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Interval immobilization technique for recognition toward a highly hydrophilic cyanobacterium toxin

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Abstract

A novel adsorption medium containing selective molecular recognition site for one of the powerful cyanobacterium toxins, Cylindrospermopsin (CYN) was developed using a special technique, namely interval immobilization technique. The adsorption medium was prepared using molecular assembly derived from an alternative-template molecule coupled with functional monomers for fixing the interval between the ionic functional groups in CYN. As results of liquid chromatographic evaluations, selective molecular recognition ability for CYN was observed as expected. Further studies proved that the association constant for CYN on this medium was slightly higher than that on blank polymer.

Keywords: Interval immobilization technique; Cylindrospermopsin

1. Introduction

Molecular imprinting technique is attractive to obtain selective molecular recognition ability for a certain compound and have been applied for some adsorption media [1–6]. However, in the traditional molecular imprinting technique, the real molecule should be required as the template molecule so that rare naturally occurring compounds and/or highly toxic compounds can be hardly utilized as the templates [7].

Therefore, some imprinting methods without real target molecule have been reported [8–11] and we have also proposed some "dummy" imprinting methods, where some alternative-template molecules were utilized instead of real target template molecules [12–16]. Although structurally close compounds to the real target molecule have been utilized as template molecules, those compounds tend to be limited to relatively hydrophobic compounds according to hydrophobic characteristics of monomers and solvents utilized in the molecular imprinting technique.

In fact, there are a lot of naturally occurring, highly hydrophilic toxic compounds in the environmental water. Especially, the toxic compounds produced by cyanobacteria bloom (blue-green algae) in the lake or pond have been one of the biggest problems because majority of these toxic compounds are only water soluble, so that these are in danger of doing harm to humans and the domestic animals through the consumption of contaminated water [17–20].

Therefore, it will be quite important to avoid the possibility in exposure to the damage by the toxins. To achieve this, the quantitative analysis as well as removal of these toxic compounds will be essential. However, a variety of foreign substances should prevent the quantitative analysis of the toxins because the concentration of toxins is typically pretty low (even ppt level) in the environmental water, and the toxicity is expressed at even ppt level.

Moreover, commonly used pre-treatment method such as solid-phase extraction (SPE) using hydrophobic adsorbent contributes little to that of the toxins due to the

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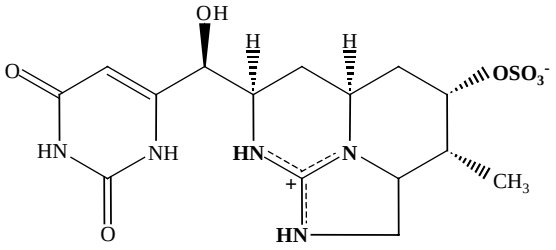

LD_{50}, 200 μg/kg (mice)

Cylindrospermopsin

Fig. 1. Structure of Cylindrospermopsin (CYN).

highly hydrophilic characteristics based on ionic functional groups in those. Therefore, an alternative pre-treatment procedure with high selectivity as well as concentration ability for the target toxins is needed. This can also realize complete removal of toxic compounds from environmental water.

In this paper, we wish to propose new adsorption medium for one of the powerful cyanobacterium hepatotoxins, Cylindrospermopsin (CYN) as shown in Fig. 1 [21–23]. CYN is one of the cyanobacterium toxins and known as powerful hepatotoxin produced by *Cylindrospermopsis raciborskii*, *Umezakia natans*, and *Aphanizomenon ovalisporum*. CYN is composed of tricyclic guanidine moiety combined with hydroxymethyl uracil and has a molecular weight of 415 Da. Moreover, the 24 h LD_{50} (mice) was found to be 200 μg/kg.

For the analysis of CYN, the useful and selective preparation method has not been established although some reports had already published [21,24,25]. Pre-treatment process by using SPE with several commercially adsorption media was utilized in above analysis methods, however, these medium have no-selectivity for CYN. Therefore, we propose that the novel adsorption medium prepared through interval immobilization technique.

In this technique, the interval between the two ionic functional groups is immobilized on cross-linked polymer using molecular assembly with appropriate alternative-template molecule coupled with some ionic functional monomers. The concept of this technique for CYN is illustrated in Fig. 2; the alternative-template molecule to be utilized also has ionic functional groups.

