Fibrocapsa japonica (Raphidophyceae) shows high extracellular haemolytic activity at low cell densities

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Abstract

Haemolysins are thought to be involved in the ichthyotoxicity of the raphidophyte *Fibrocapsa japonica*. So far, all haemolytic data were based on the analysis of intracellular haemolysins in concentrated extracts of cells at the end of the exponential or in the stationary growth phase. To gain more insight in the mechanisms of haemolysin production we studied differences in intracellular and extracellular haemolysin activity at various cell densities during exponential growth. The haemolytic activity of *F. japonica* changed significantly as a function of cell concentration. At increasing cell concentrations, extracellular haemolytic activity decreased while intracellular activity increased. Possibly, the very high extracellular haemolytic activity at low cell concentrations could be caused by growth inhibiting or allelopathic haemolysins.

Introduction

The harmful algal bloom species *Fibrocapsa japonica* Toriumi and Takano (Raphidophyceae) is potentially ichthyotoxic. The fish kills caused by *F. japonica* are ascribed to toxicity and/or mechanical damage to the fish (Khan *et al.* 1996; de Boer *et al.* 2005; Pezzolesi *et al.* 2010). Haemolysins are one of the proposed toxins that are involved in the ichthyotoxicity of *F. japonica*. Bioassay guided characterization of haemolytic fractions from the *F. japonica* Wadden Sea strain (CCRuG Cl 3) yielded the poly unsaturated fatty acids (PUFAs), C18:4n3 (OTA), 20:5n3 (EPA), and C20:4n6 (AA) (Fu *et al.* 2004). Next to these haemolytic PUFAs, potent light dependent haemolytic compounds were found in the intracellular fraction (Boer et al. 2009). A synergistic effect of the reactive oxygen species (ROS) superoxide and EPA on the ichthyotoxicity of *C. marina* was demonstrated earlier (Marshall *et al.* 2003), but also suggested to occur in *F. japonica* (Pezzolesi *et al.* 2010). Generally the haemolytic activity of *F. japonica* cells is high and comparable to other haemolytic harmful algal bloom species like *Prymnesium parvum* (de Boer *et al.* 2009). So far, all haemolytic data were based on the analysis of intracellular haemolysins in concentrated extracts of cells at the end of exponential or in stationary growth phase. Because fish mortality not only occurs during these growth phases but also at lower cell densities (Khan *et al.* 1996), extracellular haemolysins could also be involved in ichthyotoxicity. In the present study we investigated intra- and extracellular haemolysin activity during various stages of *F. japonica* growth. To distinguish between intra- and extracellular substances we used different procedures. Haemolytic activity was assessed using the earlier described erythrocyte lysis assay (ELA).

Material and Methods

In two independent experiments, growth and haemolytic activity of in total four replicate cultures of the non-axenic Dutch *F. japonica* Wadden Sea strain W420 were followed. The cultures were pre-cultured for at least 2 generations in exponential growth phase. Four 1L Erlenmeyer flasks were incubated for experiments, with an initial cell density of 700 ± 100 cells ml^{-1}. All cultures were grown in autoclaved natural sea water (Doggersbank, North Sea) adjusted to salinity of 25 with Milli-Q and enriched with f/2-Si medium. The cultures were maintained at 18 ± 1 °C in a 16:8h light:dark photoperiod with photon flux density of 45 ± 5 µmol photons m^{-2} s^{-1}. Mucocyst ejection was monitored by daily observation. Samples for cell density were taken daily in the light period, for 19-25 days. After sampling cell numbers and biovolume were immediately determined using a Coulter model ZM particle counter equipped with channelyzer 256 and 100 µm aperture tube (de Boer *et al.* 2005).

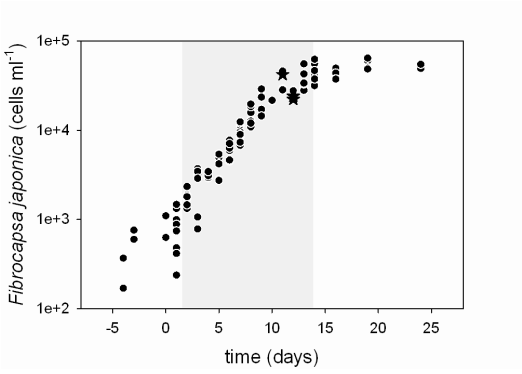

Fig.1. Growth curves of 4 *Fibrocapsa japonica* cultures. One culture was corrected for longer lag phase. Grey = exponential growth, * = samples with mucocyst ejection

Since *F. japonica* cells are easily damaged, several procedures were introduced to separate intracellular (I) and extracellular (O) haemolysin fractions. The following types of samples were used: Type I: whole, non-treated cultures containing living cells (O); Type II: whole cultures, frozen at -20 °C in order to break the cells, giving total haemolysins (I+O); Type III: filtered cells followed by extraction (I); Type IV: filtrate by standard filtration (O); and Type V: filtrate by reverse filtration (O). Samples were processed using all five procedures: for experiment 1 Type I, III and IV, and for experiment 2 Type II, III, IV and V. For Type III 10^5 cells of each culture were collected on a polycarbonate filter (2 µm, Ø 25 mm) using mild vacuum (standard filtration). Filters, cultures and filtrates were stored at -20 °C until further analysis, except Type I samples which were immediately analysed for haemolytic activity. Cell extracts (Type III) were obtained as described in de Boer *et al.* (2004). ELA was performed as described previously (de Boer *et al.* 2004). For analysis of haemolytic activity of living cell samples (Type I) the ELA buffer was adjusted to salinity of 25. The absorptions of the negative and positive control of ELA buffer were both significantly higher than the standard ELA buffer (one way ANOVA p=0.018 and p=0.006). In both ELA methods, f/2-Si medium and ELA buffer served as controls and did not show any haemolytic activity. Saponin (Sigma) was used as a reference, with an EC_{50} value of 4.3 µg ml^{-1}. The cell densities of *F. japonica* from the dilution series were plotted against the % erythrocyte lysis and were fitted by a sigmoidal curve or, alternatively, by a linear regression. EC_{50} was calculated as the concentration of *F japonica* cells (cells ml^{-1}) responsible for 50% erythrocyte lysis. Statistical analysis was performed using SPSS and Statistica.

