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Complex Forming Ability of a Family of Isolated Cyclosophoraoses with Ergosterol and Its Monte Carlo Docking Computational Analysis

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Abstract. Neutral cyclosophoraoses (unbranched cyclic β-1,2-D-glucans) produced by the *Rhizobium meliloti* 2011 were prepared by size exclusion and anion-exchange chromatographic techniques. The degree of polymerization (DP) of isolated cyclosophoraoses was determined by matrix associated laser desorption/ionization mass spectrometry (MALDI/MS) techniques. A family of purified neutral cyclosophoraoses (DP 17–27) was used as a host for the inclusion complexation with hardly soluble ergosterol. High performance liquid chromatographic (HPLC) analysis showed that it induced much enhanced solubility of ergosterol compared to β-cyclodextrin. In order to understand the molecular basis of the complex forming ability of cyclosophoraoses, a Monte Carlo (MC) docking-minimization method was used for host-guest complex formation of cyclosophoraoses or β-cyclodextrin with ergosterol. From the MC simulation we propose the 'hand-shake' mechanism for complexation of cyclosophoraoses with ergosterol.

Key words: cyclosophoraoses, β-cyclodextrin, inclusion complex, Monte Carlo simulation.

1. Introduction

Cyclosophoraoses are a class of unbranched cyclic oligosaccharides composed of β-(1→2)-D-glucans varying in size from 17 to 40 as a neutral or anionic form. They were originally found in fast growing soil bacteria, *Agrobacterium* and *Rhizobium* species as *intra-* or *extra*-oligosaccharides [1, 2]. Cyclosophoraoses are synthesized in the cytosol and transported to the periplasmic space where they play an important role in regulating the osmolarity in response to external osmotic shock [3]. Cyclosophoraoses are also known to be involved in the initial stage of root-nodule formation of *Rhizobium* during the nitrogen fixation [4]. Throughout this

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process cyclosophoraoses are suspected of being involved in complexation with various plant flavonoids. Thus, much attention has been focused not only on their biological functions but also on their potential ability to form inclusion complexes with other molecules. As for the application of inclusion complex formation, a single cyclosophoraose (cyclosophoraose with DP 17, Cys-A) was extensively used as a host for complexation with guest molecules because of its easy availability. However, the isolated Cys-A (DP 17) showed limited possibilities on industrial applications related to inclusion complex formation over various hydrophobic guest molecules [5]. Especially, Cys-A did not enhance the solubility of ergosterol at all [5]. In the present investigation we used a family of isolated cyclosophoraoses with various DP ranging from 17 to 27 as a host for the inclusion complex formation with hardly soluble ergosterol. The solubility of ergosterol was estimated from the HPLC analysis of complexed ergosterol. The molecular basis of complexation of cyclosophoraoses or β-cyclodextrin with ergosterol was also investigated with a Monte Carlo docking computer simulation.

2. Experimental

2.1. PREPARATION OF CYCLOSOPHORAOSES

Rhizobium meliloti 2011 was generously provided by Dr. R. I. Hollingsworth, MSU, E. Lansing, Michigan, U.S.A. Cells were cultured in 500 mL of GMS medium [3] to late logarithmic phase and incubated at 30 °C, 150 rpm on a rotary shaker. Cells were harvested by centrifugation (8,000 rpm, at 4 °C), washed once with a saline solution, and subjected to the hot-ethanol extraction method. Cells were then extracted with 40 mL of 75% (vol/vol) ethanol at 70 °C for 30 min. After centrifugation, the supernatant was removed and concentrated by vacuum rotary evaporator. The concentrated sample was chromatographed on a Sephadex G50 column (1.5 $\times$ 110 cm) at a rate of 20 mL/h and eluant fractions (7 mL) were assayed for carbohydrate by the phenol-sulfuric acid method. The fractions containing cyclosophoraoses were pooled, concentrated, and desalted by a Sephadex G15 column (2 $\times$ 27 cm) under previous conditions. The desalted sample was then applied to a column (2 $\times$ 35 cm) of DEAE-cellulose toseparate neutral and anionic cyclosophoraoses. The column was first washed with distilled water containing 1 mM KCl and a gradient was applied beginning with 1 mM KCl and ending with 100 mM KCl. After the cyclosophoraoses were desalted by dialysis (Spectra/PorCE (cellulose ester membrane; MWCO : 1000)), their structures were confirmed by nuclear magnetic resonance spectroscopy. The neutral cyclosophoraoses were further analyzed for the determination of molecular weight distribution with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS, Voyager, PerSeptive Biosystems) using 2,5-dihydroxybenzoic acid (DHB) as the matrix at positive ionization. Mass spectra were recorded in DHB at a mole ratio of 10^{-3} with a total loading of around 1 μg of sample. Ions were formed by laser desorption at 337 nm.

