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An extracellular hemolysin homolog from cyanobacterium *Synechocystis* sp. PCC6803

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Introduction

Cyanobacterial extracellular Cu-chelators were discovered more than a decade ago, as produced by marine cyanobacteria in response to Cu-stress (Bruland et al. 1991). The extracellular nature of the chelators suggests that the strategy of cyanobacteria against heavy metals is different from that of eukaryotic algae, in which metal-chelating proteins are accumulated inside cells (Rauser 1990). To study cyanobacterial response to Cu-stress, we used *Synechocystis* sp. PCC6803, whose genomic DNA has been fully sequenced (Kaneko et al. 1996), as model organism. In the present study, we analyzed the culture medium of Cu-stressed cyanobacterium through column chromatography, and found that Cu was associated with a protein peak that contained a homolog of hemolysin, which is known to be a bacterial exotoxin that forms aqueous transmembrane pores in mammal red blood cells (Bhakdi et al. 1996).

Materials and methods

A glucose-tolerant strain of *Synechocystis* sp. PCC6803 (Williams 1988), kindly provided by Prof. N. Murata (National Institute for Basic Biology, Okazaki, Japan), was pre-cultured in 30 ml BG-11 medium (Stanier et al. 1971) supplemented with 20 mM Hepes-KOH (pH 8.0) at 30°C with rotary shaking at 70 rpm under continuous illumination at 80 µmol photons m^{-2} s^{-1}. The pre-cultured cells at OD_{730} of 1.0 were inoculated into 900 ml of the culture medium that had been supplemented with 3 µM $CuSO_4$. The cells were grown in two 500-ml flat oblong glass vessels at 30°C with air-bubbling and illumination as above. The culture at the late log phase was centrifuged at 7,200 x *g* for 10 min at room temperature. The supernatant was filtered on a polycarbonate filter with a pore size of 0.2 µm (Nuclepore; Whatman). To stabilize Cu-chelators in the filtrate, we adopted the conditions previously applied to polyphenol oxidase (Kuwabara et al. 1997). Briefly, 1 kg of ethylene glycol, 35 g of NaCl, and 10 ml of 20% (w/v) Tween 20 were added to the filtrate, and the pH of the solution was adjusted to 6.5 with 1 M NaH_2PO_4. For purification of Cu-chelators, this solution was loaded at 4 °C on a hydroxyapatite column (1.6 cm i.d. x 8.5 cm) that had been equilibrated with 20 mM sodium phosphate (pH 6.5), 0.3 M NaCl, 0.1% (w/v) Tween 20, and 50% (w/v) ethylene glycol (buffer A). The column was washed with 50 ml of buffer A, and eluted with 90 ml of a linear gradient from buffer A to 400 mM potassium phosphate (pH 6.5) containing 0.1%

Tween 20 at a flow rate of 1 ml min^{-1}. Fractions of 3 ml were collected and subjected to quantification of Cu with a flameless atomic absorption spectrometer (AA-6800G; Shimadzu).

Results

Growth rate of the cyanobacterium in the presence of 3 μM Cu^{2+} was about half that in the normal BG-11 medium. We used this condition for purification of extracellular Cu-chelators, expecting that the cyanobacterium survive against the Cu-stress by producing the chelators. When the culture medium was subjected to hydroxyapatite chromatography, three elution peaks of Cu emerged, including the last peak of free Cu^{2+} ions (Fig. 1). The first peak matched with an elution peak of A_{280}. The fractions comprising this peak were concentrated and analyzed by SDS-PAGE (Fig. 2). Two major polypeptides were detected at 173 and 31 kDa; they are candidates for a Cu-chelator. It was rather surprising that a polypeptide as large as 173 kDa was secreted from the cyanobacrterium. Its N-terminal amino acid sequence was determined to be ALSPNVIAALQIMYTGRGVS–, which is the same as the sequence from the second residue of a hypothetical protein, hemolysin, found in the genome of the cyanobacterium (Kaneko et al. 1996). The absence of a cleavable N-terminal signal peptide suggests that the protein is secreted by a mechanism different from the Sec system, but possibly by an ABC transporting system, as is the 110-kDa hemolysin of *E. coli*.

