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Isolation of Microcystin-LR and Its Potential Function of Ionophore

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Abstract The microcystin is a cyclic heptapeptide from metabolites of cyanobacteria in the genera mycrocystis, anabaeba as a result of eutrophication. It has been known that microcystin-LR is a potent inhibitor of the catalytic subunits of protein phosphatase-1 (PP-1) as well as powerful tumor promoter. The active site of microcystin actually has two metal ions Fe^{2+}/Zn^{2+} close to the nucleophilic portion of PP-1-microcystin complex.

We report the isolation and purification of this microcystin-LR from cyanobacteria (blue-green algae) obtained from Daechung Dam in Chung-cheong Do, Korea. Microcystin-LR was extracted from solid-phase extraction (SPE) sample preparation using a CN cartridge. The cyanobacteria extract was purified to obtain microcystin-LR by HPLC method and identified by LC/MS.

The detail structural studies that can elucidate the possible role of monovalent and divalent metal ions in PP-1-microcystin complexation were carried out by utilizing molecular dynamics. Conformational changes in metal binding for ligands were monitored by molecular dynamic computation and potential of mean force (PMF) using the method of the free energy perturbation. The microcystin-metal binding PMF simulation results exhibit that microcystin can have very stable binding free energy of -10.95 kcal/mol by adopting the Mg^{2+} ion at broad geometrical distribution of 0.5~4.5 Å, and show that the K^+ ion can form a stable metal complex rather than other monovalent alkali metal ions. □

Keywords Microcystin-LR, Molecular dynamics, Metal complex

Introduction

Many microcystin derivatives have been isolated from diverse cyanobacteria in the general *Microcystis, Anabaena, Oscillatoria* in fresh and brackish water all over the world. Microcystins are greatly accelerated eutrophication of water bodies and leads to reduced oxygen contents of the water. Toxicity and carcinogenic properties of mocrocystin affects a serious health risk for mammals and the other organism of the ecosystem. [1-4]

Microcystins has a cyclic heptapeptide, cyclo[2,3-didehydro-N-methyl-Ala-D-Ala-L-X-erythro-3-methyl-β-D-Asp-L-Y-Adda-γ-D-Glu], where Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phen-yldeca-4E,6E-dienoic acid, and X and Y are two of several common L-amino acids as shown in figure1.[5-7] These microcystins are powerful inducers of tumors and have been found to inhibit on the major serine/threonine protein phosphatase (PP-1 and PP-2A).[8-9] Conformation of microcystin is important for the explanation of the difference in toxicity of the several microcystins and analogs. Structural studies

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of the free conformations of microcystin were made. The three dimensional structures of microcystin-LR in solution and microcystin-PP-1 complex have been determined by NMR and X-ray crystallographic studies.[10-11]

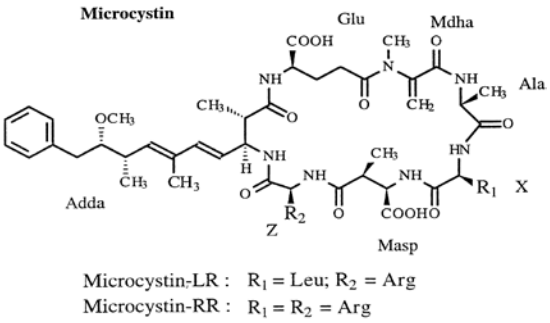

Figure 1. Molecular structure of microcystin-LR

The conformation of PP1-mycrocystin complex closely resembles that of free microcystin-LR.

In this study, we isolated microcystin-LR from cyanobacteria (blue-green algae) obtained from Dae-chung Dam in the Chung-cheong Do, Korea. The freeze dried cyano-bacteria sample mixture was extracted from solid-phase extraction (SPE) sample preparation using a CN (cyano) cartridge in order to isolate microcystin-LR. Microcystin-LR was extracted from purification by HPLC method and identified by LC-MS/MS and DAD detector.[12-14]

In order to understand the potential role of metal ions in complexation, we carried out further structural studies by using molecular dynamics Conformational changes which occur in active site of protein phosphatase-1(PP-1) are traced. Structural change and the potential of mean force (PMF) simulation using free energy perturbation of complex between the metal ions and microcystin will be able to identify the potential function in vivo role as ionophore. [15-16]

Experimental Methods

Isolation and purification of microcystin. The cyanobacteria blue-green algae sample was collected from Dae-chung Dam and the crude extract was used after lyophilization. First, CN cartridge (6mL, 1g) was activated with 70% aqueous acetonitrile 5mL and H_2O 5 mL. For extraction of microcystins, the lyophilzed cell was stirred with 10mL of 5% acetic acid during 30min and then centrifuged at 9,000rpm. After separation, pass supernatant through CN cartridge at pH 3.0. The cartridge containing microcystins was rinsed with each 5mL of 0.5M acetic acid and 5% acetonitrile. After washing, the extracts was eluted with 70% acetonitrile 15mL and evaporated to dryness.

