

Strong differences in the efficiency of digestive protease inhibitors of the cyanobacterium *Planktothrix rubescens*

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Introduction

The uptake of amino acids is essential for maintenance and growth of crustaceans and any other grazer. Amino acids serve as carbon (C) and nitrogen (N) sources, and some are essential because they cannot be synthesised by animals. The majority of amino acids in food particles is bound in proteins that have to be cleaved by hydrolytic reactions to yield amino acids or small peptides. Only these smaller units can be taken up easily by the epithelium of the gut. Proteases liberated into the lumen of the gut are responsible for the cleavage reactions. Trypsin- and chymotrypsin-like serine proteases recently have been shown to be of particular importance in the digestive processes of *Daphnia* (VON ELERT et al. 2004).

Cyanobacteria, in particular (PORTER 1973), are regarded as poor quality food due to their size- and form-related constraints on ingestion as well as their potential toxicity (DEMOTT et al. 1991, BLOM et al. 2003) and nutritional inadequacy, as a result of the lack of polyunsaturated fatty acids (AHLGREN et al. 1990) and sterols (VON ELERT et al. 2002). Diatoms and flagellates are generally considered as good quality food because of their high eicosapentaenoic acid (EPA) and corresponding high phosphorus (P) content in contrast to the cyanobacteria, which are low quality food organisms, having both low EPA and P content (GULATI & DEMOTT 1997).

Another reason for the low food quality of cyanobacteria may be their production of serine protease inhibitors. Since their first description (OKINO et al. 1993), numerous other cyclic peptides and depsipeptides, which are efficient inhibitors of either trypsin or chymotrypsin, have been found in planktonic cyanobacteria. It can be hypothesised that ingested cyanobacterial filaments are partially digested in the gut of the grazers, releasing the intracellular protease inhibitors that prevent the cleavage of proteins. As a result, the grazers become deficient in essential and nonessential amino acids.

In this study, protease inhibitors (PI) isolated from two strains of *Planktothrix rubescens* were tested against digestive trypsin-like proteases (TLP) isolat-

ed from crustaceans, collected from four Swiss lakes, that showed differences in the presence of *Planktothrix rubescens*. The goal was to test whether the crustaceans of the different lakes would adapt their digestive proteases to the *Planktothrix rubescens*-derived inhibitors.

Lakes Greifensee and Pfäffikersee are almost free of cyanobacterial populations while Lake Zürichsee exhibits a summer and autumnal population of *P. rubescens* that can be stratified in the metalimnion for months. Lake Hallwilersee is a eutrophic, artificially oxygenated lake that develops extremely dense populations of *P. rubescens* throughout the year and exhibits extensive surface scums of this cyanobacterium in early summer.

Key words: crustaceans, cyanobacteria, defence, digestion, digestive proteases, food quality, *Planktothrix rubescens*, protease inhibitors

Materials and methods

Culture of P. rubescens and extraction of PI
Planktothrix rubescens strain H9 was monoxenic and isolated from Hallwilersee; the strain A7 was axenic and isolated from Zürichsee. Both strains were grown for about 4 months in 300-ml Erlenmeyer flasks at 20 °C and 7 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light from fluorescent tubes. Chlorophyll *a* was determined in 100% methanolic extracts using the equation given by OGAWA & VERNON (1971).

The PIs from 4.1 g wet biomass of A7 and 0.58 g of H9 were extracted with 60% methanol for 2 h. To facilitate centrifugation of the cells, the culture suspension was pressurised at 20 bar in a steel vessel to collapse the gas vesicles in the filaments. The methanolic extracts were separated by centrifugation with a Sorvall® RC 5C Plus centrifuge at 10 000 \times g for 10 min. The supernatants that contained the PIs were brought to dryness in a rotary evaporator. For the inhibition experiments, the residues were taken up in 60% methanol to achieve a final concentration of 0.58 μmol chlorophyll *a* equivalents.