In fact, the resulting the molecular assembly was utilized, therefore, highly hydrophilic characteristics of the alternative-template molecule were presumably converted to be relatively hydrophobic assembly. We think that this technique can realize creation of the specific interval-immobilized and homogeneous recognition site for highly hydrophilic molecules. The alternative-template molecule to fix the interval can be designed with computer modeling with calculating the interval of ionic groups to be synthesized.

The selectivity for CYN of prepared adsorption medium was examined with high-performance liquid chromatography (HPLC). Moreover, the association constant for CYN or the number of binding sites was studied by Scatchard plot on batch condition.

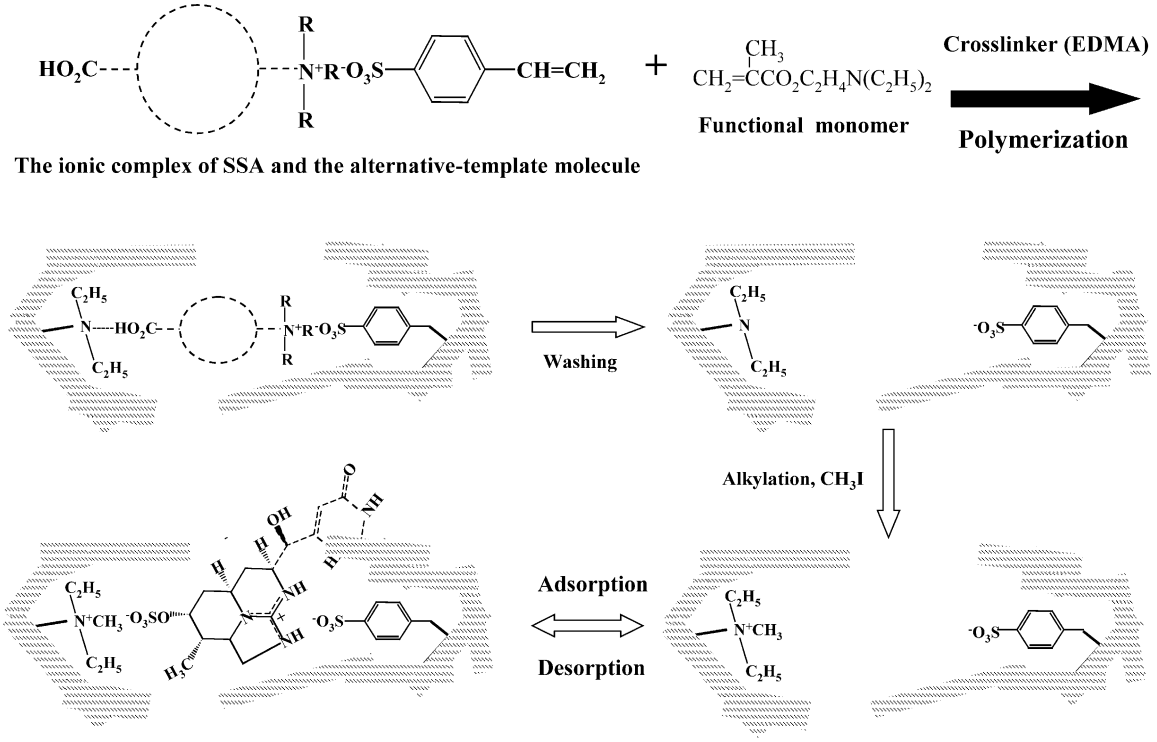

Fig. 2. Concept of interval immobilization technique for CYN.

2. Experimental

2.1. Materials

Ethylene glycol dimethacrylate (EDMA), 2-(diethylamino)-ethyl methacrylate (DAEMA) were purified by usually vacuum distillation to remove polymerization inhibitors. 2,2′-Azobis-(2,4-dimethylvaleronitrile) (ADVN), tetrabutylammonium chloride (TBA), and tributylamine were purchased from Tokyo Kasei (Tokyo Japan). Methyl 4-(bromomethyl)-benzoate was purchased from Aldrich (USA). Methyl iodide, *p*-styrenesulfonic acid sodium salt (SSA), methanol (MeOH), acetonitrile (AN), ethanol (EtOH), chloroform, dimethyl sulfoxide (DMSO) and NaCl were purchased from Wako Chemicals (Kyoto Japan) and utilized as appropriate purifications.

2.2. Isolation of CYN

A toxic strain of *C. raciborskii* (CRJ-1 = AWT 205) was obtained from Microbial Culture Collection (MCC-NIES) and grown in CT medium. Cells were separated from medium by a centrifuge, and lyophilized.