Extract samples were dissolved in 1mL AcCN and were purified by HPLC. Instrument studies were performed using an Agilent 1100 series with a diode-array detector (DAD). The separation was achieved on a YMC-Pack ODS-AQ(120Å, 300mm x6.0mm, 5um) analytical column. The mobile phase for HPLC was methanol 0.1% with formic acid (53:47) and gradient to methanol 0.1% with formic acid(76:24, at 17 min) at a flow rate of 1.5mL/min. DAD analysis was carried out at the wavelength of 238nm. MS experiments were then proceeded to identify the sample under same condition with HPLC.

Protein phosphatase-1-microcystin complex. X-ray crystal structure pdb data of microcystin bound with protein serin/threonine phosphatase-1(PP-1) from protein data bank was used for the molecular dynamic simulation. The file was supplemented and made the complete structure with the X-plor program. Separate the microcystin molecules from PP-1 and modified amino acid of microcystin data was producing basic topology and completing parameter files from Gaussian 2003 and Hyperchem7.0 as shown in figure 2.

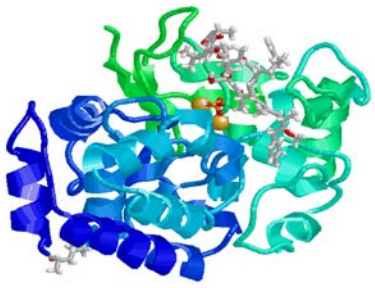

Figure 2. Active site of microcystin binding PP-1

In molecular dynamics simulations, water molecule was used as the coordinates of the 150 molecule obtained in X-ray data. The psf file of Mg^{2+}, Ca^{2+}, Na^{+}, Fe^{2+}, K^{+}, Rb^{+}, Cs^{+} ions were located on the mass center of nucleophilic atoms in microcystin using the pdf file. The 1619 water molecule was solvated in 35 Å x 35 Å x 40 Å water box.

Molecular dynamic simulations. Molecular files were initialized and conjugate gradient minimization. Langevin dynamic was applied for the atom friction coefficient (heat bath application) and atom velocity was rescaling by the Maxwell distribution. The flow diagram for the MD simulation is shown in Figure 3. Potential of mean force (PMF) simulations were calculated while maintaining constant the distance between the metal ions and the atoms. The binding free energy value to simulate the structural infor mation for each step was estimated and The flow diagram for the PMF protocol shown in Figure 4.

Results

Interaction of the cyano group of CN cartridge and guanidine group of microcystin was adopted to extract microcystins from cyanobacteria. Extracted samples containing a variety of microcystins were then purified by using HPLC experiments. The microcystin-RR and microcystin-LR were isolated and identified in the detection time of 10.158 and 15.033 respectively as shown in Figure 5. In this chromatogram, two major peaks were appeared and this is consistent with peaks obtained in TIC (total ion chromatogram). Ion chromatogram (EIC) was performed in order to confirm microcystins and two peaks were identified to be microcystin-RR and microcystin-LR, respectively. As shown in figure 6, A major peak at chromatogram of 10.16 min retention time was confirmed to be microcystin-RR by 1039.6 m/z, $[M+H]^{+}$ (molecular mass), and 520.2 m/z, $[M+2H]^{2+}$. 520.2 m/z (double charged mass) ion peaks is larger than 1039.6 m/z and it is estimated due to ionization of the guanine group of arginine. A major peak at chromatogram of 15.03 min retention time was confirmed to be microcystin-LR by 995.6 m/z due to $[M+H]^{+}$ (molecular mass), and 854.7 m/z(mass of fragment).

