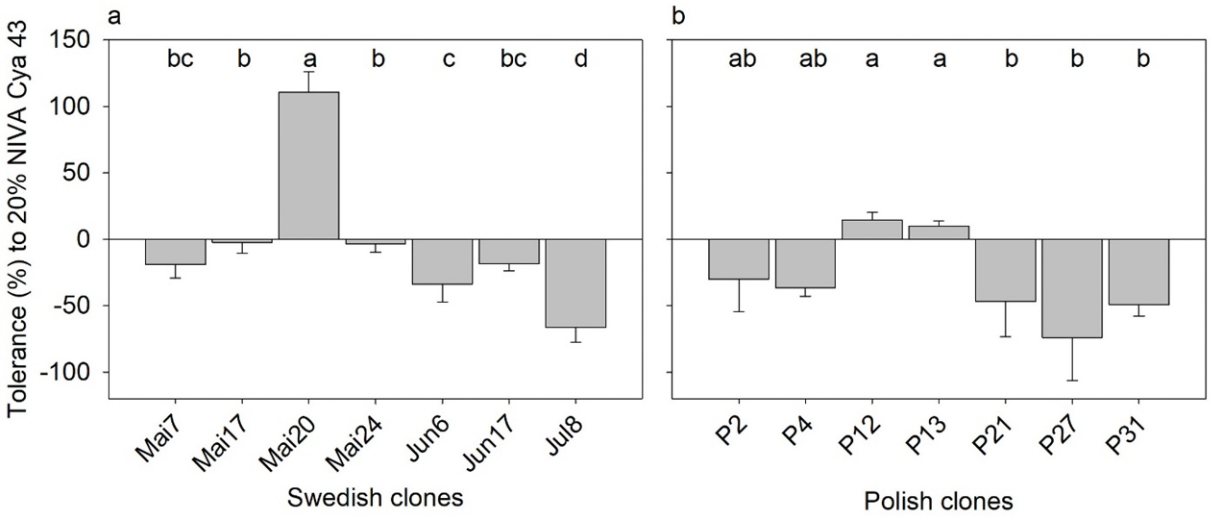

Figure S2: Tolerance of individual clones of a) the Swedish and b) the Polish population of *D. magna* clones to *Microcystis aeruginosa* NIVA Cya 43. Clones were grown on pure *C. klinobasis* ('control') and on a mixture of 20 % *M. aeruginosa* NIVA Cya 43 + 80 % *C. klinobasis* ('20 % NIVA'). For each clone tolerance (%) was calculated as the difference between population growth on 20 % NIVA and growth on the control, and this difference was normalized to population growth on the control. Positive values indicate increased, negative values indicate decreased growth on 20 % NIVA. Shown are means ± SD (n = 3) for each clone. Letters indicate differences between clones within the same population (Tukey's HSD post hoc test after one-way ANOVA $p < 0.05$: see Table S5).

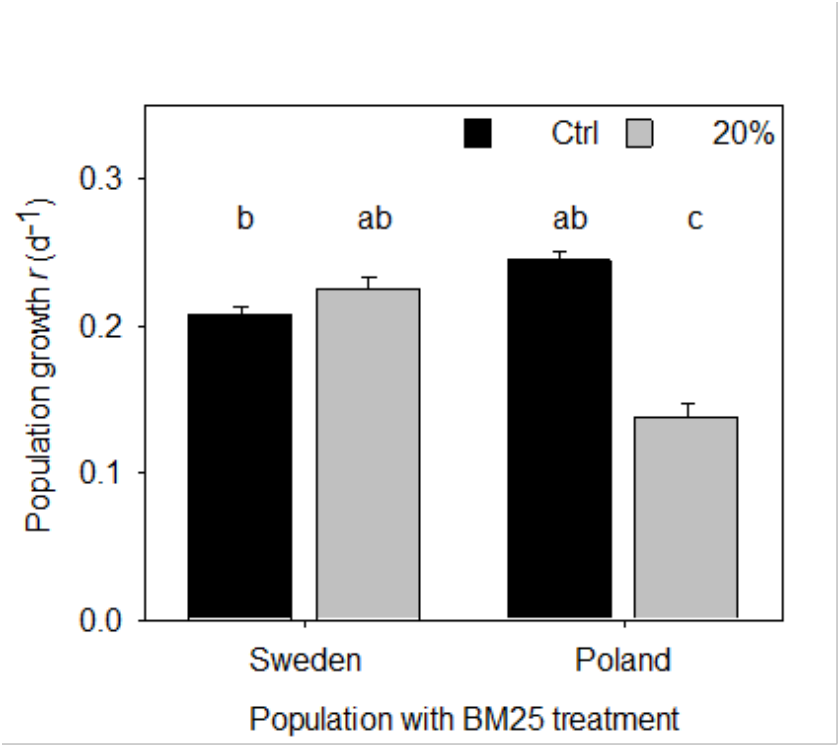

Figure S3: Population growth rate r of the Swedish (M7, M17, M24) and the Polish (P4, P27, P31) population in the absence and presence of 20 % BM25 in the diet. Clones were either grown on pure *C. klinobasis* (control, Ctrl) or on a mixture of *C. klinobasis* with 20 % of *Microcystis* sp. strain BM25 (20 %). Shown are means ± SD (n = 3) for each population. Letters indicate statistical differences between treatments within both populations (Tukey's HSD post hoc test after two-way ANOVA, $p < 0.05$; see Table S6).

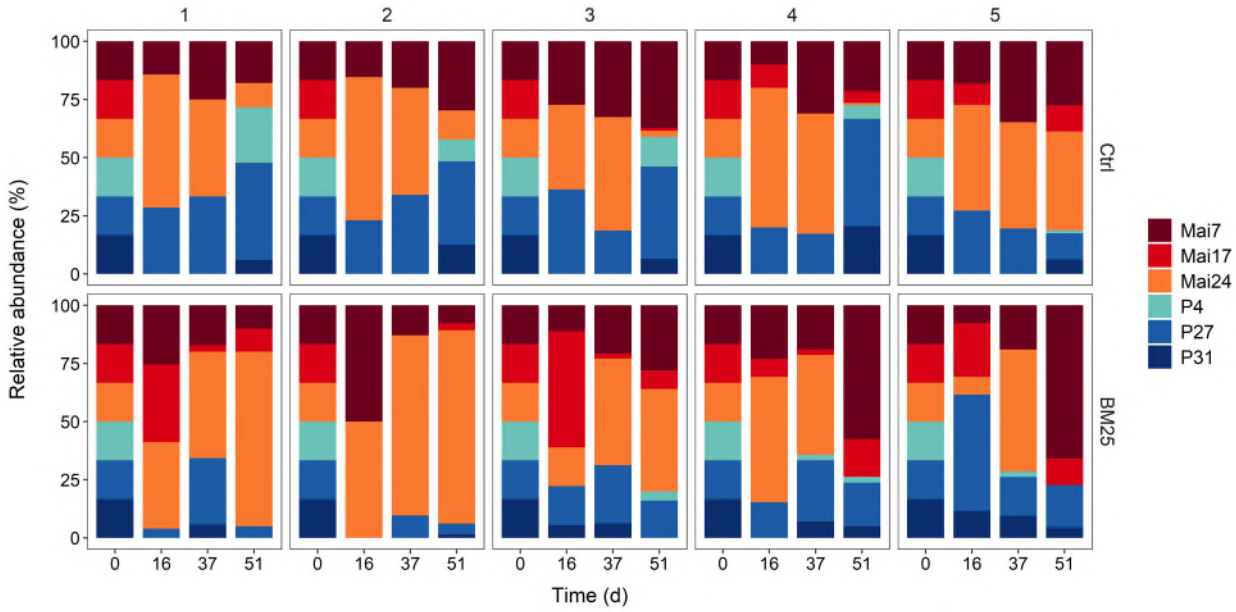

Figure S4: Relative abundances (%) of *D. magna* clones M7, M17, M24, P4 P27 and P31 in a microcosm experiment testing for the impact of *Microcystis* sp. BM25 on community composition over a duration of 51 days. Clones were tested in mixture with equal numbers of individuals in the beginning. The clones were exposed to two treatments: Control ('Ctrl'): 100 % *C. klinobasis* and 20 % BM25 ('BM25'): 20 % *Microcystis* sp. BM25 + 80 % *C. klinobasis*. Each of the two treatments was replicated fivefold, and the numbers on top indicate the different replicates.

Chapter 3

Development of a method for the measurement of elastase activity in *Daphnia magna* with respect to local adaptation

As the results presented in Chapter 1 confirmed the reclassification of CT448 from chymotrypsin to chymotrypsin-like elastase, I hypothesized that the methodology for identifying PIs via the enzyme kinetics of *D. magna* homogenates requires modification, given that the previous identification of inhibitors was conducted against chymotrypsin activity. In Chapter 3, I tested the elastase activity of *D. magna* homogenates in presence of methanolic fractions of *Microcystis* sp. strain BM25 cell extract, with and without solid phase extraction (SPE). The results demonstrate a significant inhibitory effect of methanol itself on elastase activity, but not on chymotrypsin activity. Following a methodological adaptation, assays for elastase activity were conducted using homogenates from naïve Polish and adapted Swedish *D. magna* clones, with fractions of *Microcystis* sp. strain BM25 extract obtained by SPE dissolved in dimethyl sulfoxide. A population effect between the Swedish and Polish *D. magna* homogenates was not observed. However, I could demonstrate the necessity for optimized elastase enzyme assays to establish a link between genetic adaptations and physiological responses and purification of cyanobacterial PIs.

Authors contribution

The experiment was planned and performed by me, Jacqueline Lange, as well as the data visualization and analysis. Fractionated cyanobacteria cell extracts were kindly provided by Thomas von Einem. Jacqueline Lange exclusively wrote this chapter, which was critically read by Meike Hahn and Eric von Elert.

Data availability statement

All data generated or analyzed during this study are included in the manuscript or supplements. Raw data generated during this study are available from the corresponding author upon request.

3.1 Abstract

Anthropogenic actions and global warming have increased cyanobacterial blooms, which produce toxic metabolites affecting freshwater ecosystems and the fitness of *Daphnia*. Previous studies investigated the local adaptation of *Daphnia magna* to cyanobacteria producing protease inhibitors (PIs), focusing on the serine protease gene *ct448* and its role in *Daphnia* gut homogenates. CT448 has a chymotrypsin-like structure but the substrate specificity of elastases. Prior research has primarily focused on the serine proteases chymotrypsin and trypsin, and CT448 has been incorrectly categorized as chymotrypsin and investigated without specific elastase substrates. In this context, the primary identification was that of inhibitors against chymotrypsin. Thus, it is crucial to provide optimal enzyme assay conditions for the identification of cyanobacterial PIs against elastase by bioassay guided fractionation. The isolation of PIs that have co-existed with adapted *Daphnia* might give insights to their adaptive mechanism. Therefore, elastase activity assays were conducted using gut homogenates from naïve Polish and adapted Swedish *D. magna* clones, which coexisted with the PIs producing *Microcystis* sp. strain BM25. The results indicated a significant inhibitory effect of methanol on elastase, therefore requiring the use of dimethyl sulfoxide (DMSO) as a solvent for bioassay guided fractionation of PIs from *Microcystis* sp. strain BM25. This effect of methanol did not affect chymotrypsins, which dominantly account for the digestive activity in *Daphnia*. Both tested populations of *D. magna* demonstrated inhibition of elastases by two BM25 fractions, which were obtained by SPE dissolved in DMSO. A population effect between the Swedish and Polish *D. magna* homogenates was not observed, only trends in responses to the two fractions were visible. We suspect *D. magna* population to exhibit greater differences when pre-grown on *Microcystis* sp. strain BM25, as it has been previously demonstrated that maternal effects play an important role in the response to toxic cyanobacteria. This research highlights the need for optimized enzyme assays for elastases to link genetic adaptations with physiological responses. Further, the improved measurements of elastase activity in *Daphnia*'s gut can be utilized to purify the known PIs of BM25 or harmful protease inhibitors of cyanobacteria.

3.2 Introduction

Freshwater bodies such as lakes and ponds not only function as important freshwater reservoirs for humans, but also represent complex ecosystems. Within these ecosystems the appearance of cyanobacterial blooms has increased due to anthropogenic action and global warming and is predicted to further increase in frequency (Downing et al. 2001; Paerl and Huisman 2008). Cyanobacteria produce a variety of secondary metabolites that are harmful not only to humans but also to livestock when exposed to water with high cyanobacterial concentrations (Falconer 1996; Cheung et al. 2013). Therefore, cyanobacterial mass developments are a major issue of concern in lake management (Carmichael et al. 2001).

The non-selective filter feeder *Daphnia,* which is an important link between primary producers as e.g. cyanobacteria and higher trophic levels, is more directly affected by these blooms, as cyanobacteria produce toxic secondary metabolites with negative effects on growth and reproduction rates and hence the fitness of *Daphnia* (Sivonen and Jones 1999; Rohrlack et al. 2001; Lürling 2003; Hansson et al. 2007; Gademann abd Portmann 2008; Ger et al. 2016).

In the search for cyanobacterial metabolites causing these negative effects on *Daphnia*, the cyanopeptide microcystin has been well studied in the past. In addition, the group of the so-called protease inhibitors has come into focus since these molecules are even more frequently found in surface blooms of cyanobacteria (Agrawal et al. 2001).It has been demonstrated that in *Daphnia* spp. the microcystin-free cyanobacterial strain *Microcystis aeruginosa* NIVA Cya 43 led to growth reduction (Lürling 2003) and the protease inhibitor microviridin J led to a disruption of molting (Rohrlack et al. 2004). Within the strain *M aeruginosa* NIVA Cya 43, von Elert et al. (2005) identified two chymotrypsin inhibitors, nostopeptin (BN920) and cyanopeptolin 954 (CP954) and demonstrated that each of these pure protease inhibitors (PIs) reduced juvenile somatic growth in *D. magna* similar to the effect of this cyanobacterium itself (von Elert et al. 2012). These cyanobacterial PIs of *M. aeruginosa* NIVA Cya 43 specifically inhibit digestive proteases in the gut of *Daphnia* (Schwarzenberger et al. 2010) and thus reduce growth of this grazer (Lürling 2003). Von Elert et al. (2004) demonstrated that chymotrypsin and trypsin, two classes of serine proteases, account for 80 % of the total digestive activity in the gut of *D. magna*. Chymotrypsin in gut homogenate of *D. magna* is specifically inhibited by the identified protease inhibitors BN920 and CP954 (von Elert et al. 2012).

Besides the *M. aeruginosa* strain NIVA Cya 43 a second strain of interest, *Microcystis* sp. BM25, has been demonstrated to be free of microcystin and to contain the three protease inhibitors micropeptins DR1056, DR1006 and MM978 (Schwarzenberger et al. 2013b). This strain has been isolated from a Lake in Sweden where it coexisted with *D. magna*, and *D. magna* clones originating from this lake have been demonstrated to be locally adapted to the PIs in *Microcystis* sp. BM25 (Schwarzenberger et al. 2017; Schwarzenberger et al. 2020).

For example, it has been shown that the exposure of *D. magna* to protease inhibitors of different *Microcystis* strains leads to the expression of compensatory isoforms of proteases, upregulated expression of digestive trypsins and chymotrypsins and epigenetic transfer of this elevated expression from mothers to the offspring (Schwarzenberger et al. 2010; Schwarzenberger et al. 2012; Schwarzenberger und von Elert 2013).

Schwarzenberger et. al. (2010) identified three chymotrypsin-like and trypsin-like gut peptidases of *D. magna* by LC-MS/MS based proteomics as well as their equivalent genes. Among those peptidases was the chymotrypsin-like elastase CT448. The copy number of the corresponding gene, i.e. *ct448,* varied among different *D. magna* clones. Interestingly, these copy number variations were not related to tolerance against PIs (Schwarzenberger et al. 2017). Locus-specific increased K_a/K_s ratios in *ct448* strongly suggest that the adaptation to cyanobacterial PIs in *Daphnia* results from positive selection and resultant protein modification of *ct448* rather than from adjusted gene expression levels of *ct448* (Schwarzenberger et al. 2020). To demonstrate the potential for local adaption in a Swedish *Daphnia* population to a specific *Microcystis* strain and its PIs, Lange et al. (2023) set up a multiclonal microcosm experiment with a naïve Polish and an adapted Swedish population. The Swedish clones showed higher population growth rates, a proxy for fitness, and dominated the population in the presence of dietary *Microcystis* sp. BM25, a cyanobacterium that has been isolated from the same lake as the Swedish *Daphnia* clones, but not in the absence of this cyanobacterium. Furthermore, Lange et al. (2018) demonstrated that CT448 is a chymotrypsin-like elastase of *D. magna* which has been confirmed to be a target of cyanobacterial protease inhibitors, more specifically BN920 and CP954.

In the current work, we used the Swedish and Polish *D. magna* clones that had been used in the multiclonal microcosm experiment to test for local adaptation at the protein level. It was previously assumed that the *ct448* gene and its protease were linked to chymotrypsin activity in *D. magna*. However, this was subsequently demonstrated to be inaccurate due to its elastase-like specificity. Therefore, the adaptation of *D. magna* via the *ct448* gene cannot be attributed to chymotrypsins, which are the most active digestive proteases in *D. magna*. It should be noted that the measurements for elastase proteases were not in the focus of research and were not subjected to optimization. Here, the enzymatic detection of elastases in *D. magna* gut homogenates was adapted, moreover based on the properties of CT448, which resembles one target protein for local adaptation of *Daphnia* to toxic cyanobacteria. Therefore, body homogenates of Swedish and Polish *D. magna* clones were tested for elastase activity, which also included the chymotrypsin-like elastase CT448 among other elastases, in an enzyme activity assay using the elastase-specific substrate. SPE fractions of a *Microcystis* sp. BM25 extract were investigated for their ability to inhibit the activity of the elastases in the gut homogenates. Testing the homogenates for the inhibitory fraction of the

Microcystis sp. BM25 extract will enable the subsequent identification of protease inhibitors targeting elastases, and potentially inhibiting the CT448, which has been demonstrated to be involved in local adaptation in *D. magna*. Further, we provide an improved approach for enzymatic assays for studies regarding this serine protease.

3.3 Results

In order to elucidate the effect of *Microcystis* sp. strain BM25 on the activity of proteases of different *D. magna* populations, preliminary experiments were conducted with the *D. magna* clone B, which represents an established laboratory clone. It has been conclusively determined that the methodical approach previously employed is not optimally suited for the measurement of elastases. Therefore, the activity of the digestive proteases of *D. magna* clone B was tested against extracts of two different *Microcystis* strains to compare the effect of extracts of *Microcystis sp.* BM25 with those of the well-studied *Microcystis aeruginosa* strain NIVA (Supplement 1). Inhibition of chymotrypsin and trypsin did not differ between the two cyanobacterial extracts from strain BM25 and NIVA (Supplement 1B and C). Contrarily, elastase activity was significantly less affected by BM25 than by NIVA, indicating for a dose depending effect on elastase (Supplement 1A). Interestingly, identical volumes of strain BM25 extract caused less inhibition of elastase than of chymotrypsin and trypsin in this *D. magna* reference clone, which suggests that elastase-like proteases are less sensitive to the cyanobacterial protease inhibitors. The utilized cyanobacterial inhibitors of *M. aeruginosa* NIVA and *Microcystis* sp. BM25 were extracted in methanol according to the described method. To exclude a solvent-dependent effect, the assay was repeated with dimethyl sulfoxide (DMSO) as alternative organic solvent to methanol. Solvents can have effects on solubility, stability or reaction rates of enzymes and therewith negatively influence the enzyme assay. Preliminary tests showed a sensitivity of CT448, a chymotrypsin-like elastase, towards dithiothreitol (DTT) as reducing agent (Supplement 2). Therefore, the activity of elastase and chymotrypsin was tested in the presence of equal amounts of pure methanol (MeOH) and DMSO in comparison to a methanolic BM25 extract (Figure 1). Elastase was significantly inhibited by the addition of MeOH only and by the methanolic BM25 extract (Fig. 1A; Tukey's HSD post hoc test after one-way ANOVA, $p < 0.05$), whereas there was no such solvent effect of MeOH on chymotrypsin activity. This demonstrated that it cannot be excluded that the inhibitory effect of BM25 extract on elastase was due to the solvent. Furthermore, the activity of the elastase in presence of DMSO does not differ from the elastase control without any solvent and is significantly higher than in presence of both methanolic extracts (Tukey's HSD post hoc test after one-way ANOVA, $p < 0.05$). This leads to the suggestion that inhibition of elastase by methanolic BM25 is driven by a solvent-dependent effect rather than by inhibitors

of BM25. For chymotrypsin activity no solvent-dependent inhibition was observed (Figure 1B) but a significant inhibition by the methanolic BM25 extract (Tukey's HSD post hoc test after one-way ANOVA, $p < 0.05$). Conclusively, these enzyme activity assays indicate protease specific sensitivity towards different solvents and demonstrates chymotrypsin sensitivity towards BM25 extract, probably deriving from protease inhibitors. Subsequently, extracts of BM25 were dissolved in DMSO for further tests.

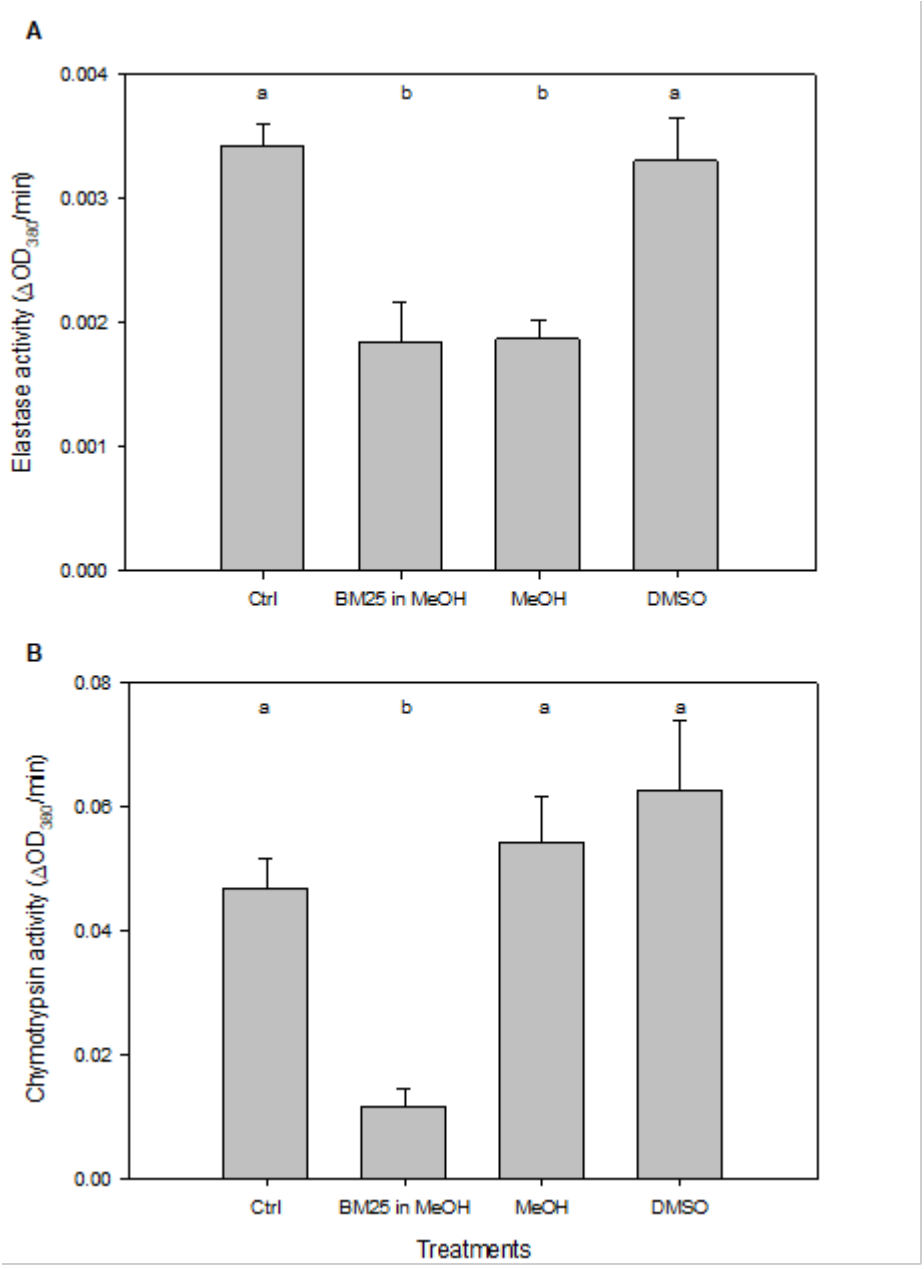

Figure 1: Enzyme activity assays of *D. magna* clone B homogenate in presence of a methanolic extract of the cyanobacteria *Microcystis* sp. BM25 and corresponding solvent controls for MeOH and DMSO. The control (Ctrl) was solvent-free. A) depicts the effects of the extract and solvents on elastase digestion of the substrate AAApNA and B) chymotrypsin digestion of the substrate AAPFpNA. Shown are means ± SD (n = 3). Tukey's HSD post hoc test after one-way ANOVA, $p < 0.05$. Letters indicate statistical differences between treatments within each activity assay.