2.2. INCLUSION COMPLEX FORMATION OF CYCLOSOPHORAOSES AND β-CYCLODEXTRIN WITH ERGOSTEROL

A family of isolated neutral cyclosophoraoses (DP 17–27) or β-cyclodextrin (Sigma) was used as a host for the inclusion complexation with ergosterol (Sigma) as a guest. When the solid ergosterol was added directly to aqueous cyclic-oligosaccharides (Cys-A or cyclodextrin) solution, it gave no clear data on the inclusion complex forming ability [5]. We prepared ergosterol in organic solvent such as chloroform or acetone and mixed it with the host aqueous solution. Both solvents gave similar results. For the purpose of efficient contacts of cyclosophoraoses with ergosterol, we used chloroform because it solubilizes ergosterol more than acetone. First, ergosterol was dissolved in chloroform to obtain a 15×10^{-3} M solution. One mL of ergosterol stock solution in a vial was added to 1 mL of aqueous neutral cyclic β-glucans(or β-cyclodextrin) solution to make various concentrations, and the mixture was shaken for 24 h at 30 °C in the dark. After equilibrium was reached, the mixture was evaporated, lyophilized and dissolved in 1 mL of water to remove insoluble ergosterol by filtration using a 0.2 μm membrane filter (Whatman), then the filtrate was lyophilized and the ergosterol concentration in the filtrate was determined by high performance liquid chromatography (HPLC 10 A (Shimadzu, Kyoto, Japan)) with ultraviolet (UV) or refractive index (RI) detector. Ergosterol was assayed at 265 nm under 55% ethanol, 45% water mobile phase composition, 35 °C oven temperature and 0.4 mL/min flow rate condition. Lyophilization was carried out with a FD-3 freeze dryer (Heto Holten A/S, Denmark). TSK gel ODS-80TM (15 cm $\times$ 4.6 mm i.d., C_{18}), ODS bonded silica gel packed column was used for HPLC.

2.3. COMPUTATIONAL METHODS

The program Insight II/DISCOVER/DOCKING from Molecular Simulations Inc. (MSI) was used to build and simulate the molecular models on an SGI R4600 platform. We used the consistence valence force field (CVFF) [6] for molecular mechanics and molecular dynamics simulations, with the following representation of the potential energy.

$$
\begin{aligned}
V(r^N) \;=\; & \sum_{\text{bonds}} \frac{k_i}{2}(l_i - l_{i,0})^2 + \sum_{\text{angles}} \frac{k_i}{2}(\theta_i - \theta_{i,0})^2 \\
& + \sum_{\text{torsions}} \frac{V_n}{2}(1 + \cos(n\omega - \gamma)) \\
& + \sum_{i=1}^{N} \sum_{j=i+1}^{N} \left(4\epsilon_{ij} \left[\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^{6} \right] + \frac{q_i q_j}{4\pi\epsilon_0 r_{ij}} \right). \qquad (1)
\end{aligned}
$$