Extraction of crustacean digestive TLP

The crustaceans were collected with a 250- μ m plankton net from four lakes situated in the northern part of Switzerland. The crustaceans from each lake were filtered through a 400- μ m screen and washed with artificial lake water to eliminate other organisms. The crustaceans were weighed and homogenised in 50 mM Tris-HCl buffer pH 7.6 (RESHEF & CARMELI 2001). The suspension was centrifuged at 7200 x g for 10 min in an Eppendorf® centrifuge and the supernatants containing the TLPs were stored frozen at -23 °C. To obtain the same activities of the extracted proteases of the crustaceans from the four lakes, the extracts had to be concentrated (in a N₂ stream) or diluted. A calibration curve of trypsin from hog pancreas (Fluka No. 93614; 16200 U/mg) was used as a reference to calculate the trypsin equivalents.

Enzyme inhibition assay

The enzyme assays were performed in microtiter plates on an absorption reader (spectra MAX 190, Molecular Devices Corp., Sunnyvale, CA 94089, USA). Samples of 120 μ l of 50 mM Tris-HCl buffer, 30 μ l of the 60% methanolic *P. rubescens* extracts and 10 μ l of the crustacean extracts were added to each well of the microtiter plate and preincubated at 25 °C for 5 min. *N*-benzoyl-L-arginine-*p*-nitroanilide was dissolved in 5% (w/v) dimethyl sulfoxide and used as substrate. To start the reaction, 40 μ l of the 2 mM substrate solution in Tris-HCl buffer was added. The absorbance change of the wells was measured at 405 nm and 25 °C for 30 min. The percentage inhibition was calculated using the endpoint method.

Nine and 11 different extract concentrations of the strains A7 and H9, respectively, were prepared from the extracts representing a range of 2.9–5800 pmol chlorophyll *a* equivalents per well (30 μ l). The 60% methanolic extracts of both *P. rubescens* strains tested against the digestive proteases extracted from the crustaceans of each lake resulted in logarithmic inhibition curves. The inhibition curves and the 50% inhibition values (IC₅₀) were calculated using the SOFTmax®PRO Formula Guide, Version 4.0 of the absorption reader software.

Results

Preliminary experiments have shown that PIs can be nearly quantitatively extracted in a single step from *P. rubescens* with 60% aq. methanol. We used the extracts from two strains of *P. rubescens*, H9 and A7, isolated from lakes Hallwilersee and Zürichsee, respectively, to test inhibitory activity on digestive TLP of crustaceans from different lakes (Fig. 1). The extracts of the two strains contained PIs that differed in their composition and concentration. For each lake, the concentration inhibition curves of the TLP of the crustaceans with the inhibitory extracts from *P. rubescens* strains H9 and A7 were established (Table 1). These curves were used to calculate the IC₅₀ values (concentration of an extract, given as chlorophyll *a* equivalents that caused 50% inhibition

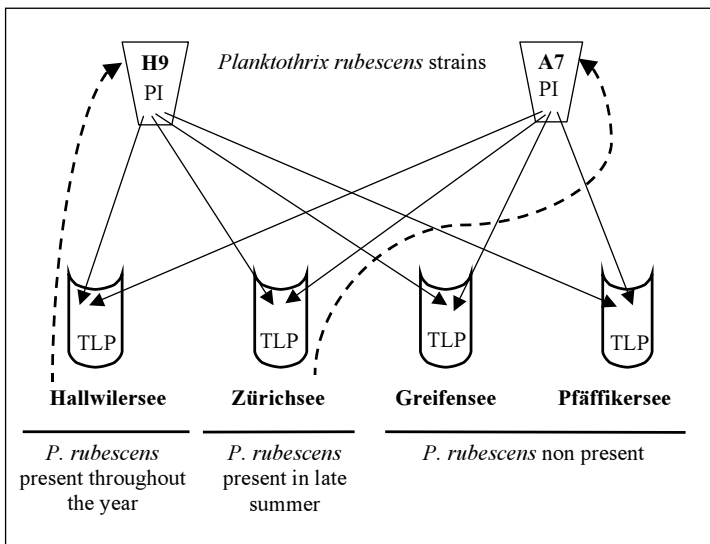


Fig. 1. Design of the test program: The PI extracts of two *P. rubescens* strains H9 and A7 isolated from Hallwilersee and Zürichsee, respectively, where tested against the TLP from the crustaceans of four Swiss lakes which differed in the occurrence of *P. rubescens*.