To identify inhibitory compounds that might drive adaptation towards them in *Daphnia*, the BM25 extract was fractionated into four different fractions by solid phase extraction (SPE) and subsequent elution steps with different concentrations of methanol. Hence, elastase activity was tested in the presence of fractions deriving from elution with 20 %, 40 %, 60 % and 80 % methanol. DMSO served as a control (Figure 2). A significant inhibition was observed for the 20 % and the 60 % fraction of BM25 (Tukey's HSD post hoc test after one-way ANOVA, $p < 0.05$). As two potential inhibitor-containing fractions could be detected, subsequent experiments were conducted with these two fractions to elucidate differences in tolerance of protease inhibitors of the two *D. magna* populations.

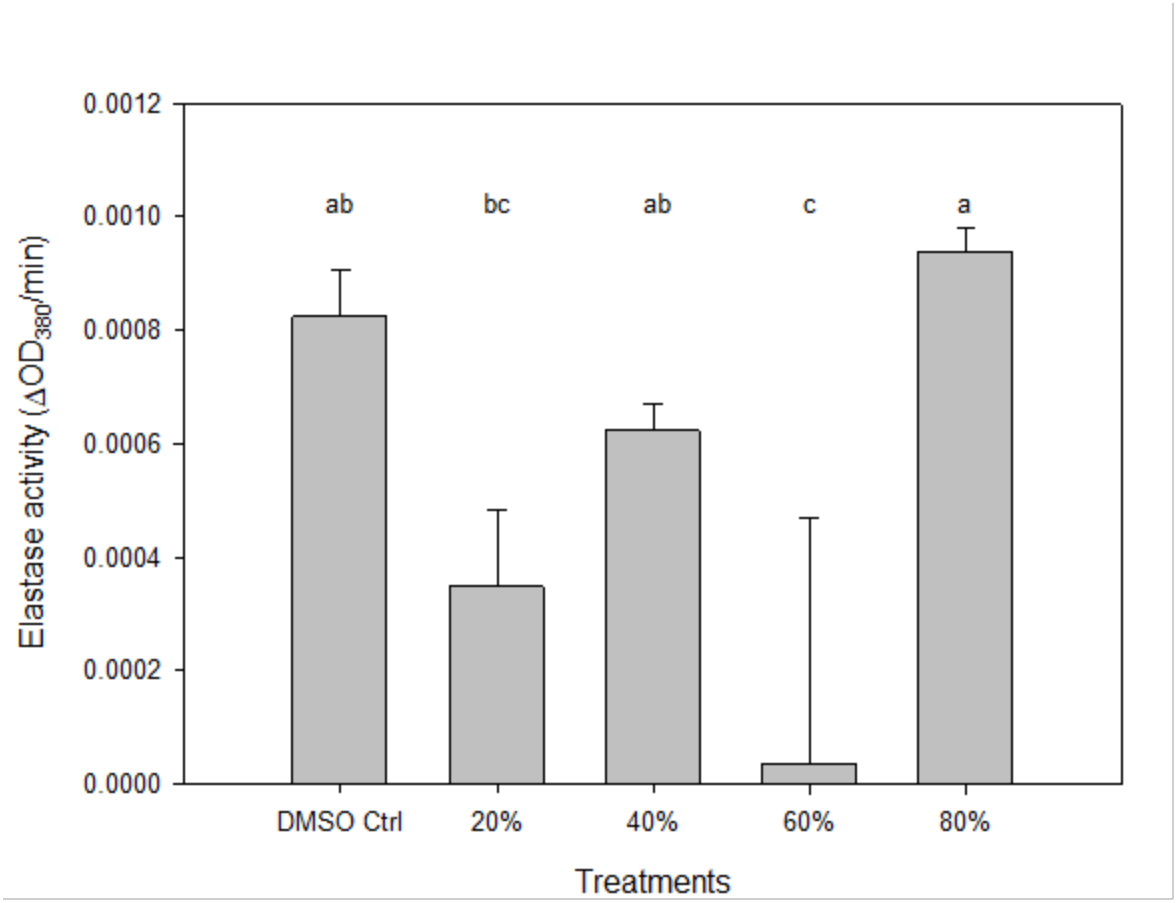

Figure 2: Elastase activity in homogenate of *D. magna* clone B in absence and presence of fractions of *Microcystis sp*. BM25 extract on AAApNA substrate. The BM25 extract was fractionated via solid-phase extraction (SPE) SPE by subsequently eluting the SPE with 20 %, 40 %, 60 % and 80 % methanol. Subsequently, the methanol was replaced by DMSO. The control consisted of DMSO addition only (DMSO Ctrl). Shown are means ± SD (n = 3). Tukey's HSD post hoc test after one-way ANOVA, $p < 0.05$. Letters indicate statistical differences between treatments.

Previously, it has been tested whether the enzymatic activity of elastases within the gut homogenates of the different clones is comparable or not. Hence, the homogenates of the

Polish clones P4, P27, P31 and the Swedish clones M7 and M24 were separately tested. The Swedish clone M17 (Lange et al. 2023) was not available for the tests. The elastase activity of the different clones did not significantly differ from each other (Figure 3). Therefore, the homogenates were pooled according to populations in equal amounts and should therefore exhibit comparable enzymatic activity. The enzymatic activity of homogenates from both *Daphnia* populations was then tested in presence and absence of the two BM25 fractions.

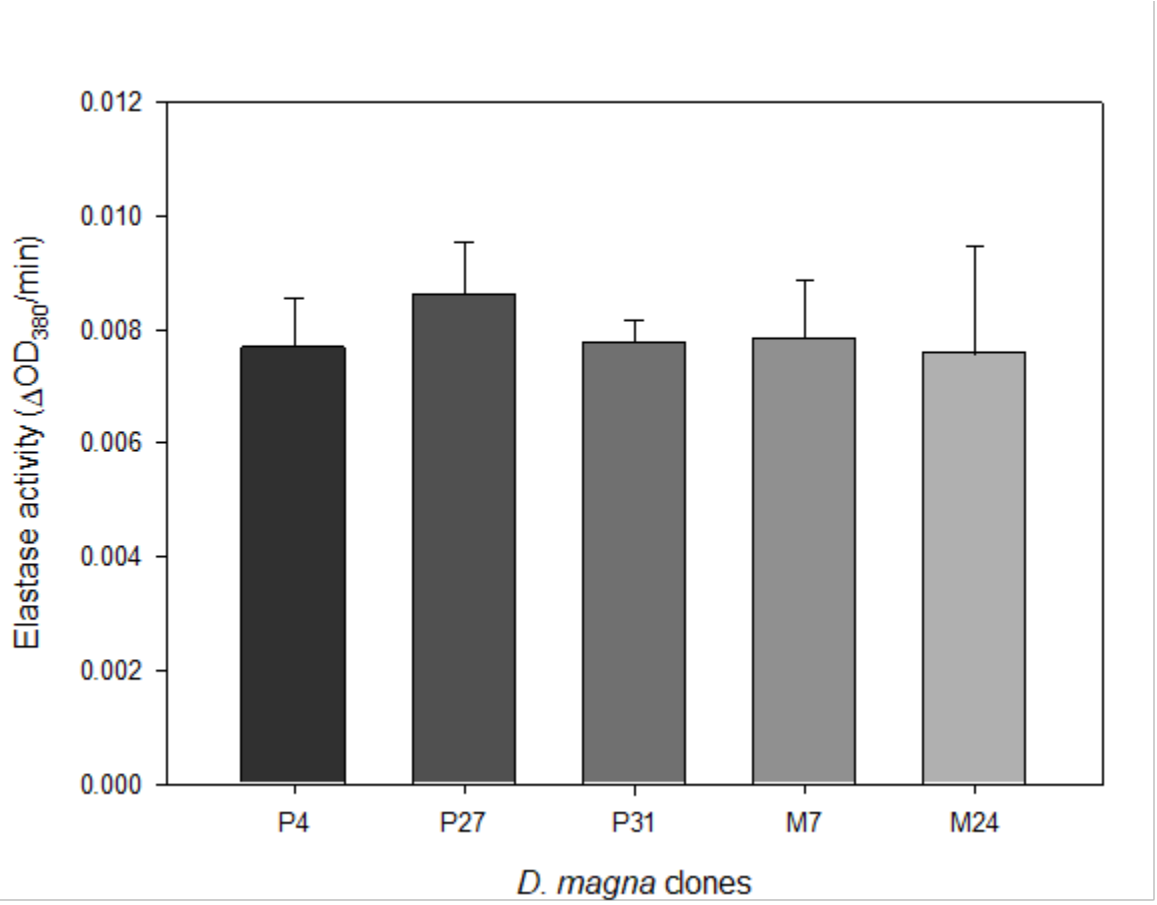

Figure 3: Elastase activity of homogenates of the different D. magna clones on. Homogenates of the Polish clones (P4, P27, P31) and Swedish clones (M7 and M24) were compared for their proteolytic homogeneity on AAApNA. Shown are means ± SD (n = 3). Tukey's HSD post hoc test after one-way ANOVA, $F = 0.402$, $p = 0.803$). No statistical differences between clones.

Elastase activity in the pooled homogenates of populations of Swedish and Polish clones was tested in the presence of identical volumes of the 20 % and 60 % SPE-fractions of Microcystis strain BM25 (Figure 4). A significant inhibition of elastase of each population was observed by both fractions (Tukey's HSD post hoc test after two-way ANOVA for population x treatment, Table 3). There was no population x inhibitory fraction interaction, which indicated that the inhibitory effects among Swedish and Polish *D. magna* populations were not distinguishable. Nevertheless, there might be trends visible for the two populations: On one hand elastases in homogenates of the Swedish clones were more susceptible towards the 20 % fraction than the

60 % fraction of the BM25 extract. On the other hand, the elastases present in the homogenate of Polish clones seems to be more inhibited by the 60 % fraction than by the 20 % fraction. This raises the question of a population-dependent reaction to the different inhibitor containing fractions.

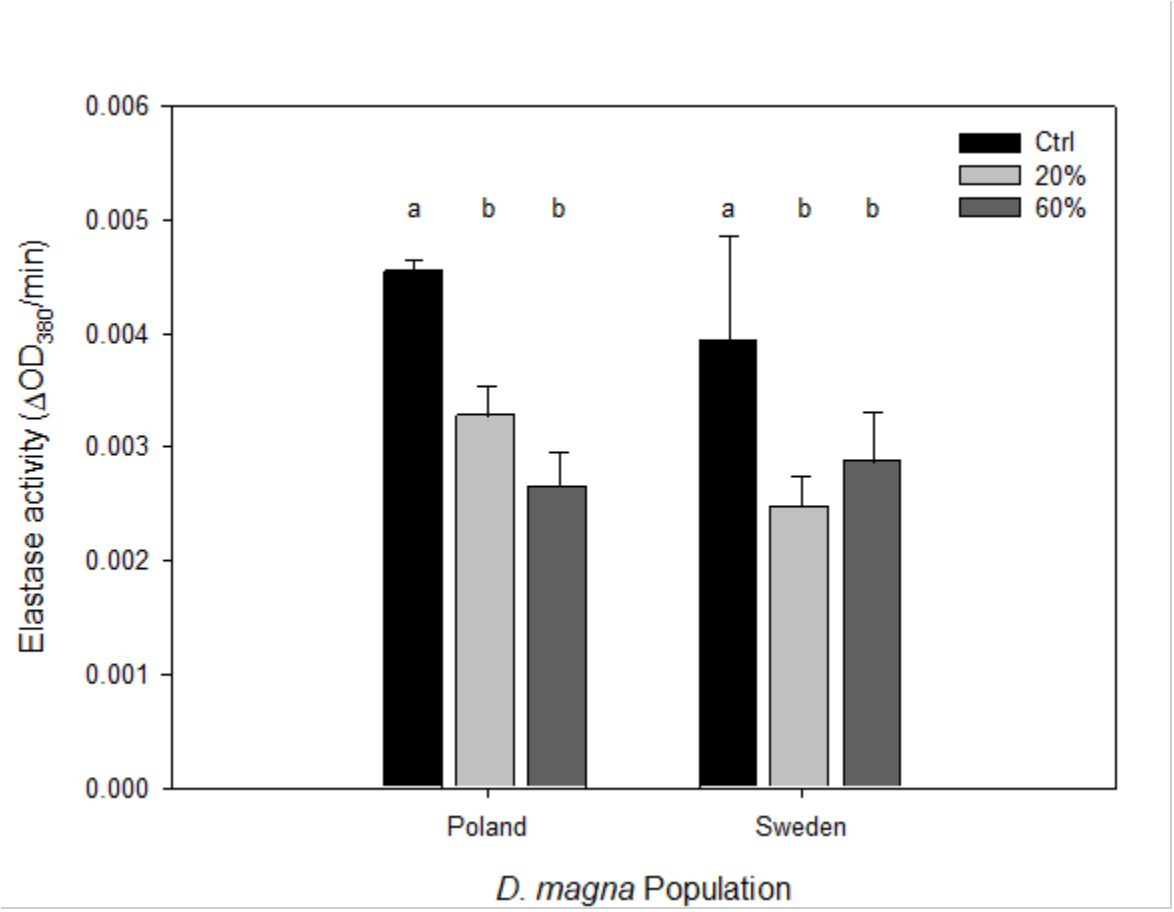

Figure 4: Inhibition of the Polish and Swedish *D. magna* clones by 20 % and 60 % methanol solid-phase fractions of extracts of the cyanobacterium Microcystis sp. BM25, redissolved in DMSO. The elastase activity was inhibited by either the 20 % or 60 % fraction of the SPE extracts, redissolved in DMSO. Shown are means ± SD (n = 3). Tukey's HSD post hoc test a two-way ANOVA for the factors 'population' and 'fraction' was performed. Letters indicate statistical differences between fractions across both Daphnia populations.

3.4 Discussion

The conducted experiments demonstrate distinct properties of elastase of *D. manga* compared to the two known serine proteases chymotrypsin and trypsin. Furthermore, solvent dependent effects on digestive elastases of *D. magna* were assessed to improve the methodical elucidation of potential inhibitor candidates in *Microcystis* sp. strain BM25.

Elastase shows different responses to extracts of two Microcystis strains, whereas chymotrypsin and trypsin are equally strong inhibited. More precisely, elastase is less strongly

inhibited by an extract of *Microcystis sp.* strain BM25 than by an extract of *M. aeruginosa* strain NIVA Cya 43 that contains the well-described protease inhibitors cyanopeptolin 954 and nostopeptin BN920 (Ploutno and Carmeli 2002; von Elert et al. 2005). Lange et al. (2018) demonstrated that heterologously expressed CT448 was inhibited by 20 µM CP954 and 20 µM BN920, with CP954 being the more potent inhibitor. The inhibitory compounds of the strain BM25 are not fully known. Schwarzenberger et al. (2013b) identified the micropeptins DR1006, DR1056 (Adiv et al. 2010) and MM978 (Zafrir-Ilan and Carmeli 2010) in extract of this strain. These inhibitors are potent chymotrypsin inhibitors, but MM978 and DR1006 do as well inhibit elastase (Zafrir-Ilan and Carmeli 2010; Adiv et al. 2010). Both micropeptins are plausible candidates as inhibitors for CT448, as it is a chymotrypsin-like protease with elastase specificity. Especially, a Leu on 5th position is a character for inhibition of chymotrypsin related serine proteases (Grach-Pogrebinsky et al. 2003).

The overall elastase activity in the Daphnia homogenates investigated has been reported to be by far lower than those of digestive trypsin and chymotrypsin. Chymotrypsin and trypsin account for 80 % of the proteolytic activity in *D. magna's* gut (von Elert et al. 2004), where trypsin is around ten times less active than chymotrypsin. However, elastase activity had not been detected in this study. Similar to the previous studies (von Elert et al. 2004; Schwarzenberger et al. 2012), we observed similar ratios of chymotrypsin and trypsin activities, extending the earlier observations by von Elert et al. (2004) we could show that as well elastase activity is present. Contrary to the previous studies, we did not add any reducing agent as dithiothreitol (DTT). DTT reduces the disulfide bonds of proteins and thus prevents intra- and intermolecular disulfide bonds formation between cysteine residues of proteins (Cleland 1964). Lange et al. (2018) showed *in silico* that CT448 harbors four disulfide bonds of which one is present on the surface and conserved in the pro-peptide. The reduction of this disulfide bridge leads to an unbound sulfide residue on the surface that may bind to other reduced cysteine residues, which may lead to the formation of aggregates and could cause a loss of activity. Hence, for *D. magna* elastase, specifically for CT448, it can be assumed that the reduction agent alters its activity and might serve as an explanation why von Elert et al. (2004) could not detect any elastase activity within the *D. magna* gut homogenate. The addition of 1 mM DTT already destabilized the CT448 significantly: the melting point of heterologously expressed CT448 was around 42.5°C. The addition of 1 mM DTT decreased the melting point to 36.67°C and the addition of 5 mM decreased it further to 35°C which gives evidence for a strong destabilization of the protein structure.

As the activity of CT448 is linked to the elastase activity measured in this study, it cannot be excluded that CT448 may be sensitive to methanol, in addition to being sensitive to reducing agents. In order to link genetic adaptations with physiological responses, such as protease activity, it is essential to optimize the corresponding measurement methods for the gene of

interest. in this case *ct448*. We showed that the addition of methanol itself, as it is present in the cyanobacterial inhibitor extracts, significantly reduces the activity of elastase. The inhibition of methanol alone is as strong as in combination with the cyanobacterial inhibitors of BM25. Different form elastase, chymotrypsin is interestingly not affected by the addition of methanolic solvents. Therefore, the standard procedure (von Elert et al. 2004; Agrawal et al. 2005; Schwarzenberger et al. 2012) for enzymatic assays that have been conducted for chymotrypsin and trypsin in *D. magna* so far needs to be adapted for the measurement of elastases.

The expression of the elastase encoding gene *ct448* of *Daphnia* is increases upon exposure to inhibitor containing cyanobacteria (Schwarzenberger et al. 2010; Schwarzenberger et al. 2012; Drugă et al. 2016; Schwarzenberger et al. 2017). This indicates that this specific gene might play a role in the adaptation of *Daphnia* to cyanobacteria. In this study, homogenates of *Daphnia,* which had not been exposed to cyanobacterial cells, were exposed to fractionated *Microcystis* BM25 extracts, and the elastase activity was not significantly different among these naïve clones regardless of the origin. This strongly suggests that upon exposure to cyanobacteria enhanced expression or even maternal transfer of increased protease gene expression (Schwarzenberger and von Elert 2013) are responsible for higher fitness of the Swedish population in comparison to the Polish population as has been observed in a common garden experiment (Lange et al. 2023). Maternal transfer of increased protease gene expression in *D. magna* has been demonstrated to benefit the offspring by an increased growth rate when the mothers were exposed to a PI producing cyanobacterium (Schwarzenberger and von Elert 2013); however, it remains to be tested if dietary exposure of the *D. magna* clones from the Swedish and the Polish population leads to increased elastase activity and to higher elastase activity in the Swedish population, which then would support the idea of local adaptation of the Swedish *D. magna* population to elastase inhibitors in the co-existing *Microcystis* sp. strain BM25. Interestingly, we observed a significant inhibition of elastase by fractionated BM25 extract in both populations and trends to different susceptibility regarding the fractions, but no significant interaction between population and treatment. Individual genotypes of *D. magna* populations have been demonstrated to exhibit differential responses to the *Microcystis* strains which contain different PIs (Schwarzenberger et al. 2021; Lange et al. 2023). Both the tolerant and sensitive *D. magna* populations contained sensitive and tolerant genotypes, but in varying proportions. Here, we pooled homogenates of sensitive and more tolerant genotypes from each *D. magna* population (Lange et al. 2023) without prior exposure to the respective cyanobacterium. This may have obscured potential evidence for the adaptation of individual clones to elastase inhibitors in *Microcystis* sp. strain BM25.

The inhibitory compounds of BM25 differ from those of *M. aeruginosa* NIVA Cya 43, which elute in the 80 % fraction and differ in terms of their physicochemical properties and structure.

The identified inhibitors of BM25 (Schwarzenberger et al. 2013b) were isolated from lyophilized powder dissolved in 80 % MeOH, and chymotrypsin assays were conducted with this methanolic extract without further SPE. The findings regarding the inhibitory properties of the PIs in BM25 cannot be transferred to CT448 or elastases, as these serine proteases are highly susceptible to methanolic solvents. While in *Microcystis* strain BM25 the potent chymotrypsin inhibitors MM978, DR1056, and DR1006 were expected to be present in the extract, they could not be assigned to individual SPE fractions due to time constraints. Consequently, it is currently not possible to assign these PIs to the observed inhibition of elastases. Further studies could be conducted by pre-incubating the *D. manga* clones with toxic cyanobacteria, e.g., *Microcystis* sp. BM25, prior to enzyme activity assays. Incubation on PI-producing cyanobacteria leads to an increased expression of CT448 which might alter the inhibitory effects of PIs. Further testing and analysis of the inhibitory fraction of the cyanobacterial extract by LC-MS might allow identification of new elastase inhibitors. Subsequently, the serine proteases of the most resistant clones can be heterologously expressed, as demonstrated by Lange et al. (2018), and their protein structure and properties analyzed to assess the role digestive proteases in local adaptation.

3.5 Methods

Daphnia cultivation

The green alga *Chlamydomonas klinobasis* (strain 56, culture collection of the Limnological Institute, University of Konstanz, Konstanz, Germany) was cultivated semi-continuously in cyanophycean medium (von Elert and Jüttner 1997) at 20°C at 130 µE m^{-2} s^{-1} light intensity, with 20 % of the medium exchanged daily. *C. klinobasis* served as non-toxic algal food to cultivate the experimental *D. magna* clones used in this study.