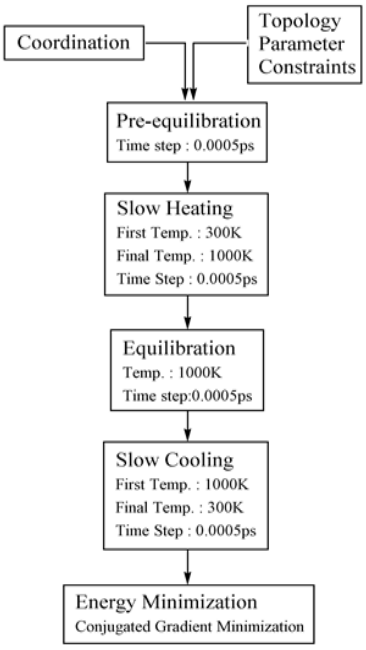

Figure 3. Flow diagrams of molecular dynamics

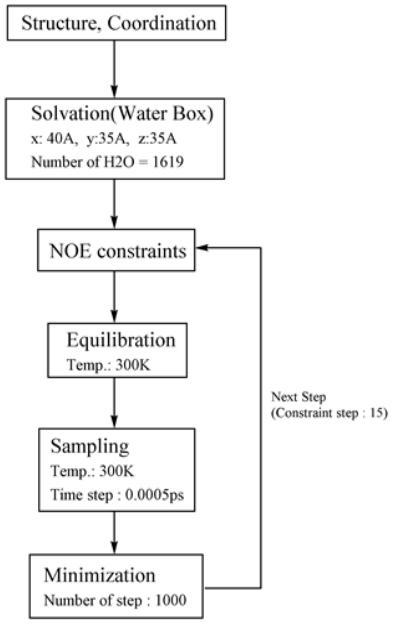

Figure 4. Flow diagram of energy perturbation PMF protocol.

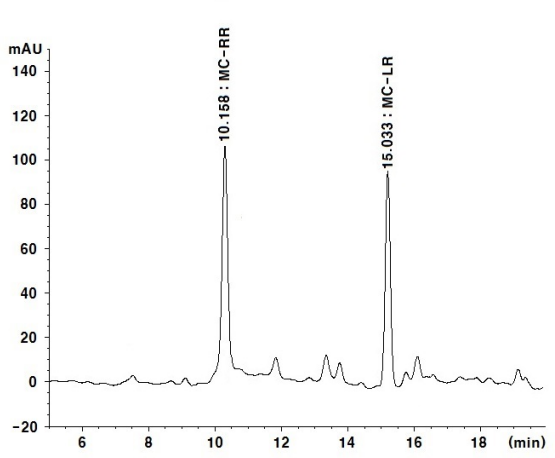

Figure 5. High performance liquid chromatogram of the microcystin-RR and LR in the extract. Column: YMC-Pack ODS-AQ 5um, 120Å 300 X 6.0 mmI.D., Mobile phase : methanol:0.1% formic acid(53:47) gradient to methanol: 0.1% formic acid(76:24, at 17 min), Flow rate: 1.5 mL/min, Detector: 238 nm

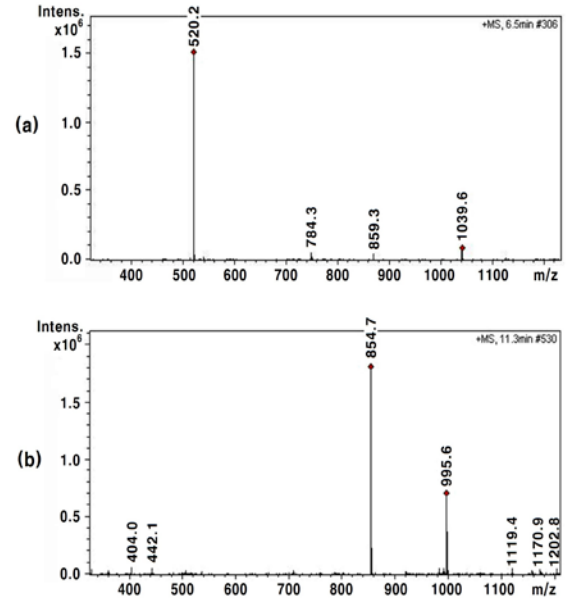

Figure 6. Mass spectrum of microcystin-RR(a) and LR(b)

As shown in figure 7, the crystal structure of PP-1 binding microcystin was adopted for molecular dynamic simulations of metal-binding microcystin complex. Microcystin forms a covalent bond with Cys-273 of PP-1 and two metal ions are located in the active site of enzyme for interaction with other substrates.[17-18] In the crystal structure, microcystin and enzyme connected with hydrogen from water molecule.