Table 1. Activity of digestive TLP extracted from crustaceans of different lakes. The activities are given as hog trypsin equivalent units per g wet biomass of crustaceans.

Lake	Sampling date	TLP activity (U/g)	Occurrence of <i>Planktothrix rubescens</i>
Zürichsee	2 Dec. 2003	370	+
Hallwilersee	26 Nov. 2003	95	+++
Greifensee	13 Nov. 2003	160	-
Pfäffikersee	13 Nov. 2003	55	-

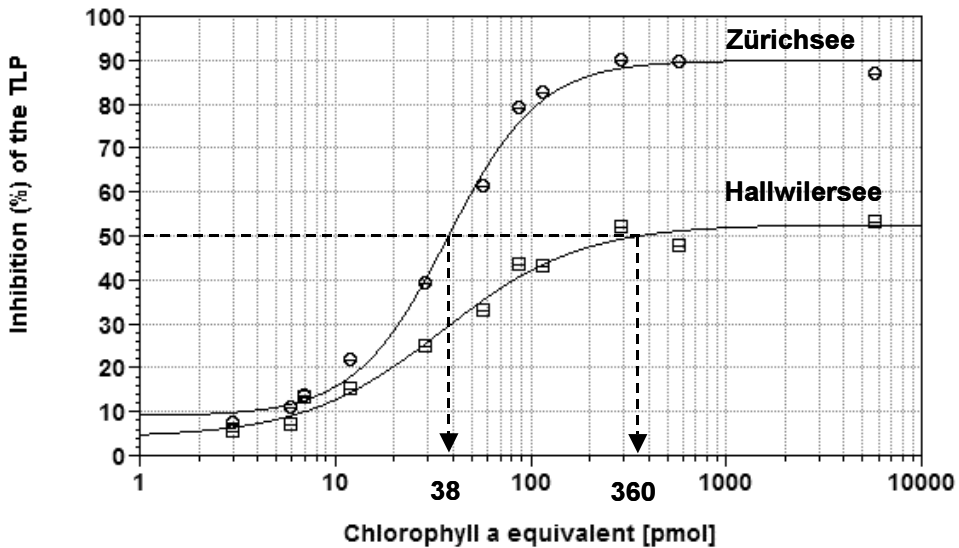


Fig. 2. Concentration inhibition curves of protease inhibitors isolated from *P. rubescens* H9. The proteases were extracted from crustaceans of Zürichsee and Hallwilersee. Values are the mean of three determinations with the \pm SD inside each symbol. The inhibitor concentrations are given as chlorophyll *a* equivalents (pmol/30 μ l).

of the TLP). The IC₅₀ values showed strong differences (Fig. 2). The maximum inhibition of TLP isolated from crustaceans of the *Planktothrix*-rich Hallwilersee was 53%, while a 90% inhibition could be achieved from the TLP from crustaceans of Zürichsee. The IC₅₀ of the inhibitors shifted from 360 pmol chlorophyll *a* equivalents to 38 pmol. Approximately 10 times higher concentrations of inhibitors were required to produce a 50% inhibition of the TLP from crustaceans of Hallwilersee. The IC₅₀ values of the TLP from crustaceans of Greifensee and Pfäffikersee showed the same sensitivity to the inhibitors as the TLP from crustaceans of Zürichsee (Fig. 3).