Preliminary tests were conducted with the *D. magna* clone B (Binnensee) (Pijanowska et al. 1993) and subsequent experiments with *D. magna* clones originated from Lake Bysjön, Sweden (Schwarzenberger et al. 2013a) and a Polish pond near Warsaw (Schwarzenberger et al. 2017). Except for clone M17, the same clones previously used for population studies in Lange et al. (2023) were used in this study for enzymatic assays. For regular cultivation of all clones, 15 *Daphnia* originating from one clutch of a single mother were cultured in 800 mL membrane-filtered aged tap water, at 19°C, at low light conditions with a day and night rhythm of 16:8 h. Every second day all animals were transferred into fresh water and fed with 2 mg POC/L of *C. klinobasis* as food alga. Third clutch neonates of one mother were kept for constant culture extension.

Cultivation of cyanobacteria

The cyanobacterium *Microcystis* sp. strain BM25 (kindly provided by Ineke van Gremberghe, Ghent University, Ghent, Belgium) originates from Lake Bysjön in Southern Skania, Sweden. The cyanobacterium *Microcystis aeruginosa* NIVA Cya 43 obtained from the culture collection of the Norwegian Institute for Water Research. Both *Microcystis* strains do not produce microcystins but contain several protease inhibitors (von Elert et al. 2005; Adiv et al. 2010; Zafrir-Ilan and Carmeli 2010). NIVA Cya 43 and BM25 were cultivated in chemostats on cyanophycean medium (von Elert and Jüttner 1997) with a light intensity of 50 µE m^{-2} s^{-1}, at a temperature of 20°C and constant air supply and a dilution rate of 0.1 d^{-1}. The concentration of POC in the cyanobacterial food suspensions was estimated regularly by measuring the extinction of the cultures at 470 nm.

Methanolic extraction of secondary metabolites

Freeze-dried (Christ LOC-1m freeze dryer, ALPHA 1-4, Merrington, Shrewsbury, Shropshire, UK) powder, equivalent to 0.5 mg C, of either NIVA Cya 43 or BM25 was dissolved in 10 mL 80 % methanol (MeOH) and resuspended over night at RT. The samples were sonicated, and again centrifuged (3 min, 4500 g). The supernatant was transferred into test tubes, evaporated to dryness using a vacuum centrifuge (RVC 2-25, Christ), and redissolved in 1 mL 100 % MeOH. The extracts were stored at -20°C until usage.

For the fractionation of the cyanobacterial extracts, 0.5 mg of the powder was resuspended in 100 mL 80 % methanol over night at RT. Subsequently, the suspension was pelleted at 15 000 g for 10 min and the clear supernatant was transferred for evaporation to dryness using a rotary evaporator and redissolved in 3x5 mL 100 % methanol. For solid-phase extraction the sample was diluted with deionized H_20 to a total MeOH concentration of 10 %. A C_{18} solid-phase cartridge (Varian Mega Bond Elut, Agilent Technologies) was equilibrated according to the manufacturer's instructions. The diluted samples were loaded onto the column and eluted with 50 mL of 20 %, 40 %, 60 %, 80 % and 100 % MeOH. The eluted fractions were separately collected and again evaporated to dryness. The single fractions were finally redissolved in 500 µl 100 % MeOH for subsequent tests and stored at -20 °C. Schwarzenberger et al (2013b). used lyophilized powder of the three *Microcystis* sp. BM25 cultures which was suspended 80 % methanol to determine the inhibitors and inhibitor concentrations of *Microcystis* sp. BM25. Within the 80 % methanolic extract the micropeptins DR1006, DR1056 (Adiv et al. 2010) and MM978 (Zafrir-Ilan and Carmeli 2010) were identified. Hence, the fraction 20 % to 80 % were utilized for the enzymatic assays. For NIVA Cya 43 only the 80 % fraction (NIVA SPE) was used in this study as von Elert et al. (2005) identified the protease inhibitors cyanopeptolin 954 and nostopeptin BN920 within the 80 % fraction of the solid phase extraction.

The solvent exchange of MeOH to DMSO for the cyanobacterial inhibitor extracts and fraction was achieved by evaporation of the MeOH to dryness and resuspension with DMSO until the initial volume was maintained.

Enzyme assays

Activity measurements of gut proteases of *D. magna* homogenate were performed with different substrates and solvents or inhibitors. For the homogenates 500 µg of fresh *Daphnia* were homogenized with 500 µL 100 mM potassium phosphate buffer pH 8.0, subsequently centrifuged for 5 min at 13 000 rpm to clear the supernatant. The supernatant was used for further enzyme assays. The homogenate was tested on three different substrates for serine proteases: N-Succinyl-alanine-alanine-alanine-para-nitroanilide (AAApNA) for elastases, N-Succinyl-alaninealanine-valine-para-nitroanilide (AAPFpNA) for chymotrypsins and Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BApNA) for trypsins (Sigma-Aldrich, St. Louis, USA). In general, 10 µL of *D. magna* homogenate was tested on 100 µM substrate (either AAApNA, AAPFpNA or BApNA) and 10 µl extract or solvent (pure MeOH or DMSO for control) in a final volume of 150 µL of 100 mM potassium phosphate buffer (pH 8.0). Enzyme activity was measured as the increase in absorbance at 380 nm within the first 30 min at RT in a Biotek Synergy H4 plate reader (Biotek, Winooski, USA). All measurements were carried out in triplicates.

For enzymatic tests for clones of the two *D. magna* populations, the elastase activity was prior tested individually for each clone (P4, P27, P31, M7 and M24) as described above. Further, the homogenates of the single clones were pooled according to their origin for enzyme assays: Polish clones were mixed in a 1:3 ratio and the Swedish clones in a 1:1 ratio. No difference in proteolytic activity was observed in the pooled homogenates of the two populations under control conditions (no solvent or inhibitory fraction).

Statistical analyses

The statistics were conducted with the software Sigmaplot 11.0 (Systat Software GmbH, Erkrath, Germany). The data were analyzed via one-way ANOVA and a post-hoc analysis (Tukey HSD). The level of significance was $p < 0.05$.

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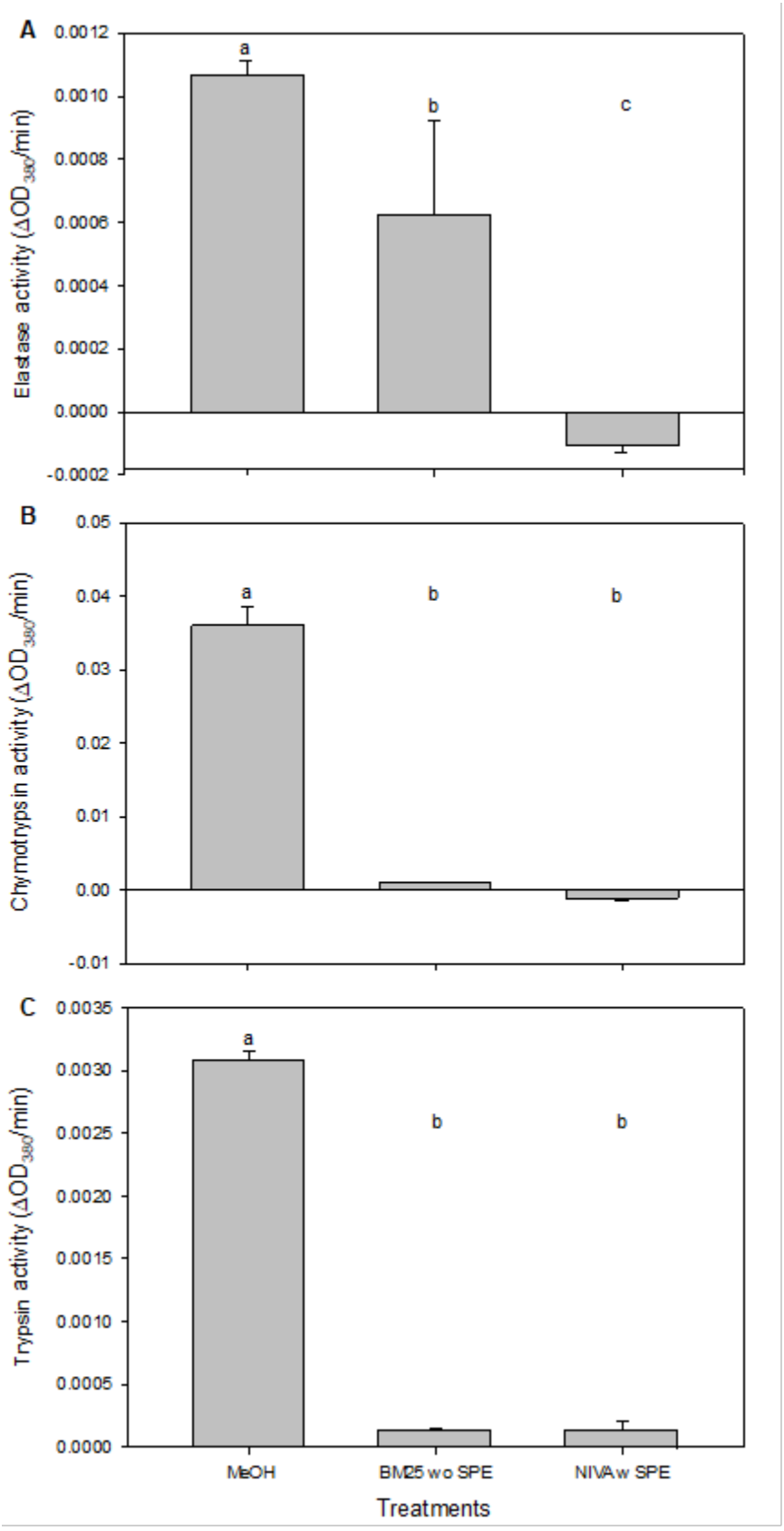
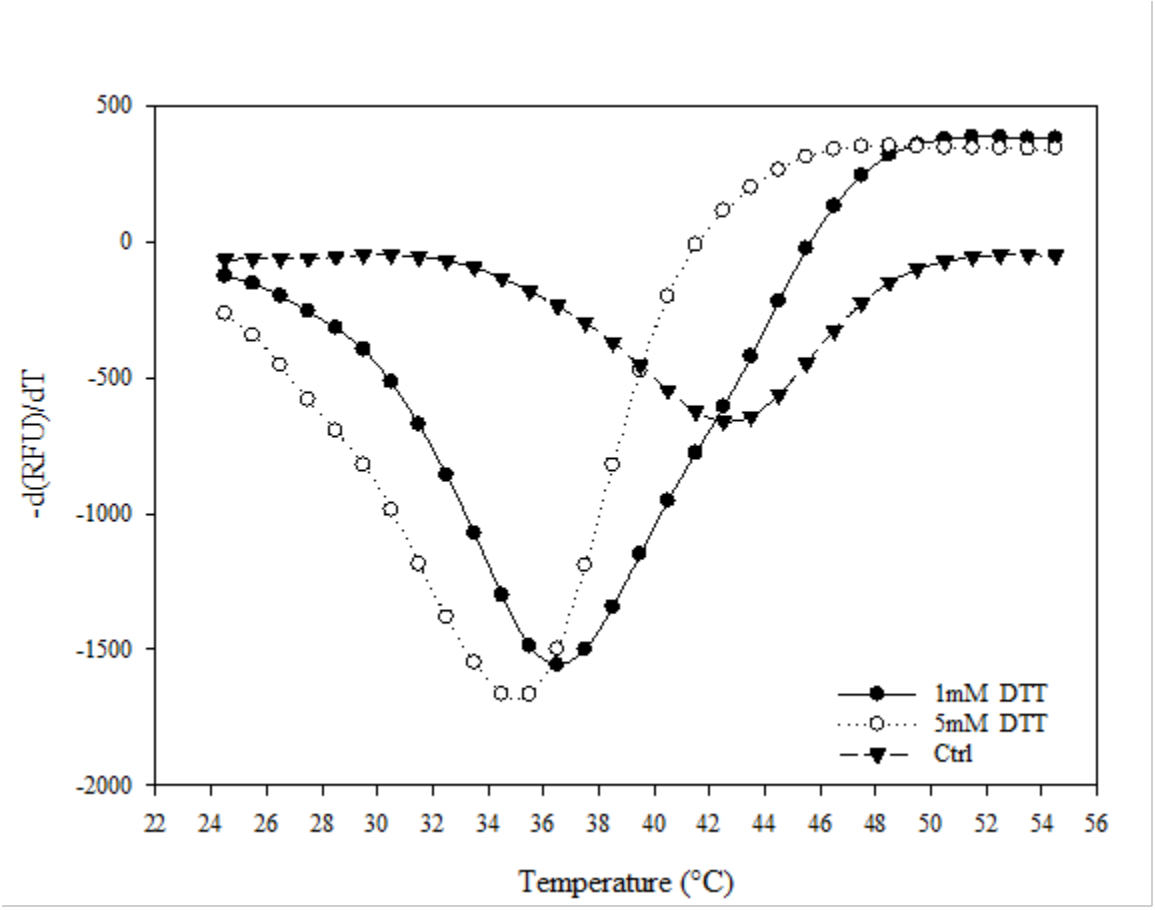
3.7 Supplements



Supplement 1: Activity assay of proteases from *D. magna* clone B homogenate with two cyanobacterial methanolic extracts. Tested were the extracts of the cyanobacterium *Microcystis* sp. BM25 without solid phase extraction (BM25 wo SPE) and *M. aeruginosa* NIVA

Cya 43 with solid phase extraction (NIVA w SPE) eluted with 80 % MeOH. The methanolic 80 % fraction was evaporated to dryness and redissolved in 100 % MeOH, BM25 extract was as well dissolved in 100 % MeOH. A) The activity (ΔOD_{380}/min) of *Daphnia* homogenates were tested on elastase-specific substrate (AAApNA) in presence of two cyanobacterial extracts, B) with chymotrypsin-specific substrate and C) with trypsin-specific substrate. Shown are means ± SD (n = 3). Tukey's HSD post hoc test after one-way ANOVA for A, B and C, $p < 0.05$. Letters indicate statistical differences between treatments within each activity assay.



Supplements 2: ThermoFluor™ assay for heterologously expressed CT448 with 1 mM and 5 mM DTT and a control without reductant. The decrease of the relative fluorescence unit (RFU) over time (dT) depicts the thermal denaturation of CT448 with increasing temperature. CT448 is less stable with increasing amounts of DTT. The methodological approach is presented in detail in Chapter 4.

Supplement 3: Pairwise comparisons of the effect of different cyanobacterial extracts on either elastase, chymotrypsin or trypsin activity in homogenate from *D. magna* clone B. Shown is Tukey's HSD after one-way ANOVA for each serine protease. Tested was either elastase, chymotrypsin or trypsin activity on pure MeOH as control, methanolic *Microcystis* sp. BM25 extract without solid phase extraction (BM25 wo SPE) or *M. aeruginosa* NIVA Cya 43 after SPE (NIVA w SPE) elution with 80 % MeOH. Respectively both cyanobacterial extracts contained 100 MeOH.

	Comparison	Diff of Means	p	q	P	
Elastase	MeOH × NIVA ´w SPE	0.00117	3	11.587	<0.001	***
	MeOH × BM25 wo SPE	0.000442	3	4.370	0.049	*
	BM25 wo SPE × NIVA w SPE	0.000731	3	7.218	0.005	**
Chymotrypsin	MeOH × NIVA w SPE	0.0372	3	45.048	<0.001	***
	MeOH × BM25 wo SPE	0.0351	3	42.514	<0.001	***
	BM25 wo SPE × NIVA w SPE	0.00209	3	2.535	0.250	
Trypsin	MeOH × NIVA w SPE	0.00294	3	91.451	<0.001	***
	MeOH × BM25 wo SPE	0.00294	3	91.308	<0.001	***
	BM25 wo SPE × NIVA w SPE	0.00000458	3	0.143	0.995	

Significance levels: *** <0.001; ** <0.01; * <0.05.

Supplement 4: Pairwise comparisons of the effect of different cyanobacterial extracts on either elastase or chymotrypsin activity in homogenate from *Daphnia* originating from different populations. Shown is Tukey's HSD after one-way ANOVA of protease from *D. magna* homogenate and different solvents or BM25 extract. Tested was either elastase (Ela) or chymotrypsin (Chym) as control on pure DMSO, MeOH or methanolic *Microcystis* sp. BM25 extract (BM25 in MeOH).

Comparison	Diff of Means	p	q	p-Value	
Ela × BM25 in MeOH	0.00158	4	10.068	<0.001	***
Ela × MeOH	0.00155	4	9.891	<0.001	***
Ela × DMSO	0.000118	4	0.754	0.949	
DMSO × BM25 in MeOH	0.00146	4	9.314	<0.001	***
DMSO × MeOH	0.00143	4	9.137	0.001	***
MeOH × BM25 in MeOH	0.0000276	4	0.177	0.999	
Chym x BM25 in MeOH	0.0351	4	8.536	0.002	**
Chym × MeOH	0.00740	4	1.798	0.604	
Chym × DMSO	0.0157	4	3.807	0.103	
DMSO × BM25 in MeOH	0.0508	4	12.343	<0.001	***
DMSO × MeOH	0.00827	4	2.009	0.522	
MeOH × BM25 in MeOH	0.0425	4	10.334	<0.001	***

Significance levels: *** <0.001; ** <0.01; * <0.05.

Supplement 5: Pairwise comparisons of the effect of the solid-phase fractions of the extract of Microcystis strain BM25 on the elastase activity of the Swedish and the Polish *D. magna* populations. Shown are results Tukey's HSD after two-way ANOVA of the Swedish and Polish *D. magna* population with the factors 'fraction' and 'population'. The factor 'fraction' consisted of the 20% and 60% solid-phase fractions of extract of *Microcystis* strain BM25; the factor 'population' consisted of the Polish and the Swedish Daphnia populations. There was no statistically significant interaction between the factors 'Population' and 'Fraction' (P = 0,171).

Comparison: **Treatment within Polish Daphnia population**	**Diff of Means**	**p**	**q**	**P**	
Ctrl x 60%	0.00189	3	7.058	<0.001	***
Ctrl x 20%	0.00127	3	4.732	0.015	*
20% x 60%	0.000624	3	2.326	0.266	
Comparison: **Treatment within Swedish Daphnia population**	Diff of Means	p	q	P	
Ctrl x 20%	0.00145	3	5.401	0.007	*
Ctrl x 60%	0.00106	3	3.933	0.041	*
60% x 20%	0.000394	3	1.468	0.568	

Significance levels: *** <0.001; ** <0.01; * <0.05.

Chapter 4

An amino acid swap in *Daphnia*'s protease CT448 might expand their cyanobacterial diet

Chapter 4 continues on the findings presented in Chapter 1 by providing a detailed examination of the properties of CT448, aimed at elucidating the structural features of this chymotrypsin-like elastase as CT448 exhibits a population-specific amino acid exchange at position 34, where Glu and Gln are present. Given that no crystal structure is available yet, analyses were conducted using the machine learning program AlphaFold 3. The predicted structure of CT448 indicates that the pro-peptide remains bound to CT448 after tryptic digestion and might be further stabilized via a salt bridge by the population-specific amino acid of Glu at position 34 (E34). It was therefore concluded that a crystal structure of CT448 should be obtained for further clarification of the structural properties, and that CT448 should be co-crystallized with a suitable PI. Nevertheless, I demonstrated via the predicted structure that special features could be visualized by AlphaFold.

Authors contribution

The experiment was planned and performed by me, Jacqueline Lange, or under my direct supervision. Data visualization and analysis were performed by Jacqueline Lange. Matthias Uthoff and Ulrich Baumann supported the protein modelling. This chapter was written exclusively by Jacqueline Lange and was critically read by Matthias Uthoff and Eric von Elert.

Data availability statement

All data generated or analyzed during this study are included in the manuscript or supplements. Raw data generated during this study are available from the corresponding author upon request.

4.1 Abstract

The combination of eutrophication and warm surface temperatures in freshwater bodies results in an increased frequency of cyanobacterial blooms, characterized by a reduction in the diversity of toxins present and the potential dominance of a few highly toxic strains. During cyanobacterial blooms, these organisms represent the dominant planktonic biomass and serve as a food source for the non-selective filter feeders of the genus *Daphnia*. In addition, many cyanobacteria produce protease inhibitors (PI), which have a negative effect on the digestion of *Daphnia*. A Swedish *D. magna* population with constant contact to protease inhibitor producing cyanobacteria was demonstrated to exhibit both molecular and local adaptation, whereas a naïve Polish population displayed a higher level of susceptibility. The serine protease CT448 exhibits positive selection at the *ct448* locus in the Swedish population, as well as a population-specific amino acid exchange at position 34, where Glu (E) is present in Swedish and Gln (Q) is present in Polish clones. Here, the properties of CT448 were further analyzed in order to elucidate the potential structural benefit of this selective amino acid exchange and the characteristics of the protease structure. In addition to maturation by trypsin, more reliable activation processes were investigated with the aim of enabling crystallization of CT448 to study the structural properties of the population-specific variants. Given that no crystal structure has been successfully obtained yet and that the tryptic maturation of CT448 displays a distinct degradation, structural predictions were conducted using the machine learning program AlphaFold 3. The predicted structure of CT448 indicates that the pro-peptide remains bound to CT448 after maturation, as a more stable binding might be achieved by a glutamic acid (E34) via a salt bridge, compared to glutamine (Q34) in the susceptible population. In order to allow for investigations and comparative analysis with different variants, a functional maturation of CT448 is needed to obtain a crystal structure of CT448 and to co-crystallize it with a suitable PI. Structural information about the CT448 gained from protein crystallization could confirm the presence of those features predicted by AlphaFold which cannot be proven in silico.

4.2 Introduction

Global warming and nutrient input have a considerable impact on lake ecosystems. Therefore, an integrated management approach, addressing both nutrient loading and climate change, is required to protect freshwater resources (reviewed by Paerl and Paul 2012). This eutrophication in combination with warm surface temperatures leads to increased frequencies of cyanobacterial blooms in freshwater bodies (Paerl and Huisman 2008; Taranu et al. 2015; Lürling et al. 2017). These blooms are accompanied by unpleasant sights and odors or turbid water. Moreover, sometimes potent toxins produced by the bacteria are a potentially life-threatening danger to humans and animals alike. (reviewed by Codd et al. 2005). Cyanobacterial blooms frequently consist of both toxic and non-toxic strains, with the toxic strains potentially benefiting more from warming and eutrophication than the non-toxic strains (Davis et al. 2009). Furthermore, the effects of temperature were the main drivers of the spatial distribution in the cyanobacterial toxins, the concentrations and toxin quota of the cyanobacterial community (Mantzouki et al. 2018). This study demonstrated that temperature-related mechanisms result in the selective development of well-adapted cyanobacteria strains, which would decrease toxin diversity and potentially lead to the dominance of a few highly toxic strains. Cyanobacteria represent the dominant planktonic biomass during such blooms, thereby providing a food source that is not discriminated against by the planktonic filter feeders of the genus *Daphnia*. This unselective feeding mechanism and a high cyanobacteria abundance leads to the uptake of cyanobacterial toxins, which have been proven to be lethal for *Daphnia* (DeMott et al. 1991; Hansson et al. 2007; Baumann and Jüttner 2008). *Daphnia* have to cope with a mixture of different toxin types, since usually several species co-occur during blooms and often each is producing multiple toxin classes (reviewed by Gademann and Portmann 2008; Janssen 2019 2019). In addition to other cyanotoxins, which are classified as hepatotoxins, cytotoxins, neurotoxins, dermatotoxins and irritants, the digestive protease inhibitors (PIs) have been demonstrated to reduce growth and reproduction rates as well as to increase mortality of *Daphnia* (Lürling 2003; Rohrlack et al. 2004; Schwarzenberger et al. 2010; von Elert et al. 2012).