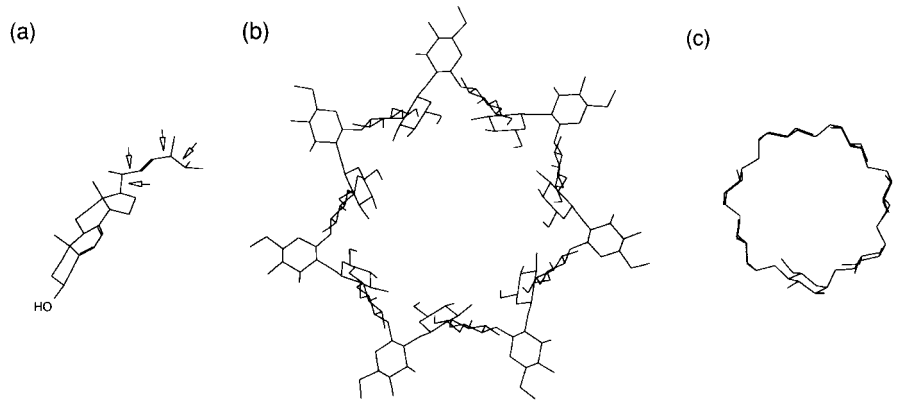

Figure 1. (a) Structure of ergosterol after simulated annealing minimization (the arrows indicate four acyclic single bonds for MC simulation); (b) molecular model of Cys-21mer proposed by Palleschi and Crescenzi; and (c) structure of β-cyclodextrin.

The first three terms of the above equation represent the energy of deformation of bond lengths, bond angles and torsion angles, respectively. The final term accounts for the *van der Waals* and electrostatic interactions.

2.3.1. *The Molecular Models for Cyclic Oligosaccharides and Ergosterol*

Because the three-dimensional structures of cyclosophoraoses are currently unavailable, we used the models of Palleschi and Crescenzi [7]. From the reported values of ϕ and ψ dihedral angles of Palleschi and Crescenzi [7], the molecular model (Figure 1(b)) of cyclosophoraose consisting of 21 glycosyl units was built and its potential energy was minimized for 1000 iterations by the conjugate gradient algorithm [8]. The molecular model (Figure 1(c)) of β-cyclodextrin was also built using atomic coordinates from the Cambridge Crystallographic Data File and energy-minimized for 1000 iterations by the conjugate gradient algorithm. The molecular model (Figure 1(a)) for ergosterol was constructed after the simulated annealing molecular dynamics. During this simulated annealing molecular dynamics, the temperature was elevated and lowered by 10 K steps from 300 to 1000 K five times. At each temperature, MD simulation was performed for 100 fs with a time step of 1 fs, so the total MD simulation time was 70 ps. A snapshot was archived every 100 fs and each of the conformations was minimized by the conjugate gradient algorithm for 100 iterations.After the conformational analyses based on the high structural and energetic similarity in the lowest-energy minima, the lowest energy one was chosen for the MC docking simulation.

2.3.2. *Monte Carlo (MC) Docking-Minimization Procedure*

From the initial molecular models, MC docking simulation started in vaccuo ($\epsilon = 1$) by minimizing this configuration for 60 iterations by conjugate gradient al-

gorithm and accepted the minimized configuration as the first frame of the MC docking simulation. The initial distance between the geometrical centers of the host and guest molecules was set to be the same for each set of host-guest molecules. In the course of developing a new configuration, the guest molecule, ergosterol, could take a maximum translational movement of 5 Å to the x, y, and z axis and a maximum rotational movement of 180° around the x, y, and z axis. To perform the flexible docking simulation, four acyclic carbon-carbon single bonds in ergosterol (Figure 1(a)) were allowed to rotate up to 180°. Each cycle began with a random translational change ($\leq$5 Å) along with 10° of rotational movement followed by conjugate gradient energy minimization by 60 iterations. The conformation of the host cyclic oligosaccharides did not change during the process of developing a new configuration, but it could be changed in the process of energy minimization. In order to accept a new configuration the Metropolis criterion was applied at 300 K [9]. To eliminate the possibility of accepting a very similar configuration, the root mean square (RMS) displacement was checked after each trial. A new configuration within 0.1 Å RMS displacement was not accepted. Docking interaction energy, defined as the difference between the energy of separate molecules and the energy of molecules docked together, was calculated as the molecules were brought near each other. During the docking process the molecules were rotated and moved relative to each other to find the minimum energy orientation. To ensure convergence, we performed several independent runs of Monte Carlo docking simulations with some parameters changed: maximum translational displacement (4–10 Å), maximum number of changes in one trial, initial configurations of host and guest, maximum dihedral angle change and maximum rotational angle change. We also carried out simulated annealing molecular dynamics simulation to search for the global energy minimum. The simulated annealing MD was performed by five cycles of gradual temperature change between 1000 and 300 K for 140 ps.