Discussion

Protease inhibitors are efficient defence molecules and affect the supply of amino acids to grazers. Because only a small percentage of the amino acids in the phytoplankton cells is present in free form, with the majority bound in proteins and other macromolecules, the assimilation of macromolecules is essential for adequate nutrition of grazers. The amino acids of proteins, however, can only be taken up in the gut when a hydrolytic cleavage mediated by digestive proteases takes place. When cyanobacteria are ingested and the integrity of the cells is lost in the gut, the intracellular PIs are released into the lumen of the gut and inhibit the

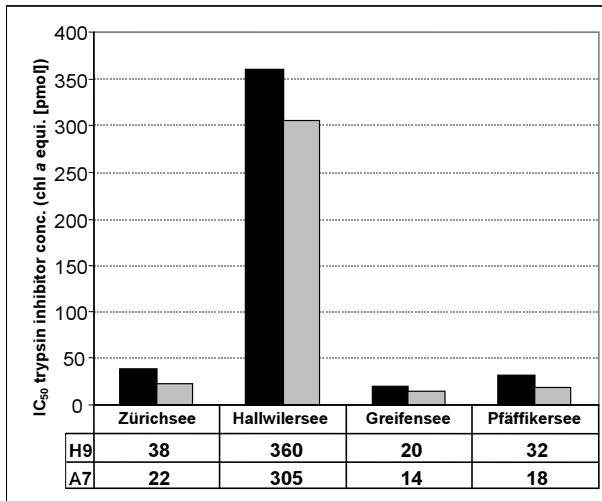


Fig. 3. IC₅₀ values of protease inhibitors from *Planktothrix* strains H9 (■) and A7 (■) for digestive TLP extracted from crustaceans of different lakes. The concentrations of the trypsin inhibitors are given as chlorophyll *a* equivalents (pmol/30 μl) representing the cyanobacterial cell mass that was extracted.

digestive proteases. This would also affect the digestion of other food particles that do not contain PIs. However, the amount and activity of the PIs has to be high enough to stop the cleavage reactions and to induce malnutrition.

Planktonic cyanobacteria frequently produce specific PIs against trypsin- and chymotrypsin-like enzymes, while other protease specificities are less common. Trypsin and chymotrypsin are the most important digestive proteases in grazers (VON ELERT et al. 2004). *Planktothrix* contains several cyclic peptides and depsipeptides (GRACH-POGREBINSKY et al. 2003). Some of them are efficient inhibitors of serine proteases and are inhibitory to either mammalian trypsin or chymotrypsin. The trypsin inhibitors anabaenopeptin A and B (HARADA et al. 1995), oscillapeptin J (BLOM et al. 2003) and the chymotrypsin inhibitor oscillamide Y (FUJII et al. 2000) were found in both extracts of *P. rubescens* (unpubl. results).

Experiments performed with proteases extracted from crustaceans from different lakes indicate that PIs from *Planktothrix* are active against the major digestive enzymes of these grazers. Recent studies (VON ELERT et al. 2004) have shown that 75–83 % of the trypsin- and chymotrypsin-like proteases extracted from *Daphnia magna* consist of digestive proteases of the gut.

Large differences were observed in the sensitivity of digestive proteases extracted from the crustaceans of the four lakes. This was particularly evident for the digestive TLP of crustaceans from Hallwilersee. To obtain a 50% inhibition of the digestive TLP of crustaceans isolated from this lake, the concentration of inhibitors had to be increased by an order of magnitude as compared to proteases of crustaceans from Zürichsee, Greifensee and Pfäffikersee. In Hallwilersee there are extended high abundances of *P. rubescens*, while in Zürichsee a seasonality of *P. rubescens* is observed. In Greifensee and Pfäffikersee, *P. rubescens* is not found. The data strongly indicate that the grazer populations adapt to the presence of PIs in their food organisms and may favour those crustaceans that express digestive proteases less sensitive to the PIs of *P. rubescens*. This conclusion leads to further questions about the adaptation of crustacean populations and/or their acclimation processes.

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