One example of PIs is the family of cyanopeptolins which have been shown to contribute to a wide range of bloom toxins (reviewed by Janssen 2019). These PIs mostly belong to the Ahp-cyclodepsipeptides and possess the design of serine protease inhibitors (Köcher 2019) that target the digestive proteases of *Daphnia*, especially *D. magna*, (Lürling 2003; Schwarzenberger et al. 2010; Lange et al. 2018). However, populations of *Daphnia* that encounter cyanotoxins over long periods are often locally adapted (Hairston et al. 1999; Sarnelle and Wilson 2005). Schwarzenberger et al. (2020 and 2017) could demonstrate that molecular and local adaptation occurred in a Swedish *D. magna* population with constant contact to PI-producing cyanobacteria, while a naïve Polish population showed higher

susceptibility. The serine protease CT448 got into focus as Schwarzenberger et al. (2020) confirmed positive selection on the *ct448* locus of the Swedish population. This adapted *Daphnia* population has been shown to dominate a community based on Swedish and Polish *D. magna* clones when the cyanobacterium *Microcystis sp*. was present in the diet, but not in the absence of cyanobacteria (Lange et al. 2023). Interestingly, this adapted population does not exhibit higher fitness (Lange et al. 2023) or somatic growth rates (Schwarzenberger et al. 2021) compared to the naïve population, when a different cyanobacterium, in this case *Microcystis aeruginosa* NIVA Cya 43, with a different set of PIs was present in their diet. It was demonstrated that demonstrated that the PIs cyanopeptolin 954 (CP954) and nostopeptin 920 (BN920) (Lange et al. 2018), identified and characterized by von Elert et al. (2005), target and inhibit the serine protease CT448. These findings indicate an adaptive tolerance of the Swedish population and point to adaptations to a locally co-occurring protease inhibitor-producing cyanobacterium. The major difference of CT448 between the two tested populations is a non-synonymous mutation of glutamic acid (E) to glutamine (Q) at position 34 (Schwarzenberger et al. 2020). Lange et al. (2018) were able to heterologously express this serine protease in *Sf*21 insect cells and characterized it *in vitro* and *in silico*. Proteomic characterization of cleavage sites (PICS) confirmed that CT448 preferentially cleaves behind small aliphatic residues like Ala, Val and Ile, thus classifying this protease as an elastase rather than a classic chymotrypsin. It can therefore be considered as a chymotrypsin-like elastase (Lange et al. 2018). Together with classic chymotrypsin, elastases are part of the chymotrypsin family, which represent serine endopeptidases and are typically found in the digestive tracts of animals. Digestive proteases are of major importance for digestion. They cleave ingested proteins into smaller peptides yielding amino acids that can be resorbed by gut epithelial cells. These digestive proteases are synthesized as inactive zymogens and require activation by proteolytic cleavage. Proelastase, the zymogen or precursor of elastase containing its pro-peptide, is activated by trypsin at its N-terminal scissile bond.

Evolutionary modifications might render the chymotrypsin-like elastase CT448 of *Daphnia* less susceptible to cyanobacterial secondary metabolites (Schwarzenberger et al. 2017; Schwarzenberger et al. 2020). The machine learning approach AlphaFold (Abramson et al. 2024) was utilized to predict the three-dimensional structures of CT448 based on the amino acid sequence. AlphaFold incorporates physical and biological knowledge about protein structure. The three-dimensional structure of a protein dictates its function. Hence, this tool was applied to estimate the structural properties of CT448 and the role of a non-synonymous mutation (Schwarzenberger et al. 2020), as AlphaFold can offer accurately elucidated structures of protein complexes (Humphreys et al. 2021; Gao et al. 2022; Bryant et al. 2022; Pei and Cong 2023).

In the current work, the characterization of CT448 based on the previous work was continued. The existence of structural properties like posttranslational modification were tested and rejected. Reliable activation processes besides the maturation by trypsin were elaborated to enable crystallization of CT448 with the focus to study the structural properties of the population specific variant. In the meantime, analyses were conducted via AlphaFold, suggesting that the pro-peptide remains bound to CT448 after processing as it is stabilized via a salt bridge by a glutamic acid (E34). Further, the *in silico* binding of CT448 and the cyanobacterial inhibitor CP 954 was modeled to elucidate the inhibitor binding.

4.3 Results

Post-translational modification

Lange et al. (2018) heterelogously expressed the chymotrypsin-like elastase CT448 in insect cells and detected species of CT448 with masses ranging from 35 to 37 kDa. It was suggested that these species were most likely the result of N-terminal truncations or post-translational modifications such as glycosylation. Two N-linkage glycosylation motifs were predicted within the pro-peptide sequence (Lange et al. 2018). Here, we tested the hypothesis of putative glycosylation of the pro-peptide. Therefore, the expressed and purified CT448 (according to Lange et al 2018) was applied to an SDS-PAGE and subsequently stained with coomassie or fuchsine (fuchsine red) which enables the detection of N-linked glycans. The CT448 of the Swedish clone M17 and the Polish E34Q variant were tested against the negative control Cpa1 and the positive control HRP (44.2 kDa) which is heavily glycosylated with galactose, arabinose, xylose, fucose, mannose, mannosamine, and galactosamine. By comparing fuchsine (Figure 1A) and coomassie (Figure 1B) stained gels, glycosylation was only detectable within the positive control HRP. Therefore, CT448 is not glycosylated despite N-glycans in *Sf*21 cells consist predominately of mannose. The observed species of CT448 are most likely attributed to N-terminal truncations as previously suspected.

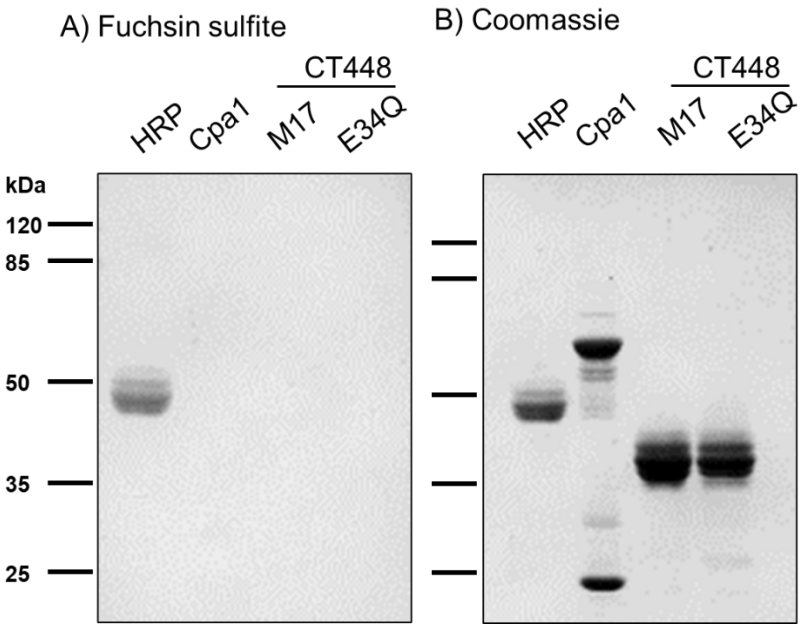


Figure 1: Glycosylation detection with fuchsin-sulfite subsequent to SDS-PAGE. Tested were CT448 (M17 and E34Q with 35-37 kDa), the positive control horseradish peroxidase (HRP with 44.2 kDa) and the negative control carboxypeptidase A1 (Cpa1 at around 60 kDa). For SDS-PAGE 5 µg of each protein was loaded and separated for each staining method. A) fuchsin staining for glycosylation detection. B) control staining with Coomassie Blue. Contrast and color adjusted for publication.

Thermostability of CT448 in presence of binding partners

Further analysis of CT448 M17 was performed to obtain information about putative binding partners like divalent cations for protein function and mechanism or crystallization. Hence, we performed ThermoFluor™ a.k.a Thermal Shift Assay to assess the stability of CT448. Typical divalent cations like Ca^{2+}, Mg^{2+}, Zn^{2+}, or Ni^{2+} were added to the solution buffer (containing 100 mM potassium phosphate pH 8.0, according to (Lange et al. 2018) with either 1 mM or 10 mM concentration. The addition of the $MgCl_2$, in both concentrations, had no effect on the stability of CT448 although 10 mM $CaCl_2$ increased the stability significantly, which is represented by a higher melting temperature (Figure 2, Tukey HSD after one-way ANOVA, $p < 0.05$). The elevation of the melting temperature from 42.67°C to 45.33°C is comparatively high, thus a noticeable alteration of stability is expected. The addition of $ZnCl_2$ and $NiCl_2$ destabilized the protein significantly.

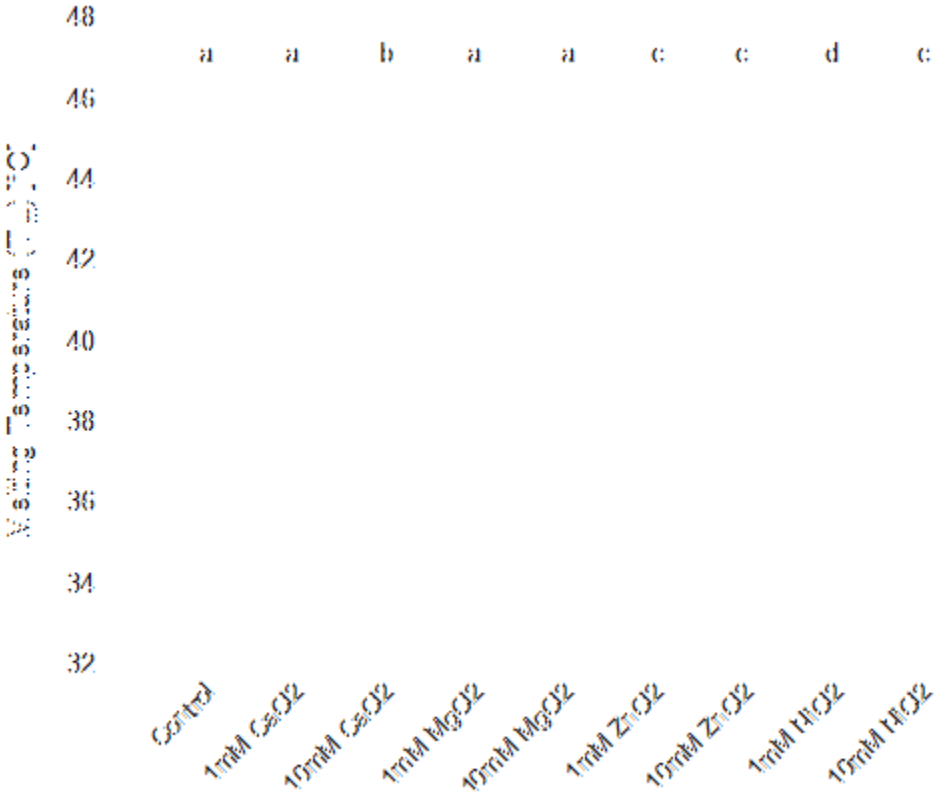


Figure 2: CT448 thermostability, e.g. protein denaturation, in dependence of various divalent cations. Depicted is the protein's melting temperature (T_m) given by the inflection point where 50 % of the protein are unfolded (Supplement 1). Tested were either calcium chloride, magnesium chloride, zinc chloride or nickel chloride in two concentrations: 1 mM or 10 mM. The control did not contain any divalent cation. Shown is the mean T_m with ± SD (n = 3). Letters indicate statistical differences between measurements at the given incubation time. Tukey's HSD post hoc test after one-way ANOVA, $p < 0.05$ (Supplement 7).

Proteolytic activation of CT448 variants

More structural analyses are needed for further investigation of this protease. Therefore, a crystal structure would provide essential information besides enzymatic comparison of the two local forms (Swedish and Polish) of CT448. Hence, proper and reliable activation, e.g. digestion, is crucial for subsequent analyses. We observed N-terminal truncation and low activity of the protein (Lange et al. 2018 and chapter 3). Therefore, a proper maturation of the heterologous protein is important to perform targeted assays. In the past, activation of the precursor has been carried out by tryptic digestion, but trypsin effects a rather unspecific and strong digestion of the entire protein. Along with chymotrypsin, trypsin is naturally present in the gut of *Daphnia* (von Elert et al. 2004). The *in silico* digestion (Expasy PeptideCutter, Gasteiger et al. 2005) of the peptide sequence of CT448 identified fifteen potential cleavage sites for trypsin and featured twenty-two for chymotrypsin (Supplement 2). Moreover, the digestion with a neutrophil elastase is predicted to result in forty-one cleavage events (Supplement 2). Of these proteases tested via *in silico* method, trypsin was considered the

most suitable enzyme for the maturation and activation of the inactive precursor of CT448. Furthermore, CT448 is likely to be hydrolyzed naturally by trypsin, given that trypsin is also present in the gut of *D. magna*. Hence, an optimization of the activation/ maturation protocol with trypsin is crucial.

Digestions with different concentrations of trypsin under various conditions were conducted to evaluate a more reliable activation of the CT448 precursor (Figure 3). The controls of CT448 without tryptic digest showed no auto-activation via fragmentation (Figure 3 upper panels) and almost no proteolytic activity (Figure 3 lower panels) on RT and 4°C throughout the incubation period of 360 min (6 h). The CT448 control at 4°C was incubated with thrombin to verify its proteolytic deficiency for CT448 as predicted *in silico* by the Expasy PeptideCutter (Supplement 2). This has been confirmed as neither fragmentation nor activity were detected (Figure 3).

The addition of trypsin resulted in a digestion of CT448 and proteolytic activity. The mature CT448 has a molecular weight of around 27 kDa (Lange et al. 2018). After 45 min of tryptic digestion, the fragmentation of the protein was detectable (Figure 3A upper panel). The proteolytic activity remained at base level 0.018 µmol*min^{-1} (Figure 3A lower panel). The highest activity was measured after 120 min maturation with 2.5 U trypsin at RT with 0.03 µmol*min^{-1} (Figure 3B lower panel, Tukey's HSD post hoc test after one-way ANOVA, $p < 0.05$) and declined after 360 min (Figure 3C lower panel). The treatments with tryptic digestions at 4°C achieved their maximum proteolytic activity after 360 min incubation (Figure 3C lower panel). The lowest overall activity of CT448 was observed when treated with 0.5 U trypsin at RT (Figure 3B and C lower panel). A less pronounced fragmentation pattern was noticeable with a distinct band at 25 kDa after 360 min incubation (Figure 3C upper panel). The unmatured and purified CT448 showed no low molecular protein species. However, the maturation by trypsin leads to strong fragmentation, i.e. multiple cleavages of CT448. Protein fragments ranged mainly from 20-25 kDa and 14-18 kDa. After 360 min, no protein with a mass of 35 kDa was present; unmatured CT448 was completely converted within 120 min at RT.

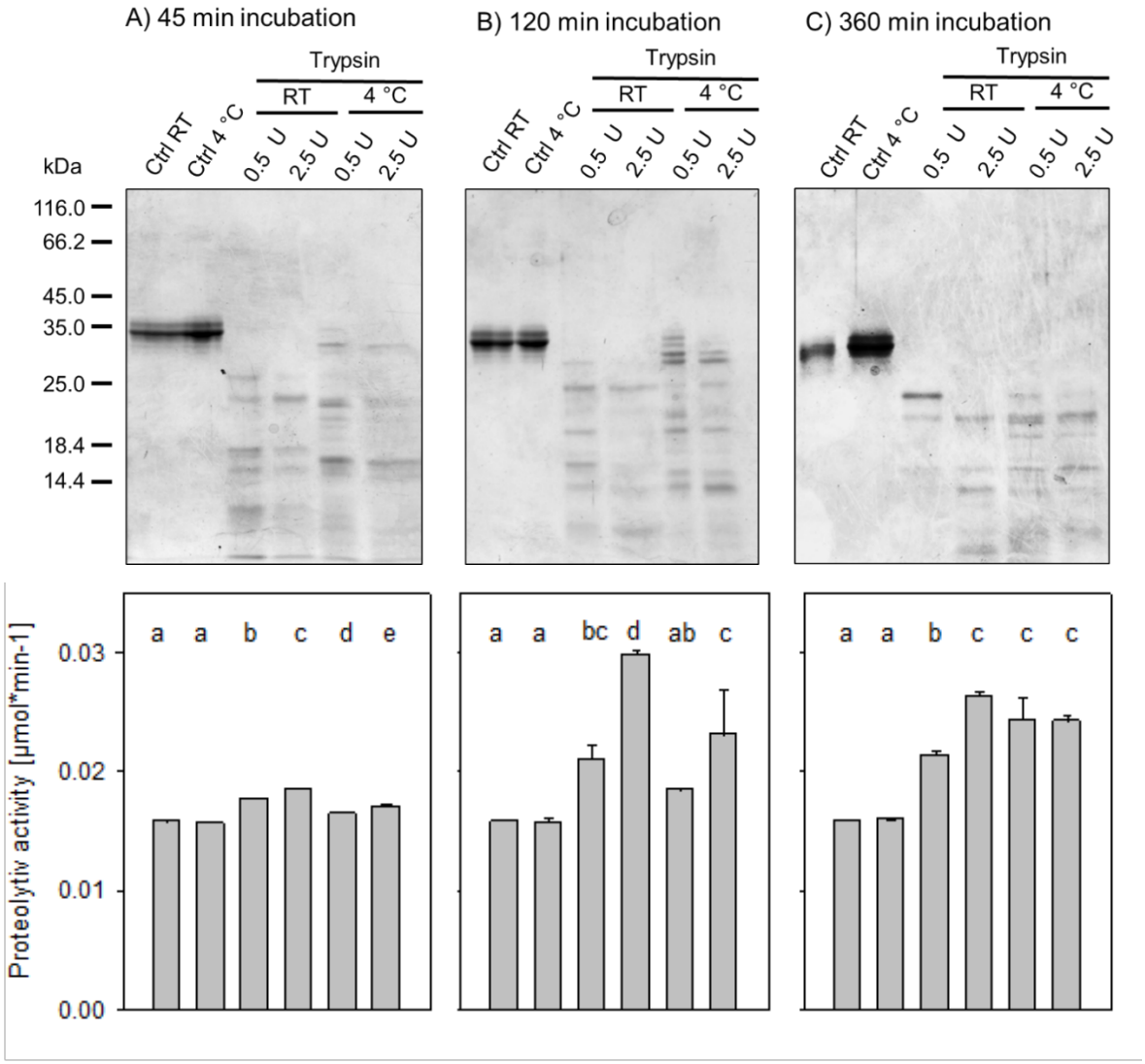


Figure 3: Tryptic treatment of CT448 and in gel analysis of CT448 maturation via band shift over time. Upper panels: SDS-PAGE of CT448 digestion within 6 h time laps. Trypsin was used for activation in 0.5 U and 2.5 U per mg CT448. As control CT448 was incubated at RT over time. The CT448 control at 4°C was additionally incubated with 2 U thrombin per mg CT448. Thrombin was included to verify its missing proteolytic activity on CT448. Hence, neither the CT448 ctrl at RT nor CT448 with thrombin at 4°C degraded over time. Samples of the digestion with trypsin for SDS-PAGE and enzyme assay were taken after A) 45 min, B) 120 min and C) 360 min. The molecular mass of the band is given in kDa. Lower panels: Proteolytic activity (µmol*min^{-1}) of CT448 after incubation with trypsin. As control CT448 was incubated at RT, while the approach with 2 U thrombin was incubated at 4 °C. N-Succinyl-Ala-Ala-Ala-p-nitroanilide was used as synthetic substrate. Proteolytic activity was measured after 45, 120 and 360 min. The plotted bars, e.g. proteolytic activity, is corresponding to the depicted SDS-PAGE lane of the upper panel. Plotted are means ± SD (n = 3). Letters indicate statistical differences between measurements at the given incubation time. Tukey's HSD post hoc test after one-way ANOVA, $p < 0.05$ (Supplement 8).

Maturation by trypsin yields more active protein but it also degrades a lot of potentially active protein. Therefore, it is necessary to establish an additional activation method that does not interfere with the subsequent enzyme assay or crystallization processes. Potential candidates

for targeted activation are tobacco etch virus (TEV) and thrombin. Both proteases are generally more specific and have no predicted cleavage sites in CT448 (Expasy PeptideCutter). Moreover, CT448's immunity to thrombin has been demonstrated above. Hence, CT448 variants with artificial cleavage sites for either TEV or thrombin were generated to achieve a successful and clean maturation. Additionally, the proteolytically inactive variant CT448 S184A was utilized to ensure for thrombin related cleavages and no interference via auto-processing. Hence, maturation processes were only detected via band shift in an SDS-PAGE. The incubation of 2 U and 4 U thrombin overnight or 24 h showed no pro-peptide cleavage, whereas the positive control PPEP 1 was digested by 4 U overnight (Figure 4). Non-protease deficient CT448 M17 with thrombin cleavage site was also digested with 10 U thrombin per mg CT448 overnight (Supplement 3). Except for the positive control PPEP1, no further digestion of CT448 was detectable within the SDS-PAGE after overnight incubation, indicating that CT448 was not cleaved and matured by thrombin (Supplement 4). An alternative approach would be the usage of a TEV cleavage site and the TEV protease instead. As the C-terminal Strep-tag is linked to the mature protease via a TEV cleavage site, a TEV digestion of the pro-peptide would allow a directed activation of CT448 and removal of C-terminal attachments. Supplement 5 demonstrates that the C-terminal Twin-Strep-tag is successfully cleaved off by TEV protease. To date, the substitution of a TEV cleavage site at the N-terminus for directed activation could not been achieved.

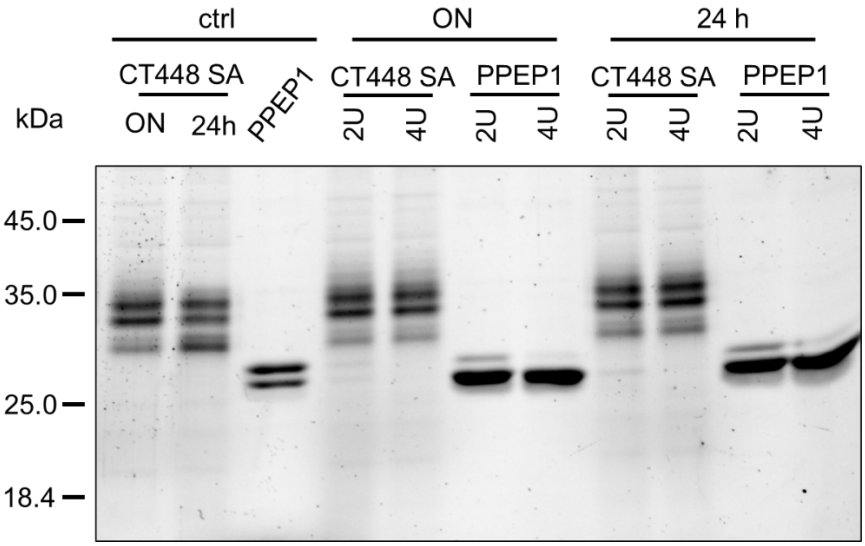


Figure 4: SDS-PAGE of thrombin digestion of mutated CT448. A thrombin cleavage site was mutated into the pro-peptide for specific activation-cleavage. Furthermore, the serine 184 of the catalytic triad was mutated to alanine to prevent CT448 induced degradation. Proteolytic activation should thereby exclusively be determined by thrombin. Thrombin was incubated overnight (ON) and for 24 h at two different concentrations (2 U or 4 U thrombin per mg CT448). The control treatments did not contain thrombin and were treated equally. PPEP 1 was utilized as positive control for thrombin digestion.