There is a cyclic hole has nucleophilic such as O,N for a good acceptor of Lewis acid and it can interact with metal ion in PP-1. This macrocyclic toxin is enough to act similar to ionophore. The molecular dynamics simulation results exhibits that there are strong electrostatic attraction with the metal ions and the ionophore of macrocyclic microcystin ring. Distance data of microcystin between metal ion and energy profiles are summarized in Table.1 and shown in figure 8.

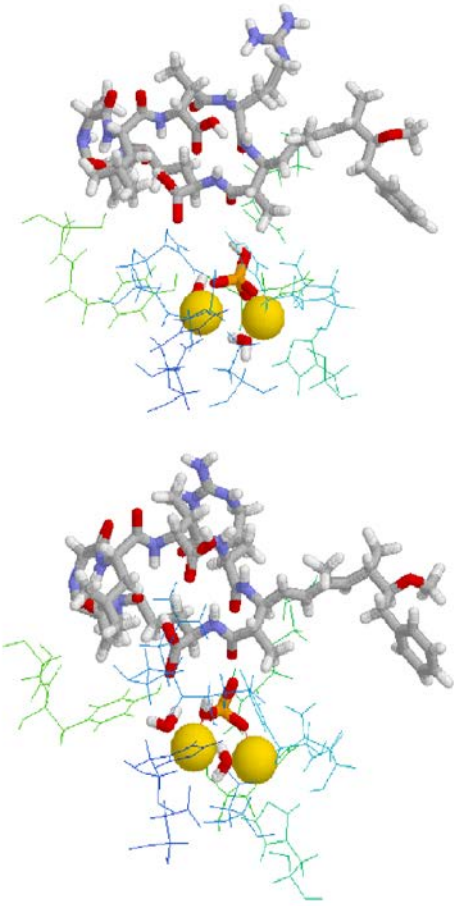

Figure 7. Ball & stick model of microcystin, metal ions and protein residues (U): Before MD simulation, (D): After MD simulation.

Table1. 1 Distance between metal ion and microcystin nucleophilic residues

Residue	Atom type[a]	Charge[b]	Before simulateon[c](Å)	After simulation[d](Å)
Masp	O1	-0.6815	4.32	4.29
	O2	-0.5995	4.86	5.92
Adda	O1	-0.6297	5.97	5.41
Arg	O	-0.6664	8.85	8.18
Glu	O1	-0.7289	8.93	8.64
	O2	-0.5839	10.65	10.41
Mdha	O	-0.6367	12.24	10.09
Ala	O	-0.60008	13.36	12.65

a. O : peptide bond oxygen, O1,O2 : acetic acid oxygen.
b. Electric potential charge is calculated from GAUSSIAN 2003
c. Before simulation distance is acquired from X-ray crystal data
d. After simulation distance is acquired from the minimized structure

Figure 9 shows PMF profile of metalbinding microcystin complexes. PMF path set to +z-direction and it was not change x,y coordination between mass center of microcystin and metal ions. Fe^{2+}, Ca^{2+}, Mg^{2+} and alkali metal ion K^{+}, Na^{+} were used with selectively test to interaction of protein. Metal binding free energy was also calculated and are shown in figure 10.

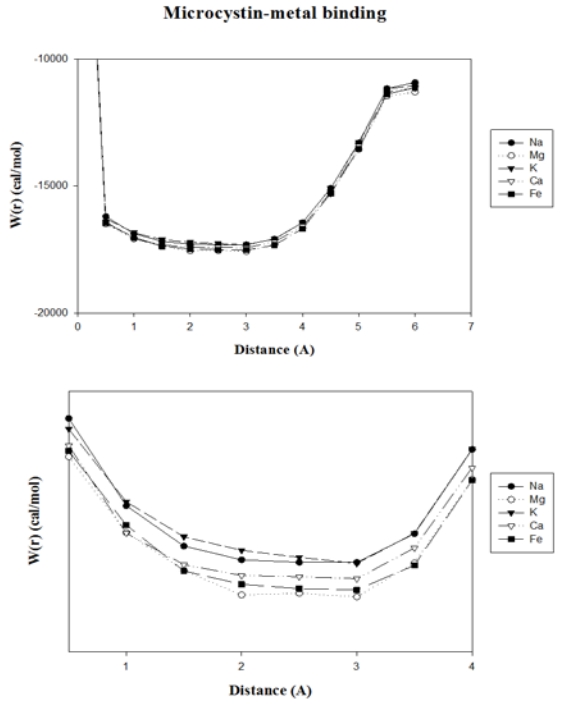

Figure 8. The PMF profile for the association of microcystin complex in water at 300K