CYN was extracted according to the method [21]. The extracted CYN was purified by HPLC on an Amide-80 column (10 mm × 250 mm, Tosoh Corporation, Japan) with 60–100% aqueous AN linear gradient for 20 min at 4.0 ml/min. The isolated CYN was identified by NMR and MS. These spectrometric data were agrees well with those of CYN [21].

2.3. Synthesis of the alternative-template molecule

Methyl 4-(bromomethyl)-benzoate (1.5 g, 6.9 mmol) and 1.0 g of K_2CO_3 were dissolved in 50 ml of AN. Tributylamine (1.0 ml, 4.2 mmol) was added slowly into the solution with stirring. After addition of tributylamine, the mixture was stirred at 100 °C for 24 h under nitrogen atmosphere with refluxing. The confirmation of formation of the purposed compound was carried out by thin layer chromatography (TLC). After the reaction was completed, the reaction mixture was separated with silica gel column chromatography ($CHCl_3$/MeOH = 9/1) to isolate tributyl-(4-methoxycarbonyl-benzyl)-ammonium bromide. Resulting tributyl-(4-methoxy-carbonyl-benzyl)-ammonium bromide was treated with 50 ml of 1.0 M HCl aqueous solution at 120 °C for 24 h under nitrogen atmosphere. After the hydrolysis of methyl ester was confirmed by TLC, the resulting compound, tributyl-(4-carboxybenzyl)-ammonium chloride (TCBA), was extracted with chloroform and purified with silica gel column chromatography ($CHCl_3$/MeOH = 6/4). The chemical yield of the compound was as high as 90.2% (calculated from amount of tributylamine). ^{1}H NMR, 500 MHz (CD_3OD) (δ, ppm): 0.97 (m, 12H), 1.36 (m, 8H), 1.76 (m, 8H), 3.13 (m, 8H), 4.78 (s, 2H), 7.42 (d, 2H), 7.97 (d, 2H).

2.4. Preparation of polymer

An ionic complex of TBA and SSA was prepared in order to dissolve SSA in organic porogen because pure SSA could not be dissolved in organic solvent at all. Preparation of the complex are achieved as follows; SSA and TBA was dissolved in water and extracted with chloroform, where the mole ratio between SSA and TBA was in 2:1, through a phase transfer effect of the molecular assembly formed. After the removal of chloroform, transferred ionic complex could be obtained. The obtained ionic complex could be easily dissolved in any organic solvent. In the case of the alternative-template molecule (TCBA), the ionic complex with SSA was also prepared. The symbols and compositions of bulk polymers prepared are summarized in Table 1. All bulk polymers were polymerized with 1.0 wt.% of ADVN at 50 °C for 24 h, after polymerization, the polymers were grinded and washed with MeOH. Then, the polymers apart from P1 were reacted with CH_3I to generate the alkylammonium groups. The reaction was carried out in DMSO at 70 °C for 24 h as shown in Fig. 2.

2.5. FT-IR spectra of the alternative-template molecule and ionic functional monomer

To confirm the interaction between the alternative-template molecule and functional monomers, a FT-IR measurement (FTIR-8100M, Shimadzu, Japan) was carried out using DAEMA, TCBA and the ionic complex of SSA and TCBA in the polymerization porogen. (AN/EtOH = 1/1).

Table 1
Composition of polymers

Symbol	Crosslinking agent	Porogen	Monomers	The alternative-template molecule
P1	EDMA (26.5 mmol)	AN/EtOH (1/1, 5 ml)	–	–
P-Non-Tem 1	EDMA (26.5 mmol)	AN/EtOH (1/1, 5 ml)	DAEMA (1.0 mmol), SSA (1.0 mmol)	–
P-Tem 1	EDMA (26.5 mmol)	AN/EtOH (1/1, 5 ml)	DAEMA (1.0 mmol), SSA (1.0 mmol)	TCBA (1.0 mmol)
P-Non-Tem 2	EDMA (26.5 mmol)	AN/EtOH (1/1, 5 ml)	DAEMA (2.0 mmol), SSA (2.0 mmol)	–
P-Tem 2	EDMA (26.5 mmol)	AN/EtOH (1/1, 5 ml)	DAEMA (2.0 mmol), SSA (2.0 mmol)	TCBA (2.0 mmol)

EDMA; Ethyleneglycol dimethacrylate, DAEMA; 2-(Diethylamino)-ethyl methacrylate, SSA; *p*-styrene sulfonic acid sodium salt, TCBA; Tributyl-(4-carboxybenzyl)-ammonium chloride.