AlphaFold Protein structure prediction of CT448

The machine learning tool AlphaFold was utilized to predict the protein structure of CT448 in order to obtain a three-dimensional protein structure, which was not realizable with crystallographic approaches. Both protein structures, Swedish (E34) and Polish CT448 (Q34), were predicted with the latest AlphaFold 3 (released May 8th 2024). The protein structures were modeled without the signal peptide and C-terminal affinity tag as precursor, i.e. zymogen, and mature protease with or without pro-peptide. As both variants of CT448 are identical besides position 34, the Swedish variant CT448 was used for the general description of the structure. The best model out of five predictions was chosen based on established metrics (Abramson et al. 2024), but the five models did not differ much anyway.

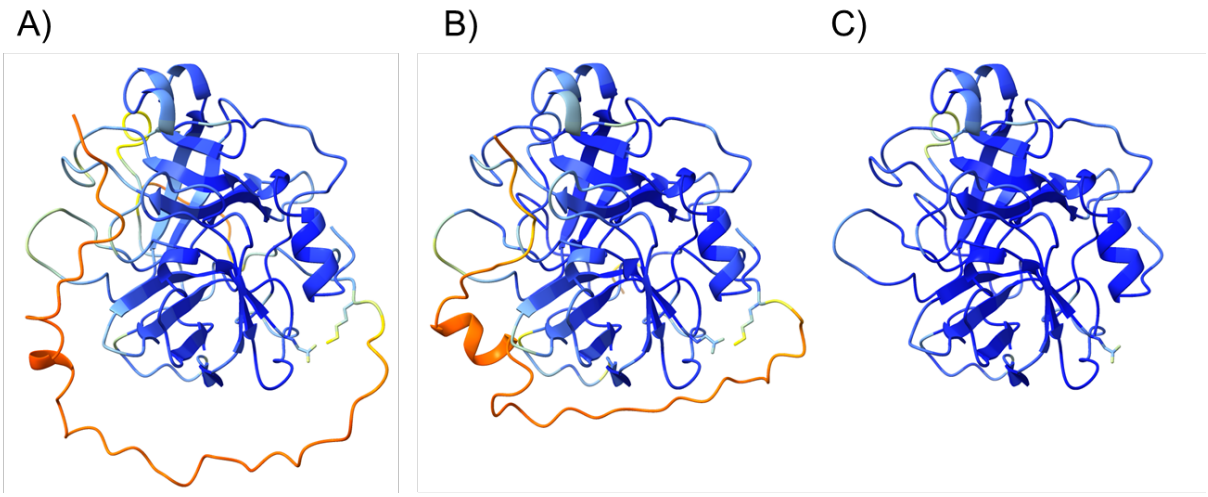


Figure 5: Model confidence of Swedish CT448 (E34) in the pre-cleavage and post-cleavage states of the AlphaFold prediction: A) Zymogen of CT448 with a pTM score = 0.82 (Supplement 6A), B) mature CT448 after trypsin cleavage at the sissle bond Arg56 with bound pro-peptide pTM score = 0.86 and ipTM score = 0.83 (measures the accuracy of the predicted relative positions of the subunits within the complex) (Supplement 6B) and C) mature CT448 without the pro-peptide with a pTM score = 0.95 (Supplement 6C). The N-terminal pro-peptide is in closer contact to the mature protein in the post-cleavage state compared to the pre-cleavage state. Proteins are displayed by cartoons, residues of interests are highlighted in sticks. Confidence bands are used to color-code the residues. Predictions colored by pLDDT (orange: pLDDT<=50, yellow: 50<pLDDT<=70, light blue: 70<pLDDT<=90 and dark blue: 90<=pLDDT<100).

The structure of Swedish CT448 was predicted as unmature (precursor) and mature state after trypsin cleavage between Arg56 and Ile57 (Figure 5). The mature protease was modeled with its bound pro-peptide (Figure 5B) and without (Figure 5C). The CT448 structures depicted highly confident protein cores while the N-termini including the pro-peptide had a predicted local distance difference test (pLDDT) below 50 and are to be considered disordered. (Figure 5). Structurally, there were hardly any differences between the protein cores with the strongest confidence in the mature CT448 without the pro-peptide Figure 5C and

Supplement 6). The highest divergence is unsurprisingly in the pro-peptides (Figure 5A and B). These unstructured regions are represented by long extended loops, especially within the zymogen (Figure 5A). Interestingly, the pro-peptides were always wrapped around the protein core, yet the predicted aligned error (PAE) indicated uncertainties in their position (Supplement 6A and B). The prediction improves from the N-terminus to the disulfide bridge Cys49-Cys108 on the surface between the pro-peptide and the protein core, indicated by the yellow and light blue ribbon there (Figure 6). Further, the population specific mutation of position 34 is located on the surface of the protease in distance to the active site.

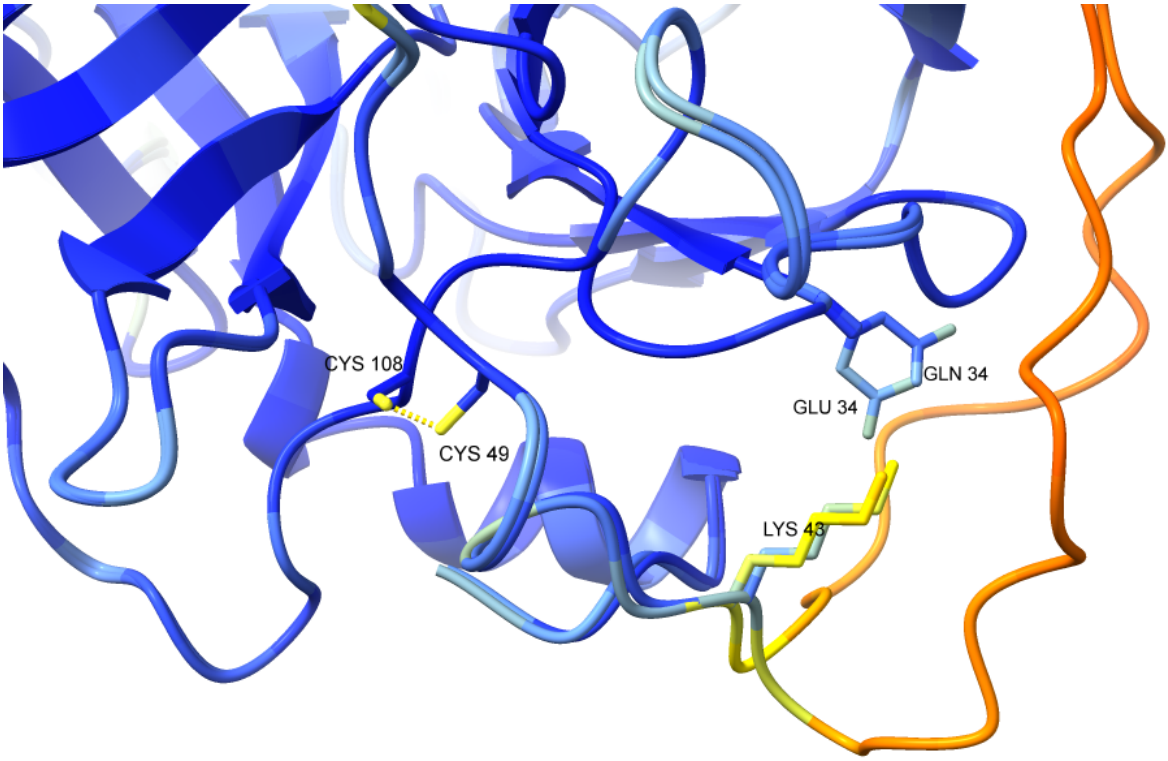


Figure 6: Close up of the overlay of Swedish and Polish CT448 in the post-cleavage, mature state with pro-peptide. Proteins are displayed by cartoons, residues of interest are highlighted in sticks. Depicted are the disulfide bridge Cys49-Cys108 (with yellow marked sulfur atoms) between core protein and pro-peptide as well as the position 34 and the Lys43 which probably forms a salt bridge. Confidence bands are used to color-code the residues. Predictions colored by pLDDT (orange: pLDDT<=50, yellow: 50<pLDDT<=70, light blue: 70<pLDDT<=90 and dark blue: 90<=pLDDT<100).

The overlay of both predicted structures of Swedish CT448 (E34) and Polish CT448 (Q34) in Figure 7 showed that the Lys43 of the N-terminal pro-peptide shares an estimated atom distance of 3.603 Å with Glu34 (E34) of the Swedish CT448 prediction. In the Polish CT448 prediction Gln34 (Q34) pointed away from Lys43 and was 5.306 Å apart. Besides, Glu34 also Asp33 (D33) is predicted to interact with Lys43 in both CT448 variants. The estimated distance of 5.3 Å of Gln34 is too far for the formation of hydrogen bonds or even for a weak electrostatic interaction (approximately 3.2-4.0 Å, (Jeffrey 1997). This model therefore depicts the impact

of the Glu34Gln mutation. The glutamic acid takes part in an interaction with the pro-peptide which will be less pronounced if mutated to the uncharged glutamine. A loss of the salt bridge could result in a more flexible pro-peptide, with a reduced likelihood of encountering the active site, as the N-terminus spans close to the active site of the serine protease (Figure 8). The catalytic triad His41, Asp87 and Ser184 are covered by the residues of Lys4, Pro5 and Tyr6, which did not interact with the catalytic triad.

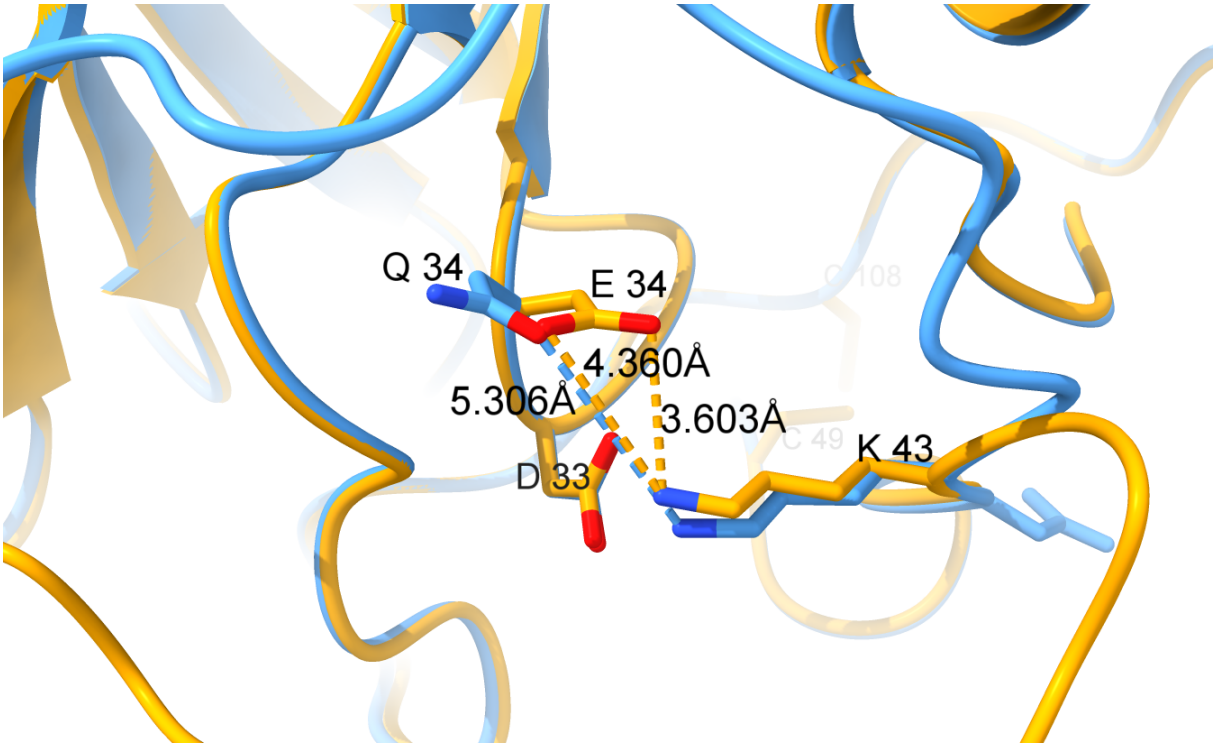


Figure 7: Overlay of the predicted matured CT448 structures. The Swedish CT448 (E34) is shown in orange, the Polish CT448 (Q34) in blue. Proteins are displayed by cartoons, residues of interest are highlighted in sticks. Highlighted as stick is the side chain of Lys43 (K43) of the N-terminal pro-peptide. Given are the estimated atom distances between these residues in angstrom (Å).

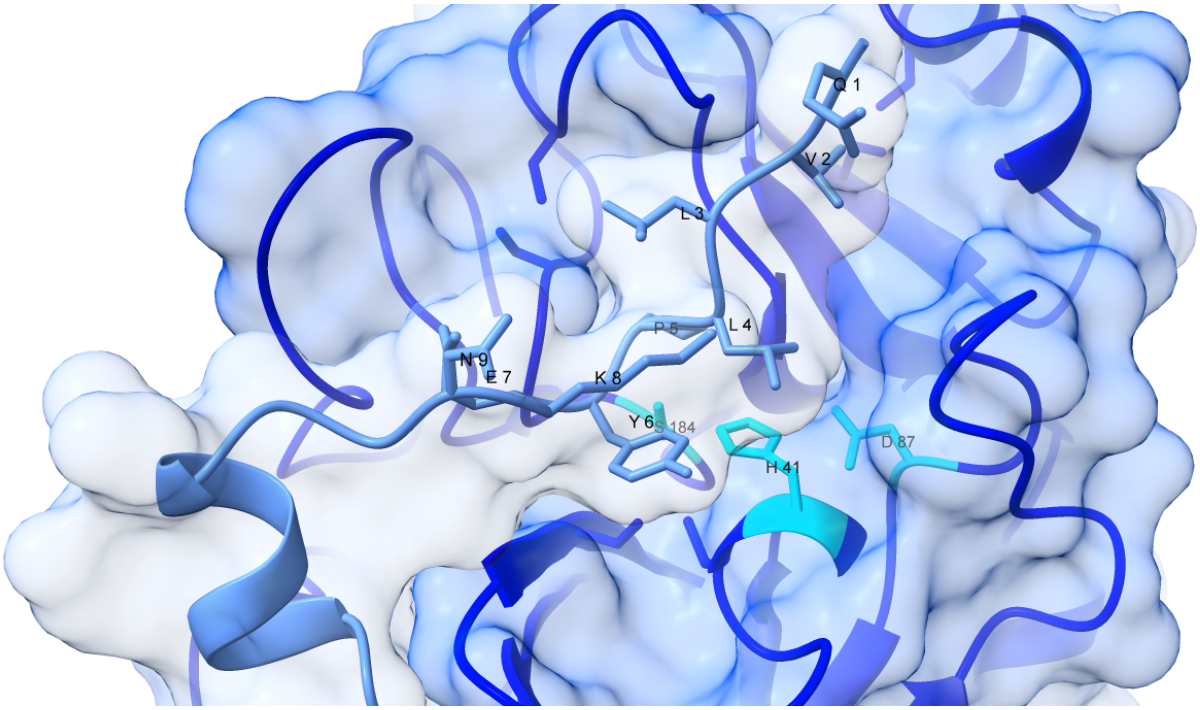


Figure 8: Overview of the binding site of the mature CT448 prediction with pro-peptide. The core protein is depicted in dark blue with the catalytic triad H41, D87 and S184 highlighted in turquoise. The pro-peptide is colored light blue. The surface structure with transparent appearance highlights the fit of the pro-peptide along the active site while the residues of Lys4 (L4), Pro5 (P5) and Tyr6 (Y6) do not interact with the catalytic triad. The residues of interests are highlighted in sticks and numbered accordingly.

The mature Swedish CT448 model without pro-peptide was utilized to test for binding of different PIs in silico. The docking tool AutoDock Vina was used to fit the known and identified PIs cyanopeptolin 954 (CP954) and scyptolin A into the substrate binding site as the inhibitors were suspected to be competitive. The binding mode of scyptolin A is known as it was co-crystallized with porcine elastase 1 (Matern et al. 2003), which resulted in a crystal structure with 2.80 Å resolution (RCSB pdb Id.: 1OKX). This inhibitor complex with porcine elastase was compared with the CT448 model and scyptolin A was docked to mature CT448 (Figure 9A). The inhibitor complex with porcine elastase with its rigid cyclic peptide structure sits in the active center pocket, but does not collide with the surface of CT448, indicating a similar fit for the active center pocket of CT448. Notably, Leu^{4} filled the oxanion hole (Figure 9). The docking of scyptolin A to mature CT448 (Figure 9B) did not result in an identical binding mode but was similar. The main discrepancy occurred at the amino acids Ala^{1} to Thr^{3} which were flipped in opposite direction to the crystallized structure of scyptolin A. Additionally, Val^{8} is located close to the active center and Ser184 but does not enter the oxanion hole. Accordingly, the accuracy of the docking is inferior to that of a crystal structure, thereby limiting the reliability of the resulting predictions.

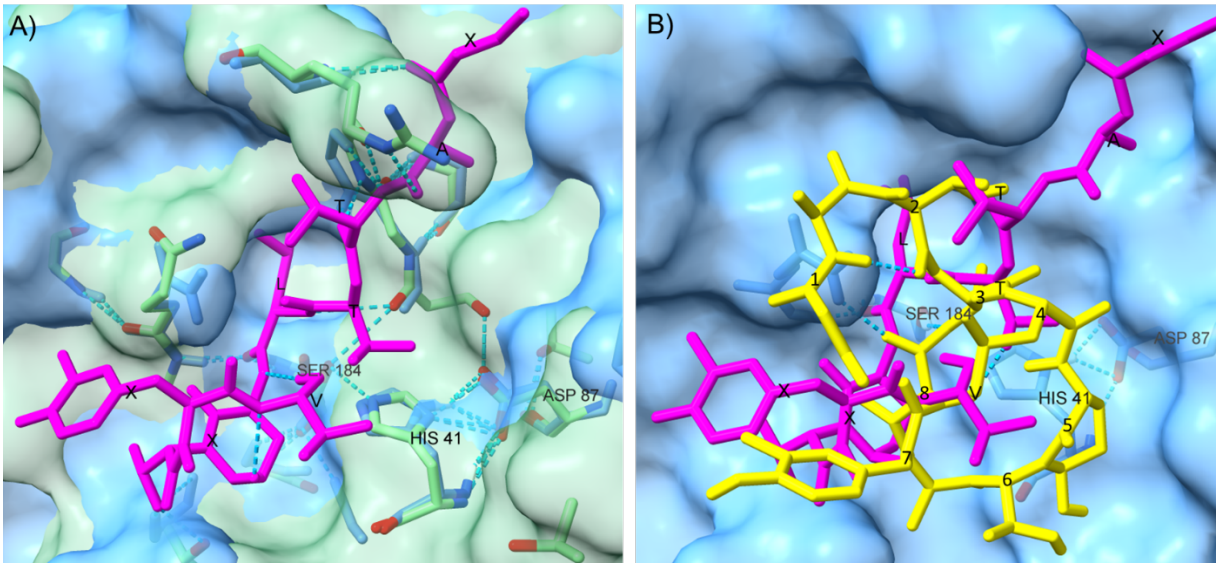


Figure 9: Stereo views of Scyptolin A binding to porcine pancreatic elastase and mature CT448. A) Stereo view of scyptolin A (lilac sticks) bound to elastase represented by its molecular surface (green) with the overlay of mature Ct448 (blue). The catalytic triad beneath the surface of both proteases is shown but only CT448 is numbered. The inhibitor with its rigid cyclic peptide structure sits snugly in the active center pocket of porcine elastase. B) Stereo view of scyptolin A docked to mature CT448 (yellow sticks) compared with the scyptolin A in binding mode from porcine elastase (lilac sticks). Numbers describe the amino acid residues of scyptolin A docked to mature CT448 (yellow sticks) according to Matern et al. (2003): Ala[1], Thr[2], Thr[3], Leu[4], Aph[5], Thr[6], cmTyr[7], Val[8]. The docking of scyptolin A resembles the binding mode of the crystallized scyptolin A, but the amino acids Ala[1] to Thr[3] flipped in opposite direction. Val[8] is located close to active center but does not enter the oxanion hole.

Furthermore, a cyanobacterial PI, cyanopeptolin 954 (CP954), was docked the AlphaFold prediction of the mature Swedish CT448 without pro-peptide (Figure 10). CP954 has been demonstrated to inhibit heterologous expressed CT448 (Lange et al. 2018). The 3D-strucutre depicts the fit of CP 954 to the binding site close to the active center (Figure 10). The cyanobacteria inhibitor covers the catalytic triad and further extends its acetylated α-NH_2 group of Gln[2] (von Elert et al. 2005) into the oxanion hole with an estimated distance to Ser184 of 3.02 Å. This is consistent with a comparable binding mode observed with sycptolin A.

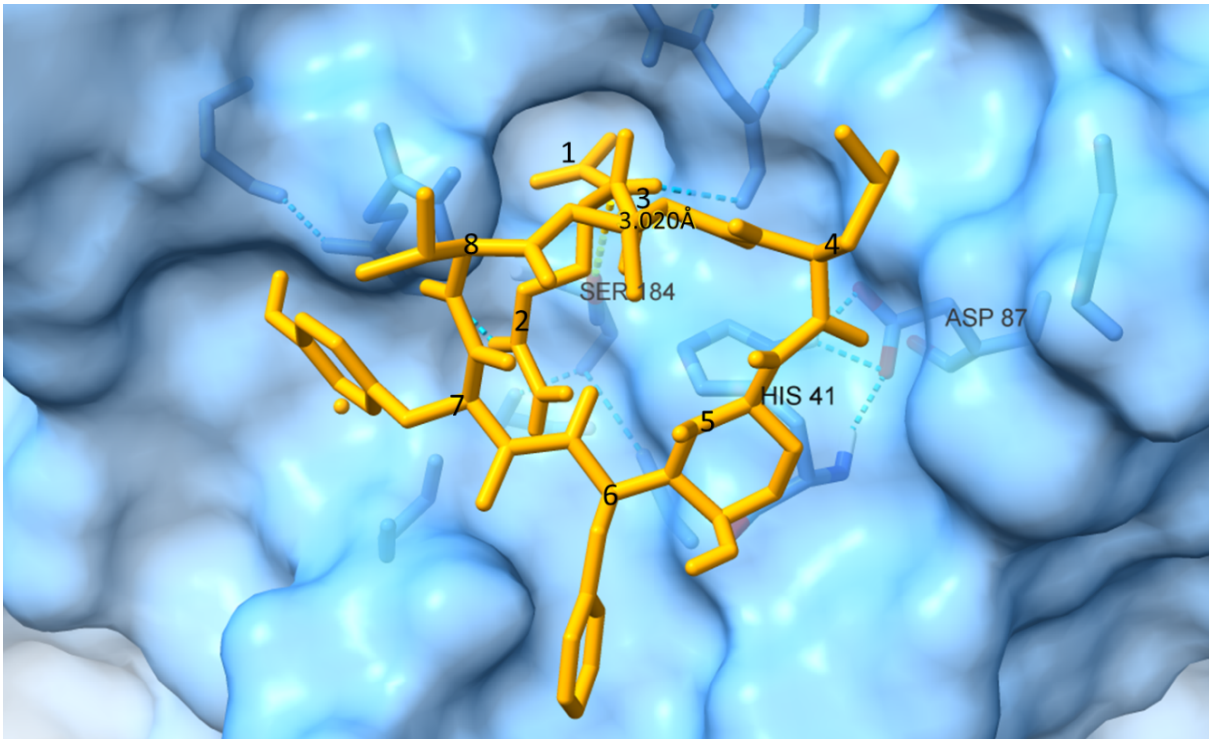


Figure 10: Stereo views of binding prediction of mature Swedish CT448 and the cyanobacterial inhibitor cyanopeptolin 954. Depicted is the substrate binding site (light blue transparent) with the docked inhibitor CP 954 (orange). The catalytic triad beneath the surface is shown, the numbers describe the amino acid residues of CP954 according to von elert 2005: Ac[1], Gln[2], Thr[3], Leu[4], Ahp[5], Phe[6], cmTyr[7] and Val[8]. The docked inhibitor is covering the catalytic triad (His41, Asp87 and Ser184), where Ac[1], Gln[2] enter the oxanion hole.