3. Results and Discussion

Neutral cyclosophoraoses from *Rhizobium meliloti* 2011 were isolated and purified using several chromatographic techniques. Gas chromatography of the alditol acetates from fully methylated cyclosophoraoses showed a single peak, which was identified as the alditol acetate from 3,4,6-tri-O-methyl-D-glucose, confirming a cyclic structure composed of (1-2)-β-D-glucose residues. Their exact structures were confirmed by HMQC (heteronuclear mutiple-quantum correlation) NMR spectrum (data not shown). Figure 2 shows the MALDI (matrix assisted laser desorption/ionization)-MS (mass spectrometry) spectrum of purified neutral cyclosophoraoses using DHB as matrix. This spectrum contains several peaks up to m/z 4401 corresponding to the [M + Na^+] pseudomolecular ions from 17-mers (cyclosophoraose with DP 17) to 27-mers(cyclosophoraose with DP 27) at m/z of 2780 + 162 n. Other very weak signals corresponding to the protonated mo-

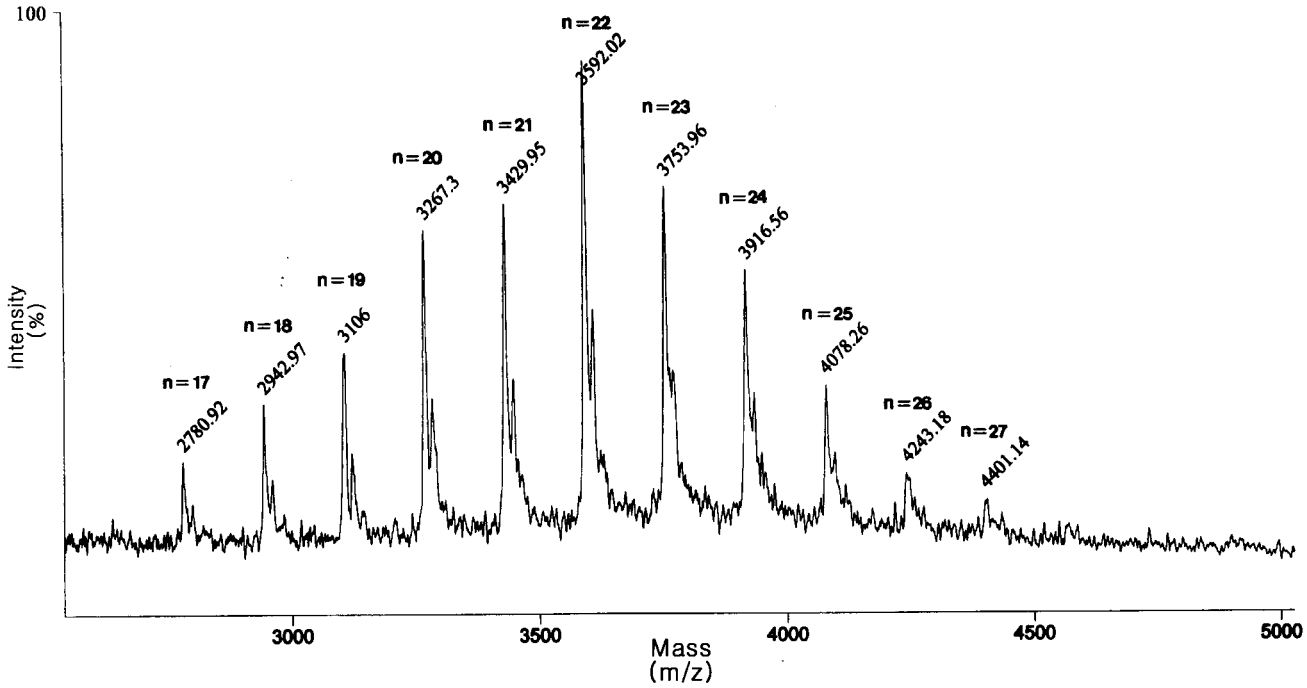

Figure 2. MALDI mass spectrum of purified neutral cyclosophoraoses. It contains several peaks up to m/z 4401 corresponding to the [M + Na^+] pseudomolecular ions from 17-mers (cyclosophoraose with DP 17) to 27-mers (cyclosophoraose with DP 27) at m/z of 2780 + 162 n.

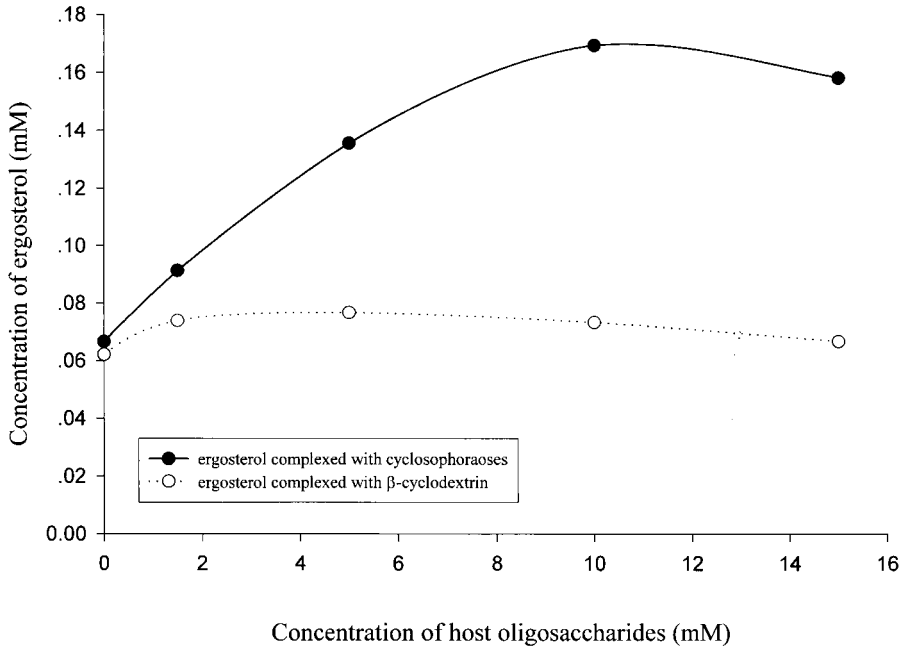

Figure 3. Solubility increase of ergosterol in cyclosophoraose and β-cyclodextrin solutions. A 15-mM ergosterol solution was mixed with 1.5, 5, 10 and 15 mM of cyclic oligosaccharides solutions. The solubility of ergosterol at each stage was analyzed by isocratic HPLC (ethanol : water = 55 : 45) at 265 nm.

lecules are also present. No fragment peaks are shown. Notice that major forms of cyclosophoraoses are ranged in 21-mers to 24-mers.

This isolated family of neutral cyclosophoraoses was used for the inclusion complex formation. With various concentrations of two host oligosaccharides; cyclosophoraoses and β-cyclodextrin, the solubility of ergosterol as a guest molecule was investigated. Figure 3 shows the solubility change of ergosterol in a family of cyclosophoraoses and β-cyclodextrin solutions. Compared with β-cyclodextrin, the solubility of ergosterol was greatly enhanced when cyclosophoraoses were used as host molecules. This enhancement of solubility might be explained by the relatively easy formation of the inclusion complex of a family of cyclosophoraoses with ergosterol. Its relatively large and various size of glucose rings would provide the basis for complexation of large hydrophobic molecules like ergosterol. If this were true, the corresponding change of thermodynamic preferential stability of complexed molecules would be expected.