2.6. HPLC evaluation for polymers

Each polymer was size classified at the range of 25–45 μm to be packed into chromatographic columns. The polymer particles were packed into stainless steel columns using slurry method and evaluated by HPLC. Aqueous MeOH solution (MeOH/1.0 M aqueous NaCl = 9/1) was used as the mobile phase in HPLC because the retention of ionic compounds was too large to be detected under non-salted or low-salt concentration mobile phase condition. Chromatographic data were acquired with a HPLC system (Shimadzu), consisting from a LC-6A as a pump, a SPD-M10A as a photodiode array detector, a CTO-10AC as a column oven.

2.7. Scatchard plot

CYN solutions were prepared with appropriate concentrations: 10^{-4} to 1.0 mM in 90% MeOH aqueous solution. Each CYN solution of 1.0 ml was added to vials containing 10 mg of P-Non-Tem 2 or P-Tem 2. After 12 h, where the vials were shaken with regular time interval at 25 °C, the amount of free CYN in the supernatant was determined with HPLC–MS using an external standard calibration. For HPLC conditions on the amount quantify for CYN, Amide-80 column (100 mm × 2.0 mm, Tosoh Corporation) was used with AN aqueous solution linear gradient (from 100 to 60%) for 20 min at 0.2 ml/min. The detection of CYN was achieved with MS-SIM at *m/z* −414.

3. Results and discussion

3.1. Design of the alternative-template molecule

To immobilize interval of the ionic functional groups on the cross-linked polymer, the alternative-template molecule was designed by a computer modeling out of consideration of the interval between the ionic groups of CYN. The structure and calculated interval of functional groups are revealed in Fig. 3.

According to the computer modeling, the interval of the ionic groups of CYN is as long as 6.242 Å. At first, although some candidates for the alternative-template molecule were searched among commercial compounds, no suitable compound was found. Consequentially, we prepared appropriate alternative-template molecule. We finally decided to select tributyl-(4-carboxybenzyl)-ammonium having the interval 6.708 Å. This interval is slightly longer than that of CYN, but this molecule involves relatively rigid phenyl group in it.

3.2. FT-IR

Results in FT-IR evaluation for the alternative-template molecule and functional monomers in polymerization porogen are shown in Fig. 4. With spectra of TCBA (2) and the ionic complex of SSA and TCBA (4), the specific adsorption band based on the sulfonyl group was observed around 1250 cm^{-1}. Moreover, SSA itself could not be soluble in any solvents except in water; however, the ionic complex of SSA and TCBA could be soluble quite easily in the polymerization porogen. These observations suggest that the ionic complex formed between SSA and TCBA was stably formed in AN/EtOH.

Additionally, with spectra of DAEMA (1), TCBA (2), DAEMA + TCBA (3) and DAEMA + SSA–TCBA (5), the specific adsorption based on a hydrogen bonding between carboxylic acid and amine was observed around 1600 cm^{-1} in (3) and (5), whereas the adsorption could not be obtained in independent DAEMA (1) or TCBA (2). These results presumably suggest that the alternative-template molecule and two kinds of functional monomers could be interacted in each other into the polymerization mixture to form the molecular assembly. This should be very important for the following molecular imprinting.

3.3. HPLC evaluation

Results in HPLC evaluations with each polymer are shown in Fig. 5. In these experiments, the aqueous solution containing 1.0 M NaCl was used as the mobile phase because the retention of the ionic solutes as well as CYN was too large to be detected as effective peaks in lower NaCl concentration conditions.

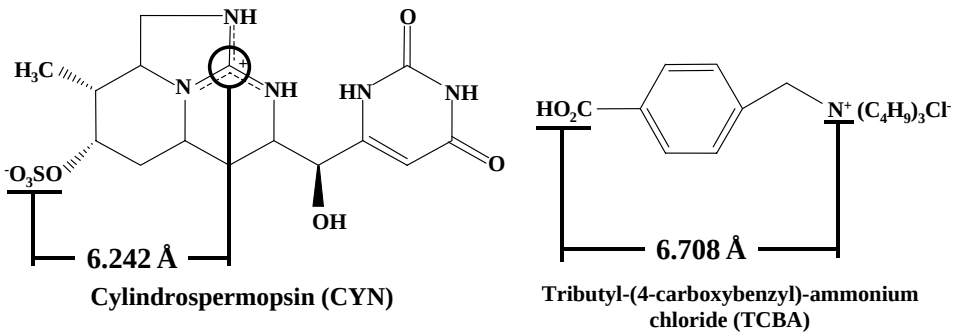

Fig. 3. Comparison of interval of ionic groups in CYN and the alternative-template molecule.