4.4 Discussion

The endopeptidases chymotrypsin and trypsin of *D. magna* account for 75–83 % of the digestive proteolytic activity, which has been shown to derive from gut homogenates when compared to crude extracts of whole animals (von Elert et al. 2004). However, elastase does not significantly contribute to the proteolytic activity in the gut of *D. magna* (chapter 3 and von Elert et al. 2004) and has not even been detected in previous studies. Nevertheless, the gene encoding this serine protease, *ct448*, has been shown to be involved in the response of *Daphnia* to toxic cyanobacteria (Schwarzenberger et al. 2010; Drugă et al. 2016; Schwarzenberger and Fink 2018; Schwarzenberger et al. 2020). Commonly, a higher expression rate of *ct448* has been observed when *Daphnia* was exposed to cyanobacterial strains of the genus *Microcystis*. Therefore, the elucidation of the function and structure of CT448 is crucial to understanding its role in *Daphnia*–cyanobacteria interactions. CT448 was successfully heterologously expressed in the insect cell model *Sf*21 to obtain pure protein in high quantities for structural analyses and characterized as chymotrypsin-like elastase (Lange et al. 2018). Within the sequence of the extended pro-peptide of CT448 (compared to mammalian homologs) putative N-linked glycosylation motifs were predicted *in silico* (Lange

et al. 2018). The hypothesis of such post-translational modifications could not be verified when CT448 was tested for glycosylation via fuchsin sulfite. The N-glycosylation of serine proteases is common and is often linked to expression, secretion or zymogen activation (Liao et al. 2007; Bence and Sahin-Tóth 2011). The human chymotrypsin C (CTRC) contains a single N-linked glycan, and a loss-of-function mutation reduced CTRC secretion from HEK 293T cells about 10-fold but had no effect on CTRC activity or inhibitor binding (Bence and Sahin-Tóth 2011). Glycosylated CT448 protease bands were not detected by fuchsin sulfite staining, indicating that the observed fragmentation of CT448 with molecular masses between 35 and 37 kDa is most likely due to N-terminal truncations and not the result of post-translational modification.

The *in-silico* digestion of *D. magna* CT448 by Expasy PeptideCutter predicts many cleavage sites for trypsin, chymotrypsin and elastase. As chymotrypsin and trypsin account for the mayor proteolytic digestion in *D. magna*, it is likely that these proteases are associated with the activation of CT448. Chymotrypsin-like proteases, like CT448, are synthesized as zymogens, inactive precursors, containing N-terminal extensions. The zymogen is activated by proteolytic digestion, releasing the N-terminal Ile16 (bovine numbering), resulting in a salt bridge of the newly formed N-terminus and Asp194 (Hedstrom 2002). A typical proteolytic activator for pancreatic zymogens is trypsin (Rinderknecht 1986). The chymotrypsin-like elastase CT448 has been demonstrated to be active after proteolysis by trypsin (Lange et al. 2018), but activity of CT448 remained very modest, and activation by trypsin was not fully reproducible. The activation tests with different trypsin concentrations, incubation time and temperature displayed that autoactivation does not occur in the given time. As autoactivation is uncommon among pancreatic chymotrypsin and elastase, this result was to be expected. The maximum activity of CT448 was achieved by 120 minutes of digestion with 2.5 U trypsin at RT. Although the tryptic digestion resulted in a strong fragmentation of CT448 over a long period, the observed proteolytic activity was comparable to that previously reported by Lange et al. (2018), suggesting a relatively low overall activity. This is consistent with its low activity compared to the major serine proteases chymotrypsin and trypsin of *D. magna*, as discussed in chapter 3 and von Elert et al. (2004).

Given the poor maturation of CT448 with trypsin, resulting from its high degree of degradation, an attempt has been made to develop an improved maturation process by the introduction of an artificial cleavage site to ensure specific digestion. Cleavage sites for thrombin or TEV protease were chosen as CT448 does not inherit a cleavage site for these proteases. The digestion sites for thrombin and TEV were designed to cleave the pro-peptide between Arg56 and Ile57 (counting without signal peptide), respectively Arg77 and Ile78 (Lange et al. 2018). Thrombin and TEV have the technical advantage that they do not interfere with the established proteolytic assay and can easily be removed for later crystallization. The thrombin cleavage site was cloned into CT448 but were not able to cleave the pro-peptide. Several activation

attempts did not result in a proteolytic activity of CT448 comparable to that obtained after a trypsin digest. We suspect that the affinity of the introduced cleavage site (LAPR/IIN) was reduced as P3 and P1'-P2' were slightly altered compared to its favored binding site. The strong preference of thrombin for arginine in position P1 and for proline in position P2 (Pozsgay et al. 1981; Lottenberg et al. 1983; Chang 1985) was conserved. As Ser, Thr or Ala are mostly preferred at position P1' (Stephens et al. 1988; Theunissen et al. 1993; Petrassi et al. 2005), aromatic amino acids such as Phe are favored at P2' (Le Bonniec et al. 1996). Basic residues are favored at P3' (Le Bonniec et al. 1996), but acidic amino acids are specifically avoided at P3 and P3' (Le Bonniec et al. 1991; Theunissen et al. 1993; Petrassi et al. 2005). Our construct exhibits Ile at P1' and P2' and Asn at P3', therefore, acidic amino acids have been avoided. Still, it has been demonstrated that the alteration of single positions, like mutations of P2-Pro and P3′-Arg, can cause 20- to 14-fold reductions of cleavage (Gallwitz et al. 2012). Even the insertion of an Asp in position P4 resulted in an almost twenty-fold drop in cleavage efficiency (Gallwitz et al. 2012). Therefore, it is likely that the alterations of the positions P1'-P3' resulted in major effects on cleavage efficiency and affinity, although *in silico* digestion of CT448 by thrombin was predicated by Expasy PeptideCutter. Furthermore, TEV was utilized as alternative protease for activation of CT448. This cysteine protease has the advantage of not interfering with our assay approach and is utilized to cleave off the C-terminal Strep-tag of the CT448 construct used for protein purification by affinity chromatography. Hence, the activation of CT448 could be combined with C-terminal tag removal in one step.

It is crucial to obtain reliable enzyme activation in order to test the different constructs, i.e. clonal variations of CT448, comparatively. In that way it can be determined whether individual mutations influence enzyme kinetics or proteolytic properties and investigated whether these might result in effects that influence physiological responses of *Daphnia* to cyanobacteria. Accordingly, it was demonstrated in the large milkweed bug, *Oncopeltus fasciatus*, that substitutions within the cardenolide target site of Na,K-ATPase α-subunits result in resistance against toxic cardenolides, which is accompanied by significantly altered kinetic properties (Dalla and Dobler 2016).

Besides more reproducible investigations of CT448 kinetics the development of a robust method for CT448 activation would allow for the crystallization of CT448. To date, attempts to crystallize heterologously expressed CT448 failed. Screenings based on sodium sulfate or sodium acetate conditions, which are widely used for crystallization of pancreatic elastases in the RSCB Protein data bank (PDB) (Odagaki et al. 2001; Weiss et al. 2002; Ruivo et al. 2016), yielded no crystals. The Thermofluor-based analysis of CT448 and divalent cations suggests a higher stability with Ca^{2+} (respectively $CaCl_2$). Weiss et al. (2002) reported that the metal-binding site of porcine pancreatic elastase mainly binds Ca^{2+} in $Na_2SO_4/CaCl_2$ crystals, but Na^{2+} in Na_2SO_4 solutions. Despite slightly different crystallization conditions, similar crystal

structures were obtained: slight rearrangement of the amino-acid residues building up the metal-binding site depending on the coordinated metals (Weiss et al. 2002). Hence, a substitution of the crystal conditions with Ca^{2+} for CT448 of *D. magna* might not alter the structure itself but result in improved crystallization. This remains to be tested.

Despite numerous efforts, proper activation and crystallization of the serine protease CT448 was unsuccessful, thereby hindering a detailed biochemical and structural characterization. Determining the structure and function of CT448 is essential for understanding its role in the response of *Daphnia* to toxic cyanobacteria. Thus, AlphaFold 3 was utilized to predict a structure (Jumper et al. 2021; Abramson et al. 2024). In May 2024 the latest AlphaFold 3 model (Abramson et al. 2024) was released, which demonstrates significantly improved accuracy over many previous specialized tools. Nevertheless, it inherits limitations with respect to stereochemistry, hallucinations and accuracy for certain targets: spurious structural order, so called hallucinations, may appear in disordered regions identified as very low confidence, but they may lack the distinctive ribbon-like appearance that AlphaFold 2 generated in such regions (Abramson et al. 2024). Still, the diffusion-based model of AlphaFold 3 enhances its ability to deal with complex protein structures and improves prediction accuracy by refining initial guesses through multiple stages of noise reduction and structural clarification, thereby allowing for better handling of ambiguous or unstructured protein regions. For these reasons, the models of CT448 and its interaction must be interpreted with caution. In particular, the variance in the flexible areas must be considered, yet AlphaFold allows for the most accurate *in silico* structure analyses currently available (Abramson et al. 2024).

The models of the zymogen and the mature CT448 demonstrate that the pro-peptide is bound to the protein by a disulfide bridge formed between Cys49 and Cys108. This bond is predicted with high confidence, indicating that the pro-peptide is likely to remain in its current conformation with similar properties. Pro-peptides serve multiple functions in the maturation process of their proteins, including folding and stabilization, sorting, and regulation of activity (Shinde and Inouye 1994; Buczek et al. 2004; Demidyuk et al. 2010). These structural elements assist in maintaining precursor proteins in an inactive or partially folded state until they reach their destination or are further processed. Additionally, they can also protect precursor proteins from premature degradation. By covering sensitive regions of precursor proteins, pro-peptides ensure their stability and functionality. Furthermore, pro-peptides can regulate the activity of mature proteins. They can act as inhibitors, maintaining proteins such as enzymes in an inactive state until a specific signal or condition triggers their removal or modification, thereby initiating their activation. This is in line with a study by Pei et al. (2024), in which AlphaFold was utilized to generate structural models for over 7000 proteins with pro-peptides of at least 20 residues either in the pre-cleavage state or in the post-cleavage state. Analysis of the residue contacts in these models revealed conformational changes for over

300 proteins before and after cleavage of the pro-peptide. In the majority of cases, post-cleavage interactions of pro-peptide and mature protein are considerably stronger than their interactions prior to pro-peptide cleavage. These results suggest that pro-peptides may play a crucial role in regulating the activity of mature proteins after cleavage. Furthermore, it has been concluded by Pei et al. (2024) that the pro-peptides serve as inhibitors of the mature protein by positioning the pro-peptides in the vicinity of the active sites of the mature enzymes. Similarly, the modeled interaction of mature CT448 with the cleaved N-terminus is stronger after cleavage as the free pro-peptide allows more flexibility. With the pro-peptide blocking the substrate binding site and the catalytic triad, inhibitors like CP954 could be prevented from binding to the mature protein.

Glu34 interacts with Lys43 fifteen residues N-terminal from the disulfide bond that connects the pro-peptide to the mature protease body. This salt bridge of Glu34-Lys43 might act as an additional linker allowing for a stronger binding of the pro-peptide in the post-cleavage state resulting in a less flexible peptide chain. Potentially, the mutation to Gln34 weakens this effect, as the loss of a salt bridge between the pro-peptide and mature protease allows for a weaker bond. It is possible that a more flexible pro-peptide increases the chance of an inhibitor binding to the active site. Hence, the pro-peptide could serve as a lock for the protease after its cleavage, inhibiting activity and moreover binding of cyanobacterial inhibitors. Activation could be achieved by the dissociation of the pro-peptide, which could occur under different physiological conditions such as pH-levels.

The gastrointestinal tract of *D. mag*na neonates was mapped with pH nanosensors, demonstrating that the pH was 5.5–6.0 at the anterior section of the gastrointestinal tract and up to 7.2 in the posterior section (Davis et al. 2020). Overall, the pH within the *D. magna* gastrointestinal tract was significantly lower than the surrounding aqueous medium at pH of 7.8. It has been observed that the pH gradient in the digestive tract exhibits variability and occurs at a lower pH-level compared to this experimental setup with a pH of 8.0 and previous studies. Elastases typically exhibit a pH optimum above 8.0. Chymotrypsin and trypsin of *D. mag*na have been demonstrated to possess an alkaline pH optimum of pH 7-10 (von Elert et al. 2004). However, it remains unclear whether neonatal and adult *D. magna* exhibit differences in pH distribution, pH range, or stressor-induced pH adaptation. Consequently, the optimal buffers and conditions for CT448 should be re-evaluated in order to yield maximum activity. Activation of the post-cleavage protease could be achieved by the dissociation of the pro-peptide, which could occur through further proteolytic processing of the pro-peptide. In this study, activity of CT448 peaked after a long incubation with trypsin. Simultaneously, the protease showed a broad degradation pattern. This was deemed insufficient as the protease is not just activated but simultaneously destroyed. However, there might be a second

mechanism or cleavage event that would release the pro-peptide altogether and thereby free the active site and reveal the true proteolytic activity. Given the crucial role that functional maturation of CT448 plays in the investigations and comparative analysis of different variants thereof, this study demonstrates the necessity of obtaining a crystal structure of CT448 and to co-crystallize it with a suitable protease inhibitor. This is due to the fact that the protein structure predicted by AlphaFold still exhibits weaknesses and does not meet the required level of confidence. Nevertheless, the structure of the pro-peptide and the population specific alteration of CT448 are notable characteristics that were displayed by the predicted structure and need further confirmation such as role of the pro-peptide in the functioning of CT448.

4.5 Methods

Glycoprotein staining

The staining for putative glycosylation of heterologously expressed CT488 was performed according to a protocol based on (Zacharius et al. 1969). CT448 was expressed as described by Lange et al. (2018) and stored at -80°C. Horseradish peroxidase (HRP) was purchased (Sigma-Adrich) and used as positive control. The kindly provided carboxypeptidase A1 (Cpa1) was expressed in *E. coli* BL21 (DE3) by Fabian Wojtalla and used as negative control. Two separate SDS-PAGEs (with 12 % polyacrylamide gels) with each 5 µg of protein (CT448 M17, CT448 E34Q, HRP and Cpa1) were conducted until proteins were separated. One polyacrylamide gel was stained with Coomassie Brilliant Blue R250 for comparative protein staining. The second gel was processed according to staining protocol for glycoproteins subsequent to SDS-PAGE modified after Zacharius et al. (1969). Accordingly, the gel was immersed in 12.5 % trichloroacetic acid (25-50 mL per gel) for 30 min and rinsed lightly with dest. H_2O. Subsequently, the immersion in 1 % periodic acid form sodium periodate (made in 3 % acetic acid) was performed for 50 min. Two washing steps for 10 min with 200 mL dest. H_2O while gently shaking were carried out, followed by an overnight wash in 200 mL dest. H_2O with a subsequent final washing step for 10 min. The immersion of fuchsin-sulfite (Schiff's fuchsin-sulfite reagent, Sigma-Aldrich) in the dark for 50 min led to the staining of the aldehyde groups of carbohydrates. A 10 min washing step with 25-50 mL (per gel) freshly prepared 0.5 % sodium disulfate was repeated for three times. Followed by a final washing step consisting of 25-50 mL (per gel) freshly prepared 0.5 % sodium disulfate for 10 min repeated for three times. The gel was stored in 3 % acetic acid. Finally, the stained gels were documented and analyzed with an ChemiDoc™ XRS+ Imaging System (BioRad).

Thermostability Assay

The Thermofluor™ assay or thermal fluorescence shift assay was conducted to evaluate the stability of CT448 (M17) in the presence of divalent cations. Therefore, heterologous expressed CT448 was mixed with either calcium chloride, magnesium chloride, zinc chloride or nickel chloride in two concentrations: 1 mM or 10 mM. This reaction mix was then gradually heated and the unfolding/denaturation of CT448 was detected by using Sypro Orange (Bio-Rad) dye. Sypro Orange binds to hydrophobic regions in denatured proteins and illuminates the presence of unstable proteins.

The following preparation was conducted with 45µl reaction containing 15 µg Protein, 10 mM or 1 mM divalent cations in 100 mM potassium phosphate buffer pH 8.0. 5 µL 10x Sypro Orange (from 5000x stock diluted with H_2O) were added and kept in the dark. The reaction was pipetted into 96-well plates. Each test was carried out in triplicates. The 96-well plate (with low profile) was inserted into a RT-PCR cycler (CFX96 Touch Real-Time PCR Detection System, Bio-Rad) with a cycling program which gradually increases the temperature by 1°C per minute in a temperature range from 20°C to 95°C. The peak of the melting curve was calculated for further comparison. The data were analyzed with Microsoft Excel (Version 2016) and Sigmaplot (SigmaPlot Version 15 Build 15.1.1.26, Systat Software GmbH). A one-way ANOVA followed by a Tukey's HSD post hoc test ($p < 0.05$) was performed to show differences between the different treatments.

Mutagenesis of CT448 variants

The acquired construct of CTT48 (M17) for *Sf*21 expression (for comparison Lange et al 2018) was modified: The N-terminal cleavage site oh trypsin was mutated for thrombin or tobacco etch virus (TEV) protease digestion. Further point mutations were introduced to obtain an inactive protease by substitution of serine 184 in the active site with an alanine (S184A) and the Polish-specific mutation of glutamate to glutamine (E34Q). A site directed mutagenesis was performed using Q5 High-Fidelity DNA Polymerase (New England Biolabs) and the Sf21 CT448 Plasmid (Lange et al. 2018) as template. A PCR reaction was prepared with 5 µL 5x Q5 Reaction Buffer, 0.5 µL 10 mM dNTPs, 1.25 µL of each 10 µM forward and reverse primer (Table 2), 10 ng template DNA, 0.25 µL Q5 High-Fidelity DNA Polymerase and added up to 25 µL with nuclease-free water. The thrombin mutagenesis was carried out with additional 5 µL 5X Q5 High GC Enhance. The PCR program consists of an initial denaturation at 98°C for 30 sec followed by 30 extension cycles at 98°C for 10 sec, corresponding annealing temperature (Table 2) for 30 sec and an extension at 72°C for 3 min. A final extension phase was performed at 72°C for 3 min. The PCR product was subsequently digested with 1 µL DpnI (New England Biolabs,) for 1 h at 37°C and used to transform chemical component *E. coli* DH5α: about 10 µL PCR product were added to the thawed DH5α and incubated for 20 min

on ice. Subsequently, a heat shock was performed at 42 °C for 60–90 sec followed by 2 min on ice. After the heat shock, 500 µL of SOC medium were added for 1 h incubation at 37°C while shaking. The pelletized and concentrated sample plated on LB-Agar plate with ampicillin. The plate was then incubated at 37°C overnight. The next day colonies were picked and incubated overnight in shaking cultures (3 mL LB medium with 3 µL ampicillin each at 37°C). For sequencing the DNA was isolated following the manual of the GeneElute Plasmid Miniprep Kit (Sigma-Aldrich). Sequenced (Table 1, pFL primer) and checked samples for correct mutagenesis were used for further baculovirus-insect cell expression and purification according to Lange et al. (2018).

The expression of different CT448 constructs for the baculovirus expression system in *Sf*21 cells was able via this method. Hence, a Polish mutant of the Swedish M17, the so called CT448 E34Q and a protease-deficient CT448 E34Q-S184A; an inactive CT448 M17, so called CT448 S184A and CT448 M17 with thrombin were generated. For the CT448 with a TEV cleavage site have been cloning attempts but no positive results to date.

Table 1: Primer pair sequences for mutagenesis of the SF21 construct CT448 M17 (Swedish population) for subsequent studies. The CT448 construct was mutated with the thrombin (and TEV) primer for activation tests. Point mutations (S184A or E34Q) were introduced for enzymatic assay approaches. The pFL primer pair (lower row) was used for sequencing of the mutated samples after transformation in *E. coli* and plasmid purification.

Construct name	Direction (5'-3')	Primer sequence	Annealing temperature
Thrombin (LAPR/IIN)	Forward	CTCGCTCCTCGTATCATCAAC	65°C
	Reverse	CGAGGAGCGAGGGGGGTGCCG	
TEV (ENLYFQ/G)	Forward	GAGAACCTCTATTTTCAGGGCATCAACGG	70°C
	Reverse	GCCCTGAAAATAGAGGTTCTCGCCGCAC	
Inactive mutant S184A	Forward	GCGACGCCGGTTCCGCTATG	68°C
	Reverse	CCGGCGTCGCCGTTGCAGG	
Polish Population E34Q	Forward	CGACCAGTGGGTGCTGACC	72°C
	Reverse	ACCCACTGGTCGGAGATCAG	
pFL sequencing primer	Forward	TAAAATGATAACCATCTCGCAAATAAATAAG	53°C
	Reverse	TTTAAAGCAAGTAAAACCTCTACAAATG	

Proteolytic Activation with trypsin

Freshly heterologously expressed and purified CT448 was used in the activity assay. CT448 variants were activated with immobilized trypsin (TPCK treated, Thermo Fisher Scientific, Waltham, MA USA) or thrombin (BD Biosciences, San Jose, CA USA). Trypsin was utilized in

0.5 U/mg and 2.5 U/mg CT448 and thrombin was tested with 2 U/mg CT448 to activate 1.5 µg of CT448. The thrombin activation was carried out only at 4 °C while trypsin was tested at both RT and 4°C. Samples for an SDS-PAGE were taken after 45, 120 and 360 min and were supplemented with Laemmli buffer, containing β-mercaptoethanol and were boiled for 3 min at 95°C. Subsequent to a standard SDS-PAGE (according to Lange et al. 2018), the gel was stained with coomassie by standard protocol and visualized a ChemiDoc (Bio-Rad Laboratories, Hercules, CA USA).

The activity of putatively maturated protease variants was assessed by providing N-Succinyl-Ala-Ala-Ala-p-nitroanilide. This is a common serine protease model substrate those degradation products absorb UV-light. 150 µM N-Succinyl-Ala-Ala-Ala-p-nitroanilide (Sigma-Aldrich, St. Louis, MO USA) was incubated with 1.5 µg CT448 for 45, 120 and 360 min with trypsin or thrombin, in a volume of 150 µL of 0.1 M potassium-phosphate buffer pH 8.0. As control CT448 was incubated at RT without any enzymatic activation. The activity was observed as the increase in absorbance at 380 nm at 27°C within the first 10 min after addition of the substrate. Samples were measured in a 96 well plate using the BioTek Synergy H4 plate reader (BioTek Instruments, Winooski, VT USA).

The proteolytic activity (µmol*min^{-1}) of the protein was calculated from the steepest parts of the activity curves. The data were analyzed with Microsoft Excel (Version 2016) and Sigmaplot (SigmaPlot Version 15 Build 15.1.1.26, Systat Software GmbH). For 45, 120 and 360 min after activation with 0.5 and 2.5 U trypsin per mg CT448 and 2 U thrombin per mg CT448 means and standard deviations were calculated, and a one-way ANOVA followed by a Tukey's HSD post hoc test ($p < 0.05$) was performed to show differences between the different treatments.