This phenomenon was further investigated with computer simulation based on the supposed molecular models of β-cyclodextrin (CD) and cyclosophoraose containing 21 glucose rings (Cys-21mer) which was proposed by Palleschi and Crescenzi [7]. We chose a Monte Carlo technique because of its proven effectiveness in a wide range of minimization problems and its random nature allowing

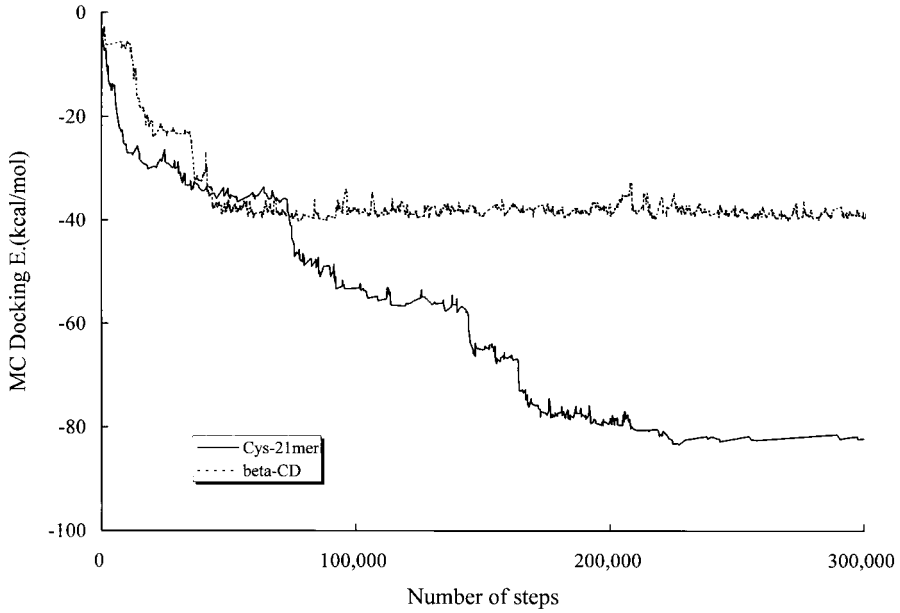

Figure 4. Energy profile of Metropolis Monte Carlo docking simulation. The MC docking energy was defined as the difference between the sum of independently calculated energy of each host-guest molecule and the energy of each configuration in the process of MC docking simulation.

for many different avenues in configuration space to be explored in a complete fashion. The MC docking energy was defined as the difference between the sum of the independently calculated energy of each host-guest molecule and the energy of each configuration in the process of MC docking simulation. Figure 4 shows the energy profile of Metropolis Monte Carlo docking simulation of Cys-21mer and β-CD with ergosterol. In the case of β-CD, docking energy became stabilized before 100,000 MC-minimization steps compared with Cys-21mer stabilized after 200,000 steps. This may indicate that there were multiple pathways for Cys-21mer to become a stable complex. The presence of several pathways for Cys-21mer during the stabilization could possibly be caused by the bigger, complicated hydrophobic hole of molecular conformation of Cys-21mer compared with β-CD. In order to ensure convergence of Cys-21mer, we performed several independent runs of Monte Carlo docking simulations and simulated annealing molecular dynamics simulation. But, only structures similar to those above were found.

Figure 5 showed snapshots of the β-cyclodextrin- and Cys-21mer-ergosterol complex, respectively, during the MC docking minimization simulation. These results may indicate that unlike β-CD, Cys-21mer itself could also rearrange the local molecular conformation inside the pocket for further energetic preferential stability. Whereas, there were no critical variations of local conformations of β-CD. Thus, it could be pointed out that cyclosophoraoses formed the inclusion complex