Discussion

The DNA sequence of the hypothetical protein hemolysin (Kaneko et al. 1996) suggests that the protein is abundant in glycine and aspartic acid, and contains no cysteine. It is interesting that the eight histidine residues in the protein are all located in the central domain (K759-F1101) within a motif represented by the sequence AGHVV (Fig. 3). Each histidine is followed by one unit of the parallel β-roll motif (Sprang 1993), suggesting that the set of the histidine-containing sequence and the parallel β-roll motif was repeatedly inserted into the protein in the process of molecular evolution. The parallel β-roll motifs are also found in the C-terminal domain (V1102-A1741), but never in the N-terminal domain (M1-S758), suggesting that the cyanobacterial hemolysin may have been made by natural molecular fusion of hemolysin and another protein, like cyclolysin of *Bordetella pertussis* (Glaser et al. 1988). The parallel β-roll motif is postulated to form a half site to bind a Ca^{2+} ion (Baumann et al. 1993). Thus, it is possible that this structure was involved in the binding of Cu to this protein; both Ca^{2+} and Cu^{2+} can take a six-coordinate structure. Another possibility is that the regularly arranged histidine residues are involved in the binding of Cu^{2+} ions. Since cyanobacteria are autotrophs, the hemolysin homolog is unlikely to be a weapon to attack other organisms. It might function as a chelator of heavy metals. We are now studying molecular characteristics of the cyanobacterial hemolysin to clarify its physiological role.

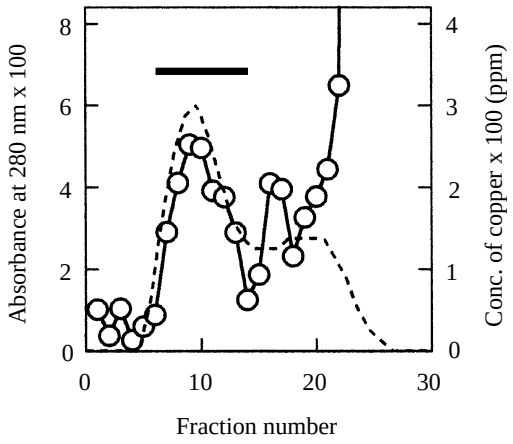

Fig.1. Hydroxyapatite chromatography of culture medium of *Synechocystis* sp. PCC6803 grown in the presence of 3 μM Cu^{2+}. Fractions indicated by bar were collected as HA preparation.

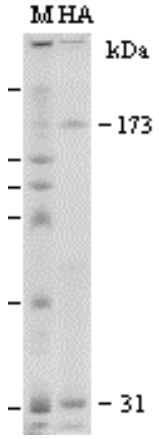

Fig.2. SDS-PAGE of HA preparation. Bars on the left indicate the positions of marker proteins in lane M (205, 116, 94, 67, 43 and 30 kDa)

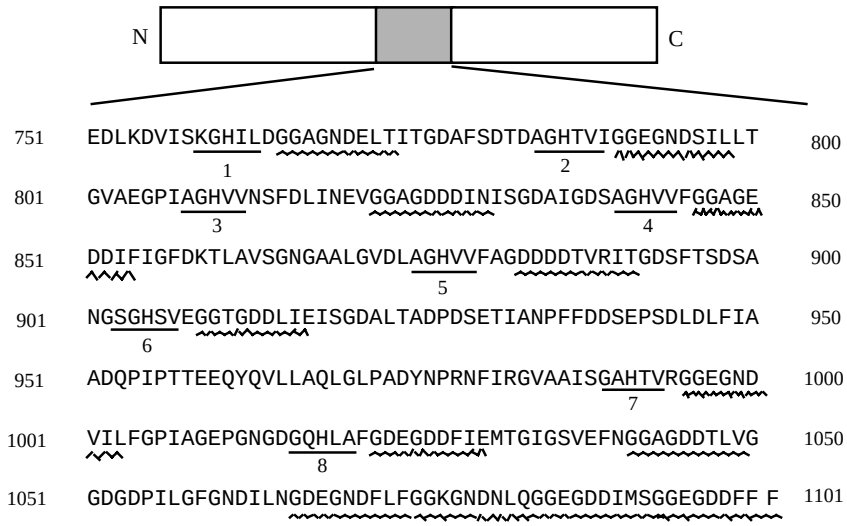

Fig.3. Amino acid sequence of the central region of the cyanobacterial hemolysin homolog. The protein is schematically illustrated with a rectangle in the upper part, with its N- and C-termini indicated. In the amino acid sequence of the central region (E751-F1101), solid lines with number indicate the 8 histidine residues in the protein and surrounding sequences. Wavy lines indicate parallel β-roll motif. Note that there are four parallel β-roll motifs in tandem at the end of this sequence.

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