Digestion of CT448 with thrombin and enzymatic assays

Protease deficient CT448 S184A was used to test for the efficiency of a trypsin-free digestion. The serine protease thrombin was utilized for this approach. Because of the mutation within the active site, this mutant CT448 is assumed to be inactive or not to display auto-activation. Proteolysis of this protease is therewith contributed to thrombin alone. Furthermore, the respective artificial cleavage site for thrombin was implemented in such a way that it cleaves the pro-peptide once.

1.5 µg protease deficient CT448 S184A were treated with 2 U and 4 U thrombin per mg protein and incubated overnight and 24 h at 4°C. To include a positive control for thrombin, the proline-proline-endopeptidase 1 (PPEP1, provided by AG Baumann, Biochemistry, University of Cologne, Cologne, Germany) was digested along with CT448. PPEP1 is known to be cleaved by thrombin as it harbors a thrombin linked his tag. Samples for an SDS-PAGE were taken after 12 h and 24 h. The gel was stained with Coomassie by standard protocol and visualized

on a ChemiDoc. Additionally, 1.5 µg CT448 M17 were incubated with 10 U of thrombin per mg CT448 at 4°C or 37°C. Samples for SDS-PAGE were taken after 0.25 h (15 min), 0.5 h (30 min), 3.5 h and 12 h. For control, 1.5 µg PEPP 1 were incubated equal to CT448 with 10 U thrombin per mg PPEP 1 at 37°C. This activation was additionally measured with an enzyme assay as previously described: 1.5 µg of CT448 M17 were incubated 10 U thrombin per mg CT448 for 3.5 h either at 4°C or 37°C. The activity assay was performed with 150 µM N-Succinyl-Ala-Ala-Ala-p-nitroanilide in a volume of 150 µL of 0.1 M potassium-phosphate buffer pH 8.0. The optical density (OD) was measured at a wavelength of 380 nm for 12 h at RT.

Digestion of CT448 with TEV

The TEV protease (Sigma Aldrich), a histidine-tagged recombinant protease, was elaborated to remove the artificial C-terminal twin Strep-tag for further crystallization procedures. The manufactures' cleavage protocol was modified as followed: 5 µg CT448 were incubated with 1:10 1:20, 1:100 and 1:200 (w/w) TEV protease in 50 µL 0.1 M potassium-phosphate buffer pH 8.0 buffer at 4 C overnight. Additionally, the reactions were conducted with 1 mM DTT to improve digestion. Subsequent samples were prepared for standard SDS-PAGE and western blotting analysis.

AlphaFold model prediction and inhibitor docking

The protein structure was predicted using AlphaFold 3 (Abramson et al. 2024), developed by DeepMind for accurate prediction of protein structures from amino acid sequences (Jumper et al. 2021). The amino acid sequences of CT448 and CT488 E34Q were submitted to the AlphaFold 3 webserver (https://alphafoldserver.com/) which generated a highly accurate 3D structure prediction of the protein. The predicted structure included confidence scores for each residue, indicating the reliability of the predicted positions. The first confidence metric is the predicted local distance difference test (pLDDT) which rewards locally correct predicted structures on a 0 to 100 scale. pLDDT scores > 70 are considered as confident, low pLDDT scores under 50 are often disordered regions with a ribbon like appearance. We aimed for structures with an overall pLDDT of > 90. The second confidence metric is the predicted aligned error (PAE), which measures the relative position of pairs of residues. If AlphaFold is confident in those relative positions PAE is low and vice versa. The confidence metrics for CT448 are given in the supplements (Supplement 6). The predicted template modeling (pTM) score measures the accuracy of the entire structure (Zhang and Skolnick 2004; Xu and Zhang 2010). A pTM score above 0.5 means the overall predicted fold for the complex might be similar to the true structure. The predicted protein structures were analyzed, and the best was chosen according to the AlphaFold 3 quality metrics. The structure of the cyanobacterial inhibitor cyanopeptolin 954 PubChem CID (11700800) was converted from SMILES (Simplified Molecular Input Line Entry System) to a 3D structure and docked via AutoDock Vina into the

structure of the best AlphaFold 3 model. The configuration with the lowest score (kcal/ mol) out of ten docking runs was chosen. The cyanobacterial inhibitor scyptolin A (PubChem CID 12967814) was processed identically for docking. Visualization and docking via AutoDock Vina (Trott and Olson 2010; Eberhardt et al. 2021) were carried out with UCSF Chimera (Pettersen et al. 2021). Afterwards, the ligand and receptor were processed in Chimera X (Meng et al. 2023).

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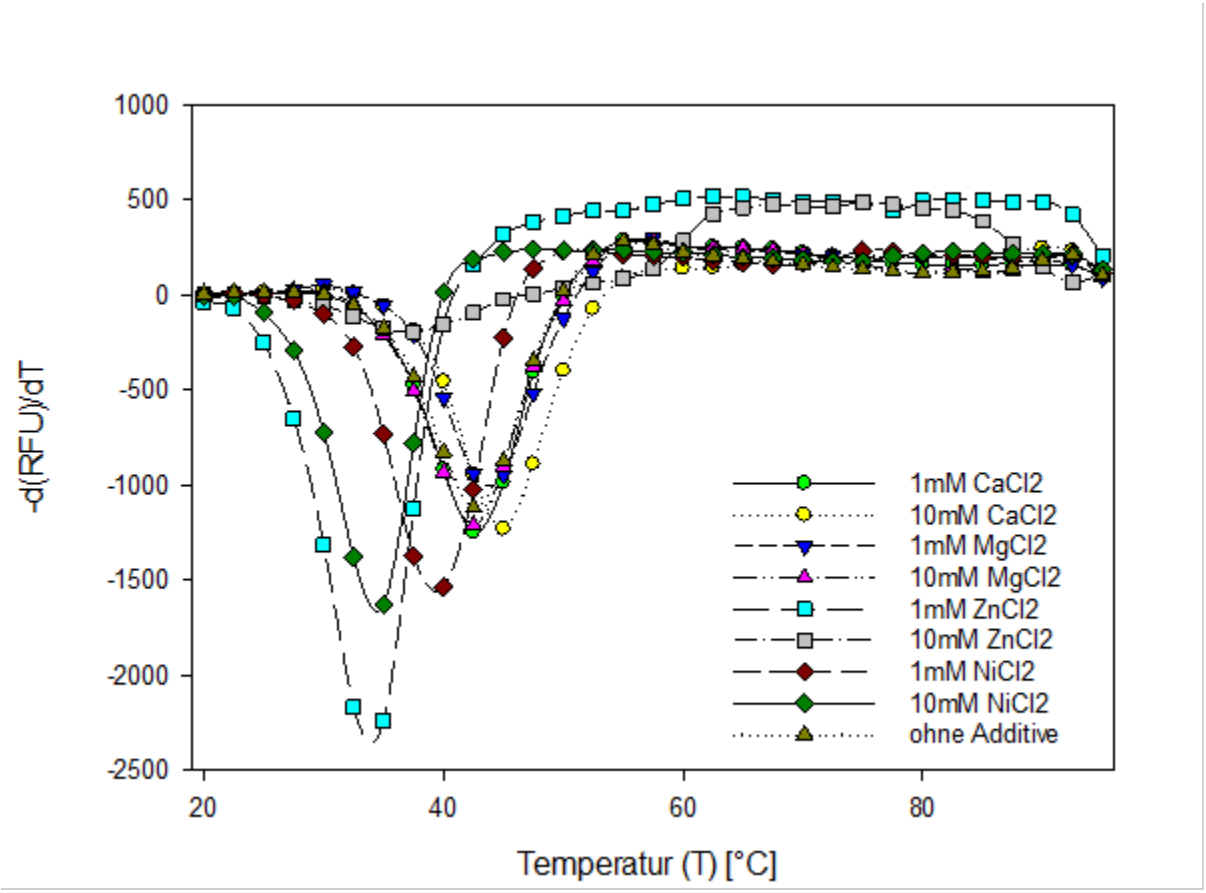
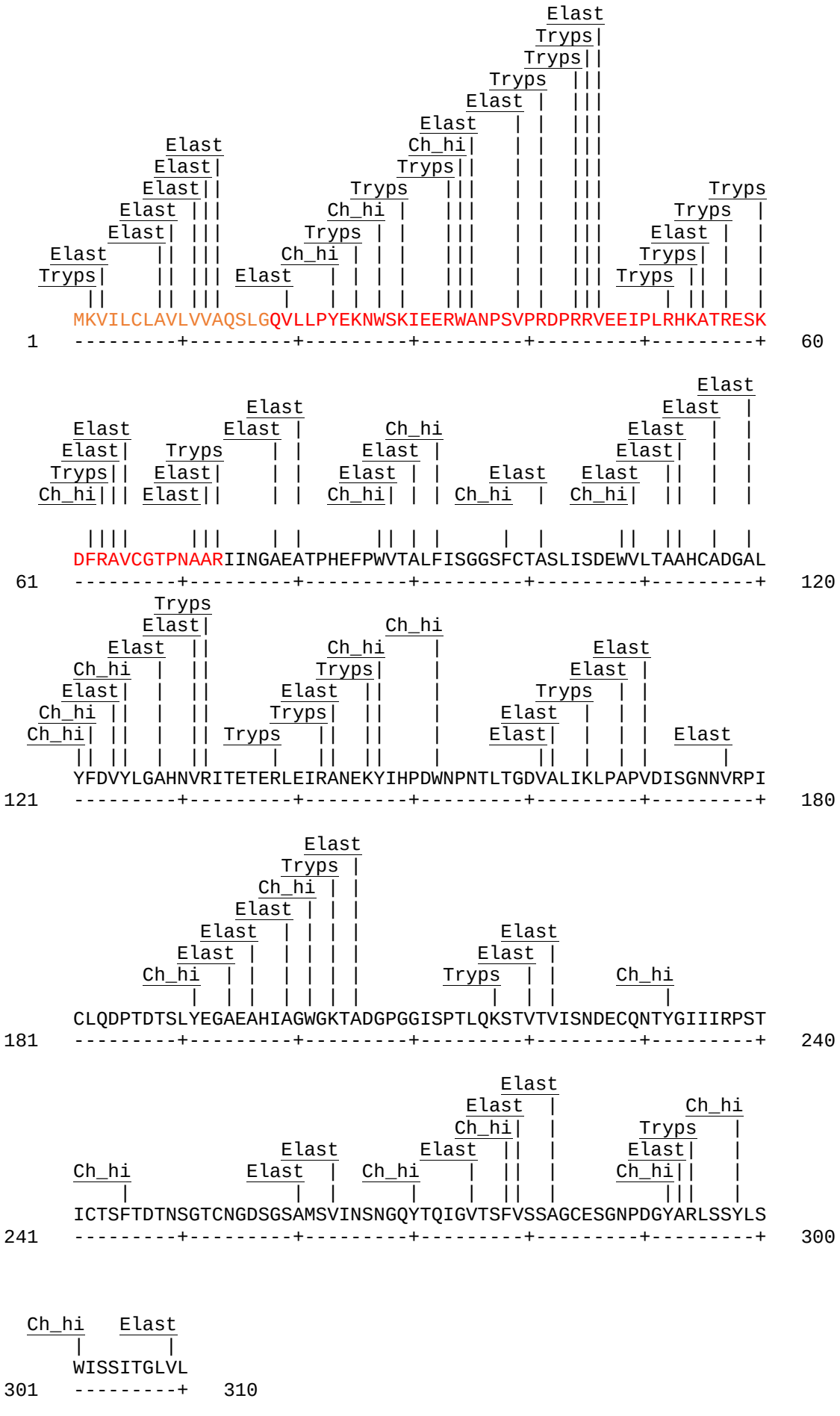
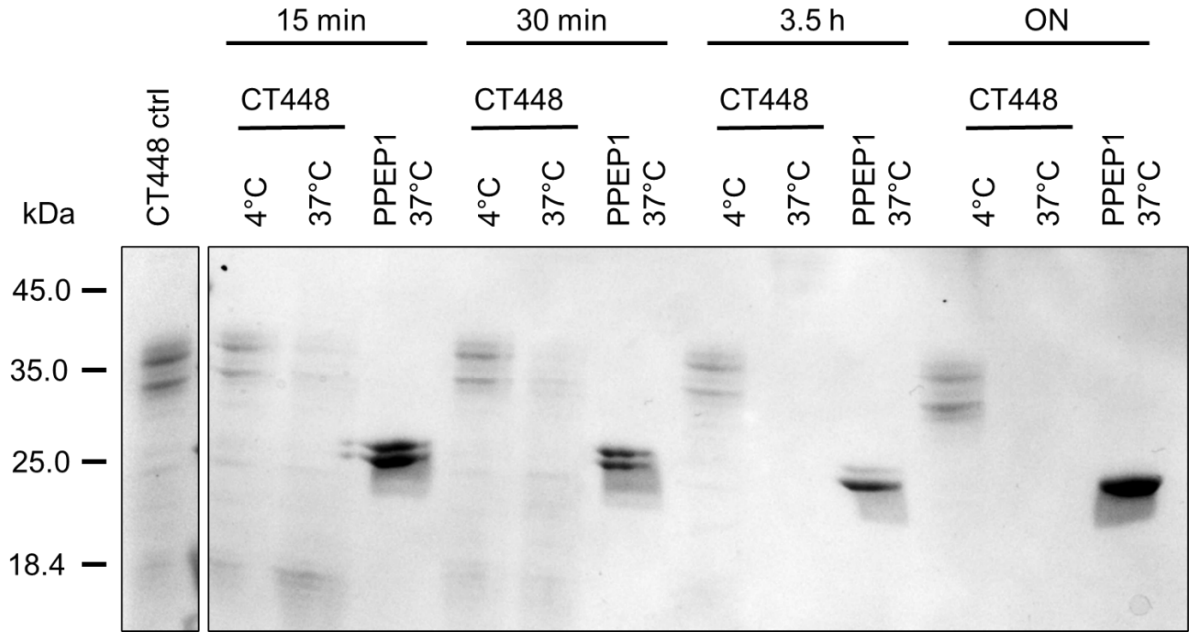
4.7 Supplements



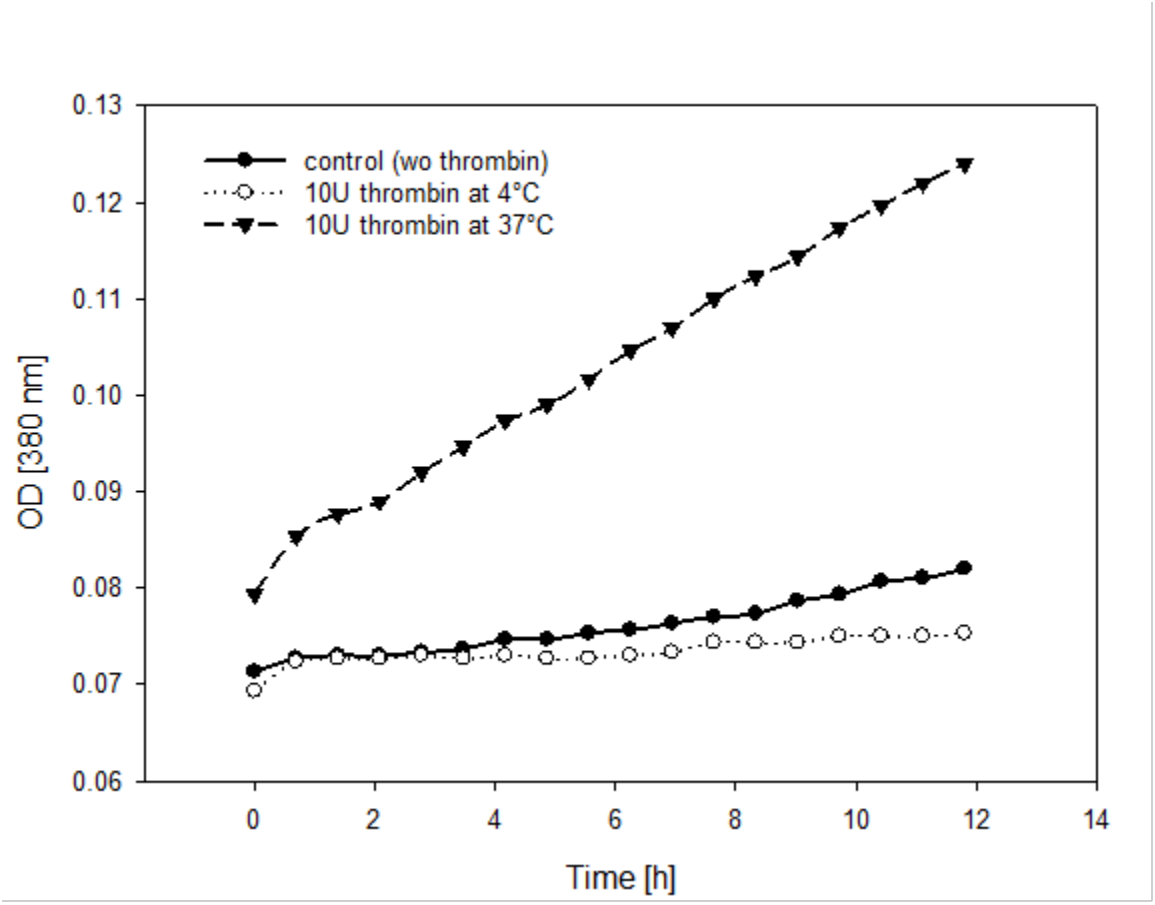
Supplement 1: Thermofluor assay profile of heterologous expressed CT448 with various divalent cations. This approach can be utilized to measure the thermostability of the folding of a protein. Tested were either calcium chloride, magnesium chloride, zinc chloride or nickel chloride in two concentrations: 1 mM or 10 mM. The control did not contain any divalent cation. Plotted is the first derivative of the fluorescence as a function of temperature (-d(RFU)/dT), where the T_m is represented as the peak of the curve. The decrease of the relative fluorescence unit (RFU) depicts the thermal denaturation of CT448 with increasing temperature. Depicted is the mean curve of the triplicates.


MKVILCLAVLVVAQSLGQVLLPYEKNWSKIEERWANPSVPRDPRRVEEIPLRHKATRESK
1 60
DFRAVCGTPNAARIINGAEATPHEFPWVTALFISGGSFCTASLISDEWVLTAAHCADGAL
61 120
YFDVYLGAHNVRITETERLEIRANEKYIHPDWNPNTLTGDVALIKLPAPVDISGNNVRPI
121 180
CLQDPTDTSLYEGAEAHIAGWGKTADGPGGISPTLQKSTVTVISNDECQNTYGIIIRPST
181 240
ICTSFTDTNSGTCNGDSGSAMSVINSNGQYTQIGVTSFVSSAGCESGNPDGYARLSSYLS
241 300
WISSITGLVL
301 310

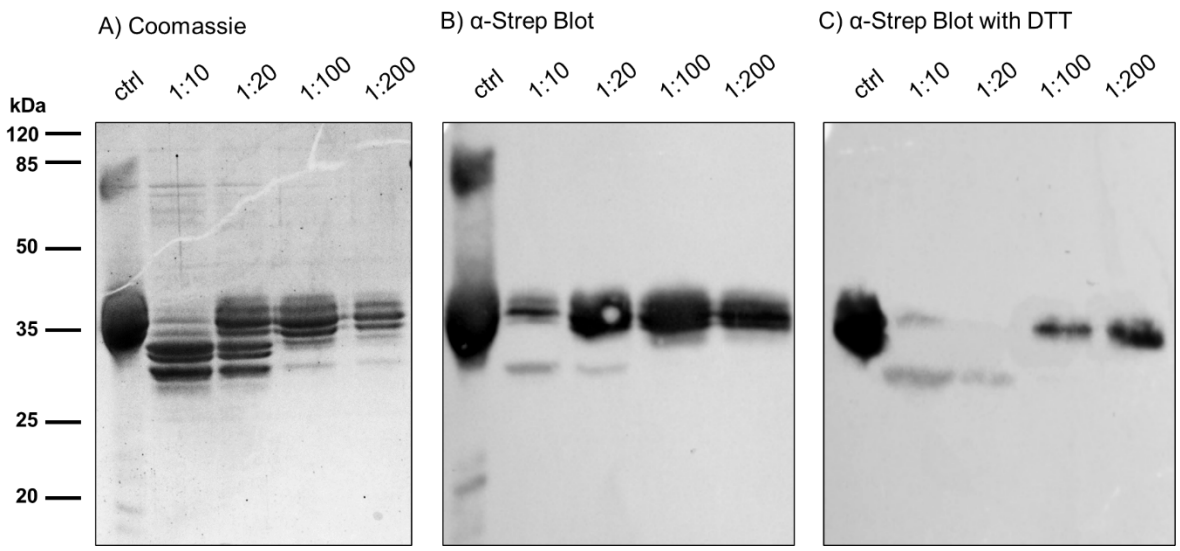
Supplement 2: Expasy PeptideCutter (SIB Swiss Institute of Bioinformatics) prediction of potential cleavage sites cleaved for CT448. Cleavage sites of chymotrypsin (CH_hi), trypsin (Tryps) and neutrophil elastase (Elast) were utilized for digestion of the CT448 construct in silico. Highlighted is the artificial signal peptide (orange), zymogen/ native pro-peptide (red) and mature sequence (black). The Expasy PeptideCutter does not predict a digestion of CT448 by thrombin and TEV protease.



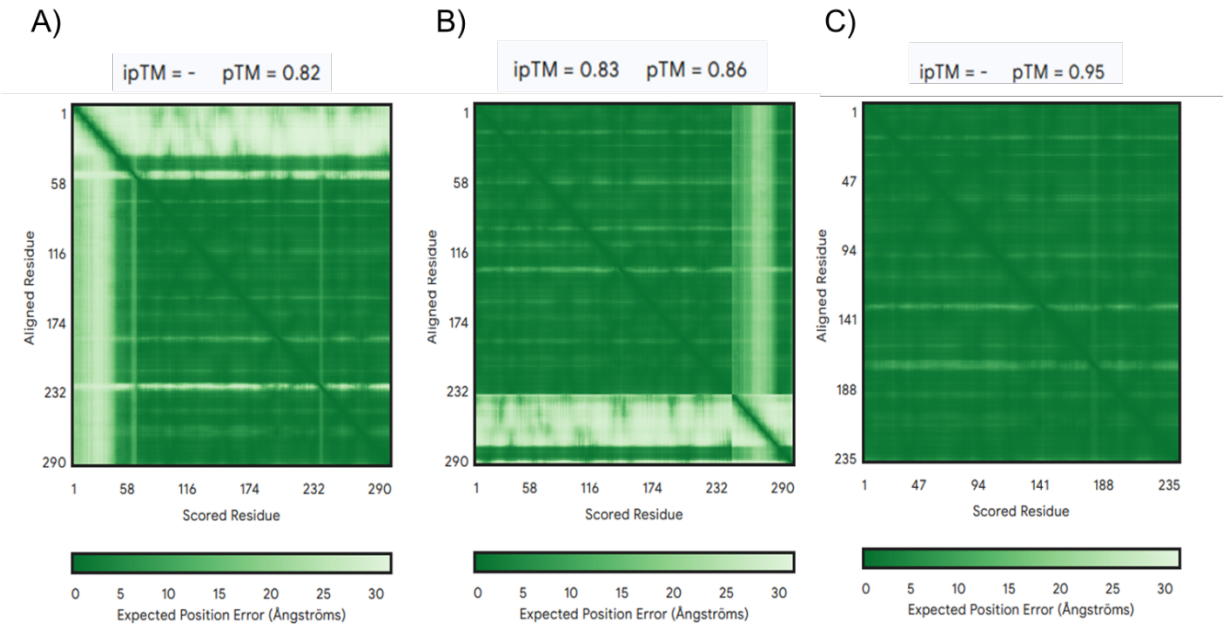
Supplement 3: SDS-PAGE of thrombin digestion of CT448 M17. A thrombin cleavage site was mutated into the pro-peptide for further directed activation. This CT448 construct was not altered regarding its proteolytic activity. Therefore, CT448 was incubated with 10 U thrombin per mg CT448 at 4°C or 37°C. After 15 min, 35 min, 3.5 h and overnight (ON) samples were taken for SDS-PAGE and an enzyme activity assay. PPEP1 was utilized as positive control for thrombin digestion.