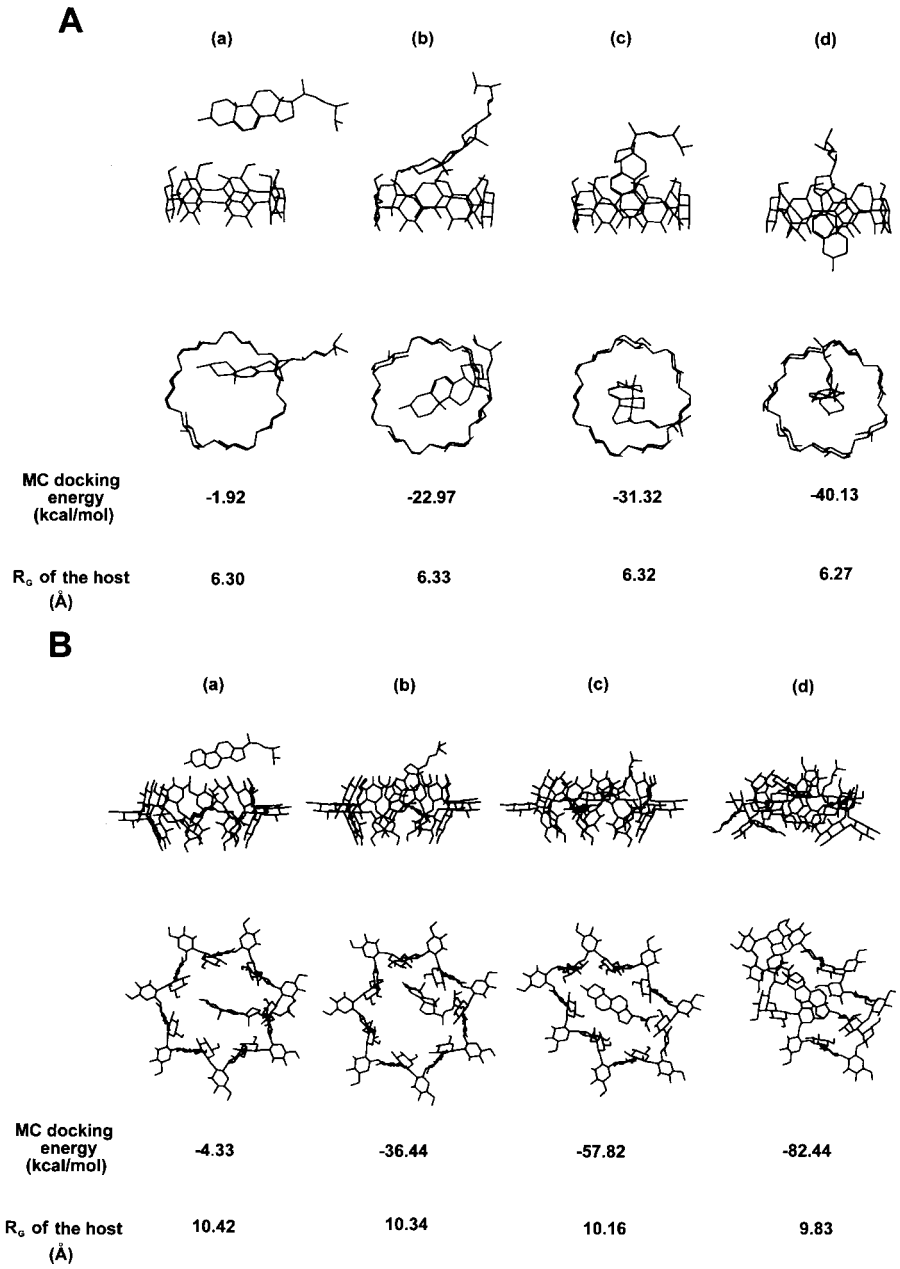

Figure 5. (A) Snapshots of β-cyclodextrin-ergosterol complex during the MC docking minimization simulation. The MC docking energies and radius of gyration (R_G) were displayed below each configuration: (a) the initial configuration; (b) step 26,220 of MC-minimization; (c) step 41,820 of MC-minimization; and (d) the lowest energy configuration of the inclusion complex (step 77,580). (B) Snapshots of cyclosophoraose-ergosterol complex during the MC docking minimization simulation. The MC docking energies and radius of gyration (R_G) were shown below each configuration: (a) the initial configuration; (b) step 53,520 of MC-minimization; (c) step 139,800 of MC-minimization; and (d) the lowest energy configuration of the inclusion complex (step 227,100).