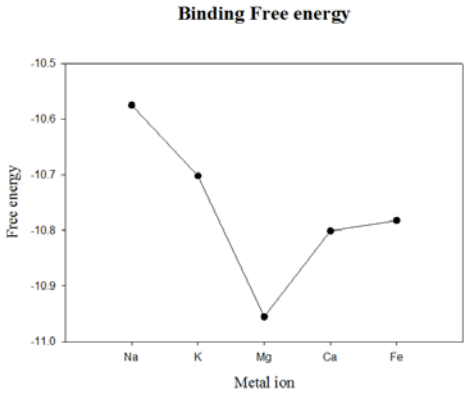

Figure 9. Absolute free energy of bonding of microsystin complex with various metal ions

In PMF results, there are not differences in the energy change for each metal ion. Metal-microcystin complexes are formed with short binding distance of all metal ions at 0.5~4.5 Å less than -15kcal/mol and with similar binding free energy. The large molecular pocket of microcystin has enough to be nucleophilic so that it can act as an ionophore metal pocket. Among metal-complexes, the Mg^{2+}-microcystin complex is more stable than other complexes with the binding free energy at -10.95kcal/mol and can release a metal ion from 6.0 Å. The conformational changes in metal complexation and its trajectory are shown in figure 10.

Discussion

Microcystin-LR and microcystin-RR forms were isolated from cyanobacteria by CN cartridge. The presence of microcystin in the extract sample was confirmed by analytical HPLC with DAD/MS.
Molecular dynamic studies with potential mean function were carried out to understand the potential function of microcystin as ionophonre. From X-ray crystallographic data and molecular dynamics simulations result of the PP-1 complex with microcystin, we were able to find that two metal ions contained in the PP-1 were significantly effects on the microcystin. Microcystin form a covalent bond

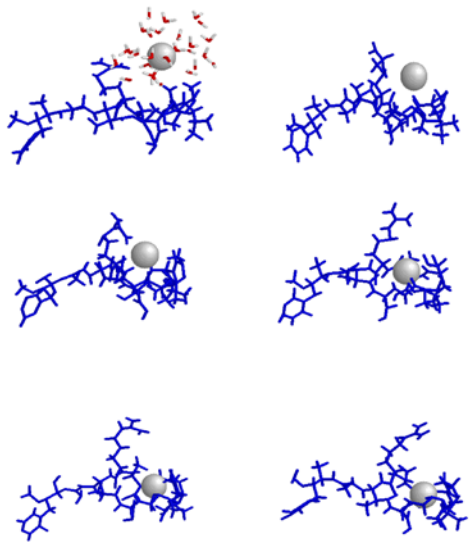

Figure 10. Representative structure of microcystin metal complex along PMF profile. The separation distance are 9, 8, 6, 4, 2,0Å, viewing the figure from top to bottom/left to right□

with Cys-273 of PP-1 by two metal ions in the active site of enzyme. The total energy of the complexes between the metal ions and microcystin were affected by electrostatic attraction.

Nucleophilicity originated from oxygen donors of macrocyclic ring and electrophilicity of metal ions are electrostatically good enough to form a metal complexes. The potential of mean force(PMF) data showed that a very low and wide range value of binding distance of all metal ions with less than -15kcal/mol caused by energy perturbation from change distance in binding free energy. Metal ions were attracted toward peptide oxygens associated with amino acid residues of Ala, Mdha, Arg rather than carboxylate oxygens. As shown in figure 10, the trajectory of metal ions firstly attracted by guanidine moiety of Arg group and subsequently capture by peptide oxygens, and this resulted in big conformational changes of metal ion pocket capture as an ionophore. Although microcystin contains a lot of nucleophilic residues, the fact that the pocket size is relatively large compared to other macrocyclic ionophores result in weak binding energy of -10 kcal/mol. Interestingly, Mg^{2+} can tightly bind to macrocyclic ring moiety with conformational changes and very low stabilization energy value at -10.95Kcal/mol, but macrocyclic ring can be easily released around 6 Å. In the alkali metal ions, K^{+} ion has relatively larger binding capacity than other metal ions, this result may be due to the size of metal ion and low ionization potential. Detail structural studies of metal complexes by NMR and the method of NMR based structure determination will be published elsewhere. [19-20]

Acknowledgements

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