Supplement 4: Activity assay of thrombin digested Ct448. Depicted are the curves of the 3.5 h incubation at 4°C and 37°C. The control was not incubated with thrombin. 1.5 µg CT448 were mixed with 150 µM N-Succinyl-Ala-Ala-Ala-p-nitroanilide in a volume of 150 µL of 0.1 M potassium-phosphate buffer pH 8.0. The optical density (OD) was measured at a wavelength of 380 nm for 12 h at RT. Even after 3.5 h incubation with 10 U thrombin per mg CT448 almost no activity was measurable. CT448 which has been incubated at 37°C showed a higher activity compared to 4°C incubation and the control but nevertheless comparable to trypsin activated CT448. Depicted is the mean curve of the triplicates. Thrombin does not hydrolyze the substrate N-Succinyl-Ala-Ala-Ala-p-nitroanilide.



Supplement 5: TEV protease cleavage of CT448 for C-terminal Twin-Strep-tag removal. Four different ratios of protease to target were tested against a control without TEV protease. Digestion was performed for 12 h overnight. Samples were collected for Coomassie staining (A), western blotting with antibody detection via StrepTactin-HRP conjugate (B) subsequent to SDS-PAGE and, α-Strep blotting with detection via StrepTactin-HRP conjugate with additional 1mM DTT for improved digestion (C). For B) and C) fading bands, e.g. absence of the StrepTactin-HRP conjugate, indicates for successful of the for N-terminal Strep-tag. Contrast adjusted for publication.



Supplement 6: AlphaFold3 confidence metrics for the Swedish CT448 (E34) protein structure for A) the zymogen, B) mature CT448 after trypsin cleavage at the sissle bond Arg56 with bound pro-peptide and C) mature CT448 without the pro-peptide. The predicted template modeling (pTM) score for the models exceeded 0.8. Values higher than 0.8 represent confident high-quality predictions, while values below 0.6 suggest likely a failed prediction. Shown are PAE (predicted aligned error) as an estimate of the error in the relative position. Higher values indicate higher predicted error and therefore lower confidence. The predicted template

modeling (pTM) score measures the accuracy of the entire structure. A pTM score above 0.5 means the overall predicted fold for the complex might be similar to the true structure. The interface predicted template modeling (ipTM) measures the accuracy of the predicted relative positions of the subunits within the complex. In this case only the mature protease was in complex with the pro-peptide in panel B.

Supplement 7: Results of the one-way ANOVA on the divalent cations for the melting temperatures of CT448 (Figure 2). The protein's melting temperature (T_m) is given by the inflection point where 50 % of the protein are unfolded and was measured in dependence with either calcium chloride, magnesium chloride, zinc chloride or nickel chloride in two concentrations: 1 mM or 10 mM. All treatments with divalent cations were conducted in triplicates (n = 3).

Source of Variation	DF	SS	MS	F	P
Treatment	8	504.019	63.002	151.206	<0.001
Residual	18	7.5	0.417		
Total	26	511.519			

Supplement 8: Results of the one-way ANOVA on the tryptic digestion of CT448 under different conditions and time (Figure 3). Trypsin was used for activation in 0.5 U and 2.5 U per mg CT448. As control CT448 was incubated at RT over time. The CT448 control at 4°C was additionally incubated with 2 U thrombin per mg CT448. Proteolytic activity was measured after 45, 120 and 360 min and each treatment were conducted in triplicates (n = 3). A one-way ANOVA was calculated for each incubation time.

	Source of Variation	DF	SS	MS	F	P
45 min	Treatment	5	0.0000186	0.00000372	289.611	<0.001
	Residual	12	0.000000154	0.0000000128		
	Total	17	0.0000187			
120 min	Treatment	5	0.000423	0.0000845	31.900	<0.001
	Residual	12	0.0000318	0.00000265		
	Total	17	0.000455			
360 min	Treatment	5	0.000303	0.0000606	99.167	<0.001
	Residual	12	0.00000733	0.000000611		
	Total	17	0.000310			

Concluding remarks and perspectives

Cyanobacterial mass developments have increased in freshwater ecosystems due to a combination of nutrient input, e.g. eutrophication, rising temperatures and high levels of CO_2 with predicted increases in frequency (Lampert and Sommer 1999; Downing et al. 2001; Paerl and Huisman 2008) along with a prevalence of potentially toxin-producing cyanobacteria (Trolle et al. 2015). The toxicity of cyanobacteria has been documented as harmful for humans and has been linked to the death of livestock when exposed to water with high concentrations of cyanobacteria (Cheung et al. 2013; Falconer 1996), since many cyanobacterial strains produce toxic secondary metabolites (Sivonen 1996). Furthermore, cyanobacterial blooms impact the aquatic ecosystem in terms of abiotic conditions, like light and turbidity (Paerl and Huisman 2008). Moreover, cyanobacteria are known to impact the fitness of the freshwater cladoceran *Daphnia* sp. (reviewed by Ger et al. 2016), an important linker between primary producers and higher trophic levels. During blooms cyanobacteria represent a large proportion of the phytoplankton and thus provide the primary food source for the non-selective filter-feeding *Daphnia*. They are of particularly low food quality (von Elert and Wolffrom 2001; von Elert et al. 2003; Martin-Creuzburg et al. 2008), poorly ingestible due to cyanobacterial colonies and filaments (Porter and McDonough 1984) and lead to in changes in *Daphnia* behavior and anatomy (Bednarska and Dawidowicz 2007). Therefore, the negative effects of cyanobacteria on *Daphnia* can be attributed, at least in part, to secondary metabolites they produce. The variety of those biologically active secondary metabolites is complex and diverse (Gademann and Portmann 2008; Janssen 2019) and some of them have been demonstrated to cause reduced growth and reproduction rates and increased mortality of *Daphnia* (Rohrlack et al. 2001; Lürling 2003b; Gademann and Portmann 2008). This study focuses on a specific group of cyanobacterial secondary metabolites, the protease inhibitors (PIs), as they are more frequently found in surface blooms of cyanobacteria (Agrawal et al. 2001; Janssen 2019) than the well-studied protein phosphatase inhibitor microcystin. It was demonstrated that *Daphnia* have increased their tolerance to toxic cyanobacteria through coexistence over time (Hairston et al. 1999; Isanta-Navarro et al. 2021) and space (Sarnelle 2005; Wojtal-Frankiewicz et al. 2013) which can be defined as local adaptation (Schwarzenberger et al. 2017). In a recent study, Schwarzenberger and colleagues (2020a) provided evidence that positive selection on specific loci has driven the evolutionary adaptation of a Swedish *Daphnia magna* population to cyanobacterial protease inhibitors.

The persistence of cyanobacterial mass developments, or blooms, is a major challenge for lake management (Paerl and Huisman 2008, revied by Huisman et al. 2018). Thus, the identification of *Daphnia* with enhanced tolerance to protease inhibitor-producing cyanobacteria would be of scientific interest, as these adapted *Daphnia* could potentially be

utilized to suppress and control toxic cyanobacterial blooms. A better comprehension of the molecular basis of local adaptation to PIs could provide insights into the inhibitory mechanism driving adaptation to cyanobacteria. This knowledge would allow for the active manipulation of zooplankton composition and altering the population in favor of less susceptible clones.

The serine protease CT448 was identified as a potential target of cyanobacterial protease inhibitors when the role of digestive proteases in relation to toxic cyanobacteria was investigated (Schwarzenberger et al. 2010; Agrawal et al. 2005a). Studies showed that cyanobacteria in the diet resulted in altered expression and activity of serine proteases in *Daphnia's* gut (Schwarzenberger et al. 2010). The most active digestive serine proteases in the gut of *Daphnia magna* are chymotrypsins and trypsins (von Elert et al. 2004). In a separate study, Schwarzenberger et al. (2010) were able to assign three trypsin and three chymotrypsin genes (i. e., *ct383, ct448*, and *ct802*) to the proteases active in *D. magna*. CT448 is as well annotated as Chymotrypsin BI (UniProtKB entry: A0A0P5W5Q6_9CRUS) by prediction. While chymotrypsin mainly cleaves behind large hydrophobic residues, elastases which are also chymotrypsin-like have a specificity for small hydrophobic amino acid residues (reviewed by Hedstrom 2002). Chymotrypsin belongs to the S1 family of serine proteases that are of major importance for the digestion of proteins in humans and *D. magna* (von Elert et al. 2004). Human elastases occur mainly in the pancreas and the phagocytes and play a pathologic role in many human disease and inflammation responses (reviewed by Bieth 2001). The proteolytic role of elastases in the invertebrate *Daphnia* has not been investigated and extensive data is lacking. The considerable taxonomic distance between mammals and invertebrates makes it difficult to correlate functional similarities between the two groups. Although, Agrawal et al. (2005b) could detect elastase activity in body homogenates of the zooplankter *Moina macrocopa*, no physiological role in response to cyanobacteria could be identified. Studies have linked the genetic variation of CT448 to tolerance to PIs (Schwarzenberger et al. 2017; Schwarzenberger et al. 2020a) and the inhibition of chymotrypsin activity (Schwarzenberger et al. 2013b; Schwarzenberger et al. 2017), leading to the assumption that higher *ct448* expression results in higher chymotrypsin activity (Schwarzenberger and Fink 2018).

However, the molecular mechanism of the increased tolerance to PIs remained unclear. So far, proteases of *D. magna* could not be analyzed, so that no specific characteristics of individual digestive enzymes could be accomplished. Therefore, an expression system of the insect cell line *Sf*21 was utilized to obtain high amounts of CT448 of *D. magna*. The results in chapter 1 showed that the single protease CT448 was successfully heterologously expressed and purified for subsequent analyses. Despite the tryptic activation of the inactive precursor (zymogen), no hydrolysis of a chymotrypsin-specific substrate by the CT448 was observed. Proteomic identification of protease cleavage sites (PICS) and hydrolysis of various synthetic substrates showed that CT448 is a chymotrypsin-like elastase with a substrate specificity for

alanine and valine. Correlations between *CT448* gene expression and chymotrypsin activity cannot be explained by the same substrate specificity of CT448 and chymotrypsin, as the CT448 hydrolyzes different substrates than chymotrypsin (Schwarzenberger and Fink 2018; Schwarzenberger et al. 2017).

Although digestive chymotrypsins present in the gut of *Daphnia* cannot be attributed to *ct448* expression, it is evident that this gene, and the respective encoded protease CT448, plays a role in the response to cyanobacteria: The CT448 gene harbors fewer copies in more tolerant *D. magna* populations (Schwarzenberger et al. 2017) and its expression is upregulated in response to cyanobacteria (Schwarzenberger et al. 2012). Additionally, *D. galeata* responded with upregulated *ct448* expression in the presence of a PI-producing cyanobacterium in its diet (Drugă et al. 2016). In *D. pulex* a significant overexpression of *ct448* has been demonstrated as well when fed with the cyanobacterium *Synechococcus elongatus* (Schwarzenberger and Fink 2018). In the more tolerant Swedish *D. magna* population, a *ct448* allele indicated positive selection as a consequence of a recent selective sweep (Schwarzenberger et al. 2020a).

The characterization of CT448 and the understanding of its role is essential to elucidate the underlying mechanisms of local adaptation as increasing frequencies, duration and intensities of cyanobacterial blooms should select for more tolerant genotypes (Ger et al. 2014). As digestive serine proteases have been identified as targets of cyanobacterial protease inhibitors in gut homogenates (Agrawal et al. 2005a), in chapter 1 it was confirmed that CT448 is inhibited by the protease inhibitors nostopeptin BN920 and cyanopeptolin 954 (CP954) from *Microcystis aeruginosa* NIVA Cya 43. However, it is assumed that the Swedish *D. magna* population investigated in this dissertation is adapted to the PIs produced by *Microcystis* sp. BM25, as it coexisted with this strain (Schwarzenberger et al. 2013a) rather than *Microcystis aeruginosa* NIVA Cya 43. Three PIs were identified in the crude cell extracts of *Microcystis sp.* BM25 (Schwarzenberger et al. 2013b), however, identification of these PIs as targets for CT448 and purification of single compounds have yet to be achieved.

Elastase activity assays were conducted in chapter 3 to develop a bioassay guided fractionation of PIs from *Microcystis* sp. strain BM25. Inhibitory effects of the cell extracts on the elastase activity could not be attributed to inhibitory compounds. The results demonstrated a significant inhibitory effect of methanol on elastase activity. Methanol is a common solvent utilized in bioassay guided fractionation with solid phase extraction (SPE) when PIs are to be extracted and identified (von Elert et al. 2005; Schwarzenberger et al. 2013b; Burberg et al. 2019; Burberg et al. 2020). Hence, the methodology was adjusted by the utilization of DMSO as solvent for the SPE-fractions instead of methanol. This highlights the necessity for the modification and improvement of elastase activity assays with the aim of identifying potential protease inhibitors against elastases, such as CT448, given that chymotrypsin and trypsin did

not demonstrate methanolic inhibition. Consequently, two SPE fractions of *Microcystis* sp. strain BM25 extract, which were obtained after elution with 20 % and 60 % methanol, were demonstrated to inhibit the homogenate's elastase activity to an equal extent in the Swedish and Polish populations. Subsequently, these two fractions can be fractionated further by HPLC, thus allowing subsequent elastase activity assays to be conducted in order to identify the inhibitory compounds of interest. One plausible candidate might be the inhibitor DR1006 (Adiv et al. 2010), which was obtained from a different *Microcystis* strain, as it has a leucine at the fifth position of the C-terminus, which is an indicator of a chymotrypsin-related protease inhibitor (Grach-Pogrebinsky et al. 2003). Accordingly, elastase was inhibited by the micropeptin DR1006 with a half-maximal inhibitory concentration (IC_{50}) of 13.0 mM, whereas no inhibition was observed with the micropeptin DR1056 (Adiv et al. 2010), which is also identified in BM25 (Schwarzenberger et al. 2013b). The well-studied elastase inhibitor scyptolin A from the cyanobacterium *Scytonema hofmanni* also has a leucine in the fifth position from the C-terminus and has been co-crystalized with porcine elastase (Matern et al. 2001; Matern et al. 2003). The investigation of the micropeptin DR1006 is further suggested, as it features elastase inhibitor properties, but it is likely that new protease inhibitors can be identified in *Microcystis* sp. BM25. Hence, here an adapted method has been provided to identify and purify protease inhibitors against elastases of *D. magna*. Following the identification of elastase inhibitors, larger quantities of the purified compound can be employed in co-crystallization with CT448 to elucidate the binding modes of the protein-ligand complex through the investigation of the crystal structures. Therefore, in Chapter 4, the optimization of the maturation of CT448 was investigated to improve the crystallization conditions for this protease and enzyme assays for comparison of the homologues in the Polish and Swedish Daphnia populations, which vary in susceptibility to cyanobacteria. Previous attempts to crystallize CT448 were unsuccessful (Lange, data not published). The observed strong degradation of CT448 by trypsin is suspected to hinder further activity. Besides the scissile bond Arg56-Ile57, there are 14 putative cleavage sites for tryptic digestion. This enables more targeted activation, which is necessary to compare the two CT448 variants, Swedish and Polish. A more specific maturation could be achieved with a cleavage site for TEV (tobacco etch virus) protease, as one digestion step could result in only two cleavages: between the scissile bond at position 56 and 57 and the cleavage of the affinity tag for further crystallization.

The latest AI driven protein modelling tool, AlphaFold (Abramson et al. 2024), was utilized to predict the three-dimensional structure of CT448. Although the protein model is not based on a crystal structure, certain features of CT448 could be analyzed. Of particular interest is the pro-peptide of CT448, which wraps around the core protein in both the pre- and post-cleavage states according to modeling by AlphaFold. In comparison to other elastases (see Chapter 1), the pro-peptide is relatively elongated and linked to the mature protease via a disulfide bond

on the protease's surface. In particular, the model predicts that the N-terminus of the pro-peptide covers the active center of CT448, possibly preventing protease activity in the post-cleavage state. This modulatory activity of pro-peptides is well known (reviewed by Demidyuk et al. 2010; Pei et al. 2024).

The potential involvement of the pro-peptide in the protease's functionality may be further supported by the documented population-specific mutation at position 34, as reported by Schwarzenberger et al. (2020a): An amino acids substitution was observed between the Swedish (Glu/E) and Polish (Gln/Q) *D. magna* populations. However, it should be noted that this substitution did not occur near the active center, as had been postulated (Schwarzenberger et al. 2020a). The three-dimensional structure of CT448 modeled by AlphaFold predicts the position of residue 34 on the surface of the core protein in close proximity to Lys43 of the pro-peptide. The positive charge of Glu34, which can be found in the Swedish *D. magna* population, facilitates the formation of a salt bridge with Lys43, which is attaching the pro-peptide to the surface of the core protein. The CT448 homologue found in the Polish D. magna population does not allow for this salt bridge, as the Glu is exchanged to a Gln at position 34. Consequently, it can be hypothesized from the *in silico* 3D modeling that the binding of the pro-peptide to the mature CT448 is stronger when residue 34 is Glu, as opposed to Gln. A whole-genome expression microarray of *D. pulex* was implemented by Asselman et al. (2012) to identify pathways in response to *Microcystis* stress. The study revealed differential gene expressions, indicating that *Microcystis* influences protein folding and may contribute to the accumulation of misfolded proteins (Asselman et al. 2012). Given that numerous pro-peptides assist in the correct folding of immature proteins (Baker et al. 1993; Yabuta et al. 2001; Buczek et al. 2004), it is plausible that the function of the pro-peptide of CT448 is to facilitate proper folding and stability and might prevent misfolding that might result from cyanobacterial stress. As discussed above, the expression of CT448 increases when cyanobacteria are present in the diet of *Daphnia*. This may suggest a molecular mechanism of enhanced tolerance to PIs, as overexpression of a digestive protease that is also functionally expressed under bloom conditions and possible accompanying misfolding of proteases may maintain digestion in *Daphnia*. Investigation of this hypothesis demands for information about the protease's structure which is more methodologically sound than in silico prediction like that gained from protein crystallization.

From the perspective of lake management, the identification of *Daphnia* with an increased tolerance to cyanobacteria that produce inhibitors would be of significant interest. These *Daphnia* could potentially be employed as a biological tool for the suppression and regulation of cyanobacterial blooms containing protease inhibitor producing species. A microcosm experiment was set up to investigate the effect of a locally adapted population in absence and the presence of a Microcystis sp. BM25 over a period of 51 days. Chapter 3 demonstrated the

superiority of the adapted Swedish *D. magna* population over the Polish population in the presence of Microcystis sp. BM25 under prebloom conditions. These findings indicate that the Swedish population has undergone a process of local adaptation in response to a concurrent cyanobacteria species, as suggested in previous studies (Schwarzenberger et al. 2017; Schwarzenberger et al. 2020a; Schwarzenberger et al. 2021). Under conditions where no cyanobacterial stress was applied, this fitness advantage was not continuously present. In absence of cyanobacteria the shares of *Daphnia magna* originating from the Polish and the Swedish population did not differ significantly at the end of the experiment. The shift of clone frequency is as well observed in nature where more resistant clones to cyanobacteria typically become more prevalent towards the end of the warm season, when cyanobacteria are most prevalent, and vice versa (Schaffner et al. 2019). Interestingly, a recent study suggests that opposing selection and maintaining resistance is costly for *Daphnia* as reduction in nutrient input, accompanied by a reduction of cyanobacteria, resulted in the reappearance of highly susceptible genotypes and subsequent loss of grazer resistance (Isanta-Navarro et al. 2021). These findings indicate that tolerant clones, such as the Swedish population, do not constantly dominate the community, instead their dominance is determined by the phytoplankton composition as tolerance cyanobacteria might be costly.

As demonstrated in Chapter 3, the Swedish *Daphnia* population, characterized as genetically adapted to local conditions, has also been observed to dominate over a mixed population while *Microcystis* is present in their diet. Moreover, tolerant *Daphnia* clones were observed to suppress the growth of cyanobacteria in nutrient-enriched mesocosms, whereas those sensitive to cyanobacteria did not (Chislock et al. 2013). This finding is corroborated by the work of Sarnelle (2007), who described the ability of *D. pulicaria* to suppress an already developed cyanobacterial bloom. From the perspective of water management an increased biomass of *Daphnia* is important for the effective suppression of cyanobacterial blooms (Wright and Shapiro 1984; Leibold 1989). Nevertheless, the possibility of reducing algal blooms in eutrophic lakes through herbivory seems to be limited, particularly when cyanobacteria have reached high biomass and large cladocerans have been depleted (Ghadouani et al. 2004). Consequently, research on co-evolutionary dynamics that enhance zooplankton tolerance should concentrate on the identification of individual factors to shift the limitation of more tolerant clones, with the aim of reducing cyanobacterial blooms.

This dissertation characterizes a serine protease from *D. magna* and investigates its properties. I investigated yet unknown specificity and properties of the chymotrypsin-like elastase CT448. Therefore, I utilized sophisticated biochemical techniques, including the proteomic identification of protease cleavage sites (PICS), and established a methodology for the heterologous expression of the *D. magna* serine protease CT448 in *Sf*21 insect cell line. In consideration of yet unknown properties exhibited by CT448, I adapted the methodology for

enzymatic elastase assays for the bioassay-based fractionation of cyanobacterial inhibitors, involving solid-phase extraction (SPE). To analyze structural characteristics of CT448 without a crystal structure I utilized the machine learning tool AlphaFold to predict the three-dimensional structure. Hence, I could visualize the population specific amino acid exchanges at position 34, with glutamic acid in a population tolerant to toxic cyanobacteria and with glutamine in a susceptible population. With this data on CT448, the correlation between chymotrypsin activity within the gut of *Daphnia magna* and the gene expression of *ct448* is invalid which emphasizes the necessity for future studies on the role of *ct448* and elastases in tolerance and response to toxic cyanobacteria. Further, the functional maturation of CT448 is needed with respect to its role in comparative analyses with different variants. This study demonstrates the need to obtain a crystal structure of CT448 and to co-crystallize it with a suitable PI, along with further bioassay approaches. In the context of a rising prevalence of toxic cyanobacteria due to global warming and human activity, an accurate understanding of the principles enabling *Daphnia* to develop tolerance to cyanobacterial toxins is advantageous for effective strategies for the management of cyanobacterial blooms.

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Eidesstattliche Erklärung (gemäß §7 Absatz 8)

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht.

Köln, den 07.10.2024 Jacqueline Lange

Teilpublikationen

Lange, J., Demir, F., Huesgen, P. F., Baumann, U., von Elert, E., & Pichlo, C. (2018). Heterologous expression and characterization of a novel serine protease from *Daphnia magna:* A possible role in susceptibility to toxic cyanobacteria. Aquatic toxicology, 205, 140-147.[1]

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[1]entspricht Chapter 1

[2]entspricht Chapter 2

Danksagung

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