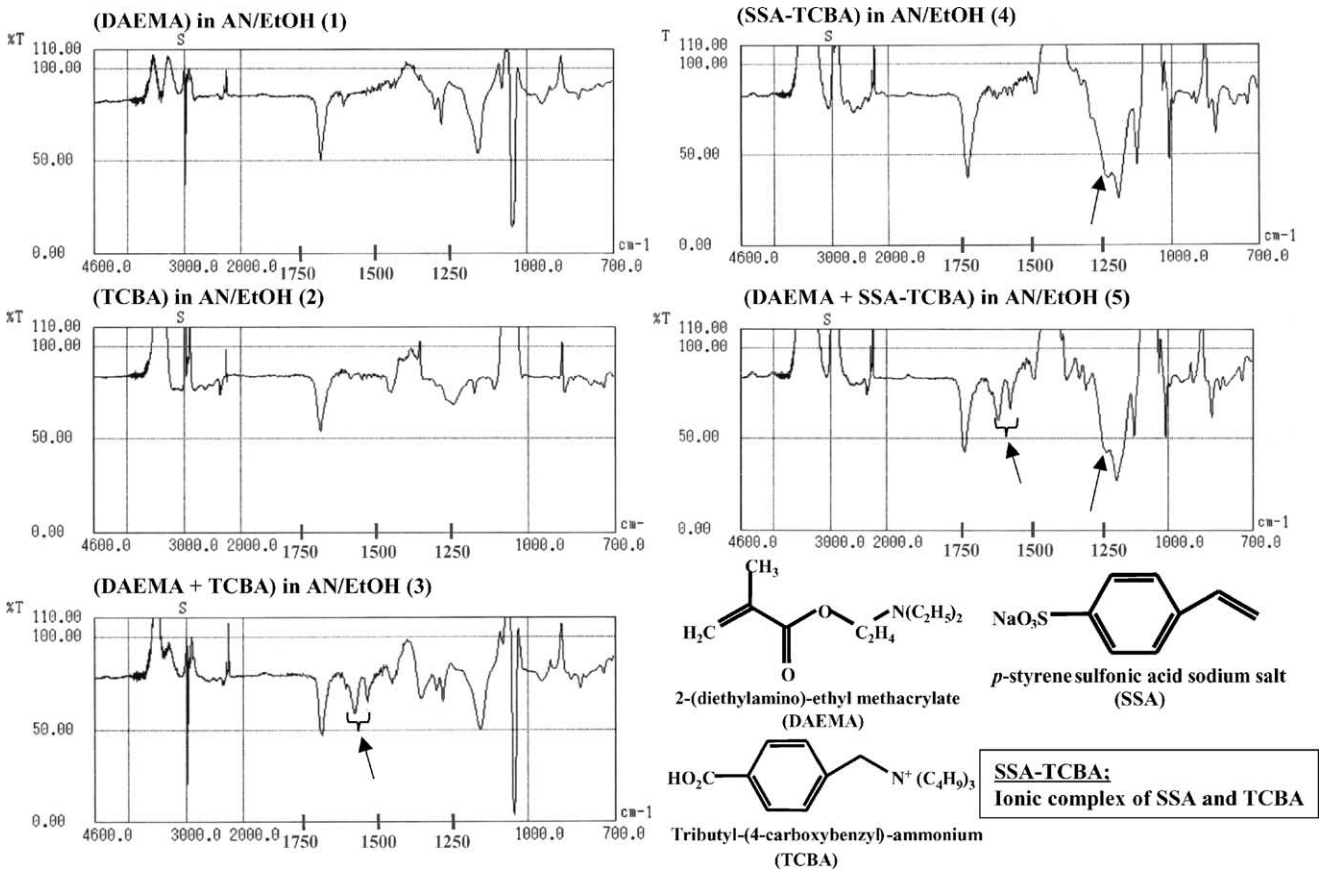

Fig. 4. FT-IR spectra of the alternative-template molecule and ionic functional monomers.