more dynamically than β-CD. This flexibility of cyclosophoraoses appears to be essential for effective inclusion complex formation. Theoretical calculations of the Radius of Gyration (R_G) from the atomic coordinates of the Cys-21mer and β-CD during the MC-minimization process shows that the molecular conformation of Cys-21mer became contracted and indented in an irregular doughnut-like form, while that of β-CD kept almost constant (Figure 5). It should also be noted that R_G calculated from the molecular model of Cys-21mer decreased from 10.4 to 9.8 Å compared to β-CD from 6.30 to 6.27 Å during the simulation. The calculated R_G of Cys-21mer was further reduced when molecular dynamic simulations were performed without guest molecules (data not shown). The flexibility of the spacious glucose rings in complexed Cys-21mer allowed R_G changes for further energetic stability of its conformation.

Recent research into the conformation of cyclosophoraoses in aqueous solution with SAXS (small angle X-ray scattering) indicated that its molecular conformation was an irregular doughnut-like ring with a thick cylindrical shape [10–12]. This irregular doughnut-like ring structure could be interpreted as a time average snapshot during the dynamic docking mechanism. The MC-minimization simulation indicated that Cys-21mer could form a thermodynamically more stable inclusion complex with ergosterol than β-CD, as expected by HPLC analysis.

In the present investigation, we tried to show that a family of cyclosophoraoses purified from *Rhizobium meliloti* 2011 could successfully make a better complex with ergosterol compared with β-CD. This is in contrast to the result of Cys-17mer with which no complexation was observed. We suggest from the MC docking simulation that the complex formation may be caused by the preferential thermodynamic stability of complexed cyclosophoraoses. The selection of Cys-21mer for the simulation was determined, as it was one of the major forms in a family of cyclosophoraoses from the MALDI-MS analysis. Our study on the cyclosophoraose complex suggests that a family of cyclosophoraoses can be more useful in complexation with hardly soluble molecules than a single isolated one.

Conclusively, we propose a “hand-shake” fit in induced-fit paradigm for complexation of cyclosophoraoses with ergosterol, which suggests induced complementariness and the flexibility in the host compared to the relatively static type observed in β-CD.

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References

1. M. Abe, A. Amemura and S. Higashi: *Plant Soil* **64**, 315 (1982).
2. A. Amemura: *Agric. Biol. Chem.* **48**, 1809 (1984).
3. M. W. Breedveld, L. P. T. M. Zevenhuizen and A. J. B. Zehnder: *J. Gen. Microbiol*. **136**, 251 (1990).
4. H. R. G. Clarke, J. A. Leigh and C. J. Douglas: *Cell* **71**, 191 (1993).
5. K. Koizumi, Y. Okada, S. Horiyama, T. Utamura, T. Higashiura and M. Ikeda: *J. Incl. Phenom*. **2**, 891 (1984).
6. P. Dauber-Osguthorpe, V. A. Roberts, D. J. Osguthorpe, J. Wolff, M. Genest and A. T. Hagler: *Proteins: Structure, Functions and Genetics* **4**, 31 (1988).
7. A. Palleschi and V. Crescenzi: *Gazz. Chim. Ital*. **115**, 243 (1985).
8. R. Fletcher: *Understanding Optimization*, Vol. 1, John Wiley, New York (1980).
9. N. Metropolis, A.W. Rosenbluth, M.N. Rosenbluth, A.H. Teller and E. Teller: *J. Chem. Phys*. **21**, 1087 (1953).
10. W. York, J. Thomsen and B. Meyer: *Carbohydr. Res.* **248**, 55 (1993).
11. I. Andre, K. Mazeau, F. Taravel and I. Tvaroska: *Int. J. Biol. Macromol*. **17**, 189 (1995).
12. M. Mimura, S. Kitamura, S. Gotoh, K. Takeo, H. Urakawa and K. Kajiwara: *Carbohyr. Res*. **289**, 25 (1996).