Results

All cultures showed similar growth curves (p=0.64) with initial lag phase, followed by exponential growth phase between days 1 and 11-14 (Fig. 1). Specific growth rates at the applied conditions were also comparable (p=0.68), and on average 0.34 ± 0.02 d^{-1}. The exponential growth phase of the replicate cultures was defined between 1.3 ± 0.15. 10^3 and 4.2 ± 0.3. 10^4 cells ml^{-1}. Mucocyst ejection started at a cell concentration of 2.2. 10^4 cells ml^{-1}.When cell concentrations were below 0.5. 10^4 cells ml^{-1}, no significant differences in haemolytic activity were found between filtrates Type IV and V (p=0.59) nor between filtrates and whole culture (Type I) (p=0.64, Fig. 2). Above this cell concentration, maximum lysis of erythrocytes was mostly not sufficient to calculate extracellular haemolytic activity. This indicates that below 0.5. 10^4 cells ml^{-1} the calculated extracellular haemolytic activity was not caused by damaged or broken cells during sample processing. Highest extracellular haemolytic activity was 20 x higher (EC_{50} = 600 cells ml^{-1}) than maximum intracellular activity from Type III samples (EC_{50} = 1.2. 10^4 cells ml^{-1}, Fig. 3).

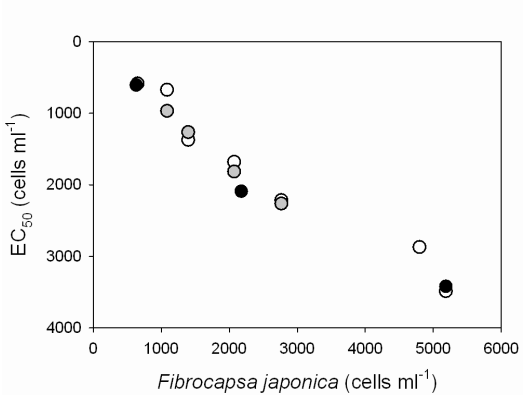

Figure 2. Effect of cell concentration on haemolytic activity (EC_{50}) with different sampling methods. White: filtrate by standard filtration; grey: filtrate by reverse filtration; black: whole, non-treated cultures containing living cells.

The cell concentration was positively correlated with the intracellular haemolytic activity and negatively correlated with extracellular haemolytic activity (Spearman rank correlation; R^2=0.97 and R^2=-0.65). Mucocyst ejection did not affect the haemolytic activity neither inside nor outside the cell. Only the maximum erythrocyte lysis of the frozen samples (Type II) of days 16 and 19, was sufficient to calculate haemolytic activity (data not shown). These EC_{50} values were not different from Type III samples (p=0.09).

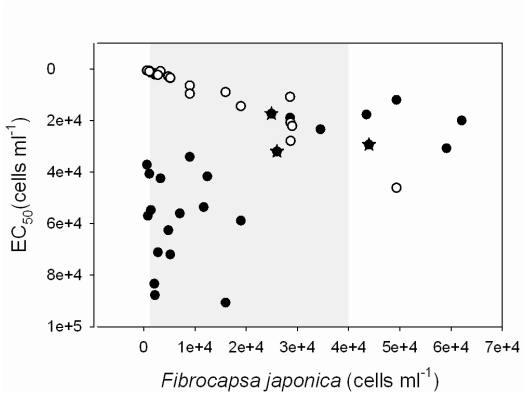

Fig. 3. Effect of *Fibrocapsa japonica* cell concentration on haemolytic activity (EC_{50}) measured intracellular (black) and extracellular (white) Grey = exponential growth phase; * = samples with mucocyst ejection.

Discussion

Different procedures for estimating extracellular haemolytic activity showed similar results. This implies that, although cells of *F. japonica* break easily, standard filtration may be used for distinguishing between extracellular and intracellular haemolysin fractions. When cells were damaged by freezing, results were similar to intracellular activities derived from filtered cells. It is unclear why high extracellular haemolytic activity was not observed in the frozen samples. Haemolytic activity of *F. japonica* significantly changed as a function of cell concentration during exponential growth, i.e. when growth rate was maximal and constant. Very high extracellular haemolytic activity at low cell concentrations could have ecological advantages by improving the competitive abilities. More specifically, the haemolysins might be the same growth inhibiting or allelopathic compounds released by *F. japonica* at low cell concentrations during exposure to bacteria (van Rijssel *et al*. 2008).

The intracellular haemolysins showed highest haemolytic activity at high cell concentrations when nutrients were getting scarce and mucocysts were released. The intracellular haemolytic activity could be attributed to the PUFAs and haemolytic compounds other than PUFAs, as described earlier (Fu *et al*. 2004; de Boer *et al.* 2009). So far, the mechanism of the ichthyotoxicity of *F. japonica* was ascribed to the production of these PUFAs (Marshall *et al.* 2005, de Boer *et al.* 2009, Pezzolesi *et al.* 2010), however in the present study we have shown that extracellular haemolysins might have different structural and ichthyotoxic characteristics.

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