According to the result shown in Fig. 5, it seems that the ionic functional groups were arranged within the polymer structure on the polymers apart from P1 because the retention factor k' of the ionic solutes was basically larger than those on P1. (On P1, all ionic solutes were not retained at all.) On the other hand, k' of non-ionic solute, benzene was almost same on all polymers. Moreover, the retention effect observed above was much larger on the adsorption medium, P-Non-Tem 2 and P-Tem 2, which were prepared with twice ratio of the functional monomers against P-Non-Tem 1 and P-Tem 1.

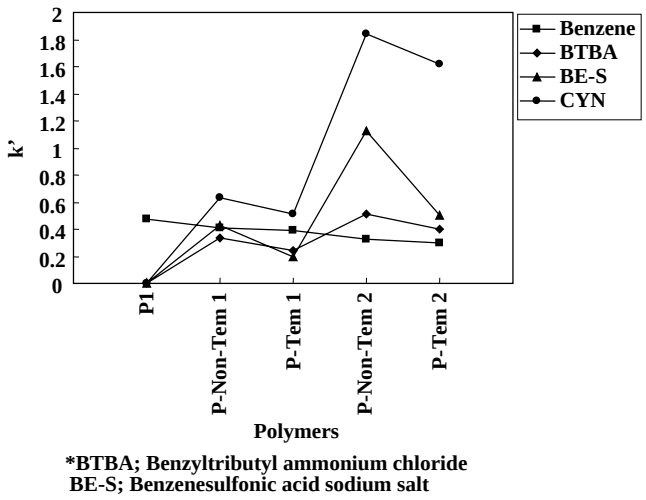

Fig. 5. Comparison of k' on each polymer. HPLC conditions: mobile phase, MeOH/1.0 M aqueous NaCl = 9/1; column size, 100 mm × 4.6 mm (i.d.); flow rate, 0.5 ml/min; detection, photo diode array; temperature, 30 °C.

In addition, the selectivity for CYN was also much larger on P-Non-Tem 2 and P-Tem 2. If we take a look at retention factor k' itself, which directly suggest retention power, it seems that the effect of usage of the alternative-template molecule was negative, where the retention of ionic solutes was found to be smaller on the adsorption medium prepared using the alternative-template molecule like in P-Non-Tem 1 and P-Tem 1.

Consequentially, the comparison of the separation factor α values of CYN against another solutes is the better way to show the selectivity for CYN. The comparison is shown in Fig. 6. In comparison of the continuous lines between P-Non-Tem 1 and P-Non-Tem 2, as well as P-Tem 1 and P-Tem 2, it is understood that the introduction of both ionic functional monomers into the polymers enlarged the retention selectivity for CYN. This is probably because CYN involves two opposite ionic moieties in it.If we compare the effect of the alternative-template through the differences in α between P-Non-Tem 1 and P-Tem 1 as well as

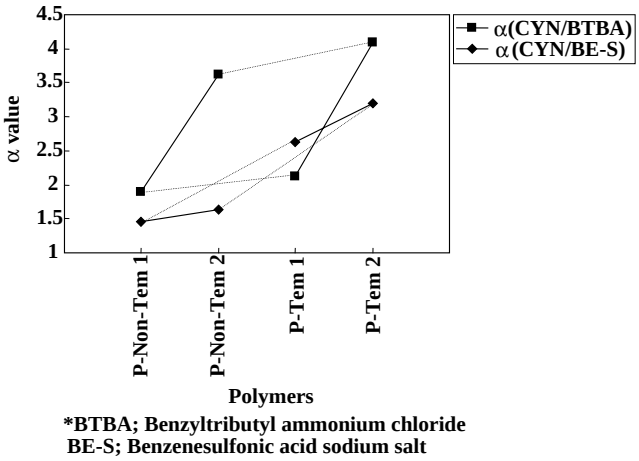

Fig. 6. Comparison of α value on each polymer. HPLC conditions: mobile phase, MeOH/1.0 M aqueous NaCl = 9/1; column size, 100 mm × 4.6 mm (i.d.); flow rate, 0.5 ml/min; detection, photo diode array; temperature, 30 °C.

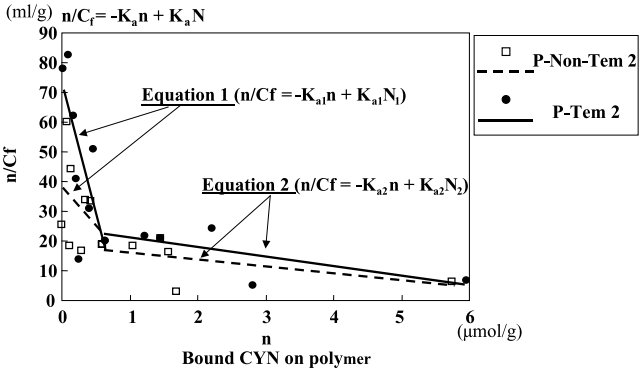

Fig. 7. Scatchard plot for CYN on P-Non-Tem 2 and P-Tem 2 (adapted from [29], Fig. 4). The association constant (K_a) and number of binding sites (N) was determined from the slope and y-intercept, respectively, of fitted line ($n/C_f = -K_a n + K_a N$) obtained by least squares regression.

P-Non-Tem 2 and P-Tem 2, it is clearly found the effect of the alternative-template molecule. Especially, the effect was even larger on the value against benzenesulfonic sodium salt (BE-S) by using the alternative-template molecule. Therefore, it strongly suggests that these results indicated the immobilization of DAEMA on polymerization by the hydrogen bonding between carboxylic acid of the alternative-template molecule and amine of DAEMA.

Additionally, more detail results of comparison for separation factor are show in Table 2. As shown Table 2, the relative comparison concerning with or without the alternative-template molecule was slightly higher in P-Tem 2/P-Non-Tem 2. Therefore, these results presumably suggest that larger number of accurate recognition sites for CYN were formed according to use larger amount of the alternative-template molecule.

3.4. Scatchard plot

To obtain more detailed information about recognition effect for CYN, the Scatchard plot on batch condition was carried out because this evaluation was often operated for examination of association constant in molecular imprinting [26–28]. In facts, the results of Scatchard plot have been described in our previous paper [29]. Based on the results and analysis, the association constant K_a and number of binding sites N for CYN were calculated according to Langmuir model based on Scatchard plot. The results of Scatchard plots are shown in Fig. 7 and the results are briefly summarized in Table 3.

Table 3 shows that the association constant and number of binding sites was similar in each other on both polymers, and the distribution of plots was much broader at higher CYN concentration conditions. On the other hand, P-Tem 2 had slightly higher association constant for CYN and narrower distribution of plots than those on P-Non-Tem 2 in lower CYN concentration conditions. These notable differences suggest that the polymer prepared through interval immobilization technique, P-Tem 2 has somewhat of high-affinity binding sites for CYN illustrated in Fig. 2.

According to these results of Scatchard plot, it seems that certain interval (length) of specific binding sites for CYN were formed onto P-Tem 2 by interval immobilization technique for ionic groups so that high association constant was obtained on the polymer, P-Tem 2.

The N value was larger on P-Non-Tem 2 than that on P-Tem 2. But, Scatchard plot based on batch adsorption strongly suggests that the specific recognition sites for CYN were formed within the polymer prepared through interval immobilization technique as in P-Tem 2, while on P-Non-Tem 2, non-specific binding sites might be dominantly formed.

In consequence, lower retention ability for CYN on polymers prepared with the alternative-template was observed in HPLC evaluations. It follows that the specific recognition site were constructed with two ionic groups by the interval immobilization, therefore, total binding sites for CYN on the polymer prepared with the alternative-template molecule

Table 2
The relative ratio of α value

	P-Tem 1/P-Non-Tem 1	P-Tem 2/P-Non-Tem 2
α(CYN/BTBA)	1.11	1.13
α(CYN/BE-S)	1.79	1.96

BE-S: benzenesulfonic sodium salt; BTBA: benzyltributyl ammonium chloride.

Table 3
Binding parameter determined by Scatchard plot (adapted from [29])

	K_{a1} ($\times 10^4$ M^{-1})	K_{a2} ($\times 10^3$ M^{-1})	N_1 (μmol/g)	N_2 (μmol/g)
P-Non-Tem 2	2.7	2.3	1.4	8.1
P-Tem 2	8.9	3.2	0.82	7.6

was less than those on non-template polymers so that the retention of ionic solutes was decreased.

4. Conclusion

The novel adsorption medium for cyanobacterium Cylindrospermopsin, through the "interval immobilization technique" that is specific immobilization method of certain ionic functional monomers into the cross-linked polymer structure with the alternative-template molecule, recognized CYN accurately on evaluations of HPLC and Scatchard plot.

The target molecule utilized in this study, CYN involves opposite ionic group in it. Therefore, the immobilization of functional monomers onto the polymer matrix was not so easy. However, this technique can be expected as novel selective pre-treatment media for highly hydrophilic natural toxic compounds and useful purification method for the environmental water. We will examine for other compounds through this technique in the future.

Acknowledgements

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References

[1] G. Wulff, A. Sarhan, Angew. Chem. 84 (1972) 364.
[2] B. Sellergren, B. Ekberg, K. Mosbach, J. Chromatogr. 347 (1985) 1.
[3] K. Nilsson, J. Lindell, O. Norrlow, B. Sellergren, J. Chromatogr. A 680 (1994) 57.
[4] M. Kempe, K. Mosbach, J. Chromatogr. A 691 (1995) 317.
[5] M. Kempe, L. Fischer, K. Mosbach, J. Mol. Recognit. 6 (1993) 25.
[6] D. Spivak, K.J. Shea, J. Org. Chem. 64 (1999) 4627.
[7] J. Haginaka, H. Sanbe, Anal. Chem. 72 (2000) 5206.
[8] J. Matsui, Y. Miyoshi, O. Doblhoff-Dier, T. Takeuchi, Anal. Chem. 67 (1995) 4404.
[9] J. Matsui, Y. Miyoshi, T. Takeuchi, Chromatographia 17 (1996) 332.
[10] P. Martin, I.D. Wilson, D.E. Morgan, G.R. Jones, K. Jones, Anal. Commun. 34 (1997) 45.
[11] J. Jodlbauer, N.M. Maier, W. Lindner, J. Chromatogr. A 945 (2002) 45.
[12] K. Yoshizako, K. Hosoya, Y. Iwakoshi, K. Kimata, N. Tanaka, Anal. Chem. 70 (2) (1998) 386.
[13] K. Hosoya, K. Yoshizako, H. Sasaki, K. Kimata, N. Tanaka, J. Chromatogr. A 828 (1–2) (1998) 91.
[14] K. Hosoya, Y. Iwakoshi, K. Yoshizako, K. Kimata, N. Tanaka, H. Takehira, J. Haginaka, HRC J. High Resolut. Chromatogr. 22 (5) (1999) 256.
[15] K. Hosoya, K. Yoshizako, T. Kubo, T. Ikegami, N. Tanaka, J. Haginaka, Anal. Sci. 18 (2002) 55.
[16] T. Kubo, K. Hosoya, Y. Watabe, T. Ikegami, N. Tanaka, T. Sano, K. Kaya, J. Chromatogr. A 987 (2003) 389.
[17] K. Kaya, M.M. Watanabe, Microbiol. Cult. Coll. 10 (1994) 53.
[18] G.A. Codd, J.S. Metcalf, K. Kaya, J. AOAC Int. 84 (2001) 1626.
[19] P.R. Hunter, J. Med. Microbiol. 36 (1992) 301.
[20] G.H. Elder, P.R. Hunter, G.A. Codd, Lancet 341 (1993) 1519.
[21] K. Harada, I. Ohtani, K. Terao, Toxicon 32 (1) (1994) 73.
[22] P.R. Hawkins, N.R. Chandrasena, I.R. Fralconer, Toxicon 35 (3) (1997) 341.
[23] R. Li, W.W. Carmichael, S. Brittain, K. Kaya, M.M. Watanabe, Toxicon 39 (2001) 973.
[24] R.L.G. Norris, G.K. Eaglesham, G.R. Shaw, P.R. Senogles, R.K. Chiswell, M.J. Smith, B.C. Davis, A.A. Seawright, M.R. Moore, Environ. Toxicol. 16 (2001) 391.
[25] J.S. Metcalf, K.A. Beattie, M.L. Saker, G.A. Codd, FEMS Microbiol. Lett. 216 (2002) 159.
[26] P. Sajonz, M. Kele, G.M. Zhong, B. Sellergren, G. Guiochon, J. Chromatogr. A 810 (1998) 1.
[27] M. Quaglia, K. Chenon, A.J. Hall, E. De Lorenzi, B. Sellergren, J. Am. Chem. Soc. 123 (2001) 2146.
[28] T. Takeuchi, T. Mukawa, J. Matsui, M. Higashi, K.D. Shimizu, Anal. Chem. 73 (2001) 3869.
[29] T. Kubo, N. Tanaka, K. Hosoya, Anal. Bioanal. Chem. 378 (2004) 84.