Study on Arsenic Methyltransferase Expressed in Recombinant E. coli

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Abstract

This study aimed to facilitate efficient arsenic detoxification by inducing the microbial methylation of inorganic arsenic. Recombinant Escherichia coli strains K63 and KC42 were transformed to overexpress arsenic methyltransferase, and the methylation of inorganic arsenic was evaluated using the enzyme extracted from these strains. To ensure continuous reactions by maintaining enzymatic activity, the extracted enzyme was immobilized in microcapsules (MC) to catalyze the methylation of inorganic arsenic. The total yield of methylated organic arsenic compounds in strain K63 was 32.9% (after 2 h of incubation at pH 7.0 and 35°C), of which trimethyl arsenic compounds (TMAC) accounted for 8.3%. The total yield in strain KC42 was 35.9% (after 2 h of incubation at pH 6.5 and 35°C), of which TMAC accounted for 10.8%. When arsenic was methylated using MC prepared with the crude enzyme solution, the total yield of methylated organic arsenic compounds was 12.6% and 5.7% in strains K63 and KC42, respectively. The residual enzymatic activity was calculated to be 53.1% and 48.8% in strains K63 and KC42, respectively. Future studies should aim to increase the residual enzymatic activity, thus elevating the yield of organic arsenic compounds, by optimizing the conditions for enzyme immobilization in MCs.

Key words: Detoxification of inorganic arsenic, Arsenic methyltransferase, Enzyme-immobilized microcapsule, Recombinant E. coli, Cellulomonas sp. K63, Bordetella petrii KC42

1. Introduction

Toxic inorganic arsenic found in natural environments (such as groundwater, soil, and factory wastewater) are removed using various treatment methods including aggregation/coprecipitation, adsorption, and reverse osmosis membranes. However, these approaches simply concentrate dissolved inorganic arsenic without eliminating its fundamental toxicity. Therefore, inorganic arsenic species must be eventually separated from our living environment via solidification or landfilling, necessitating special storage facilities and long-term monitoring. Therefore, a novel technique to eliminate its fundamental toxicity would be ideal to ensure the safety of the environment.

Since the latter half of the 1970s, it has been reported that the toxicity of arsenic compounds greatly differs depending on the difference in chemical structure and form. Among them, a methylation pathway for inorganic arsenic has been reported in marine organisms and mammals, supposedly accounting for a detoxification mechanism. Furthermore, it was suggested that the toxicity of arsenobetaine (AB) (a type of methylated organic arsenic) was 1/300 of that of inorganic arsenic. Currently, studies are evaluating the conversion of inorganic arsenic to AB. However, the problems associated with its low methylation efficiency and high cost still remain.

Arsenic methylation by bacteria is a well-known process. However, few microorganisms that produce AB or trimethylarsine oxide (TMAO), which is a precursor of AB, from inorganic arsenic have been discovered to date. Only Pseudomonas putida, Klebsiella oxytoca, and Xanthomonas sp. have been observed to produce TMAO in the culture medium. However, the yield of TMAO by these bacteria was <3%. Therefore, research on arsenic detoxification using microorganisms has not progressed.
We considered that the method using microorganisms, which can be prepared in large quantities and are easy to extract, is the most feasible in terms of cost for the methylation of inorganic arsenic. To date, arsenic-methylating bacteria (Cellulomonas sp. K63 and Bodetella petrii KC42) that can effectively convert inorganic arsenic to methylated organic arsenic have been isolated from arsenic-free soil in the Miyazaki Prefecture, Japan\textsuperscript{16–18}. These bacteria showed unprecedented high efficiency of arsenic methylation, and the production of AB could not be confirmed because of low conversion efficiency to TMAO. Consequently, the methylation of inorganic arsenic to the precursor TMAO is the most important for AB production. Furthermore, to explore the possibility of practical use, the cells were immobilized in a carrier and arsenic methylation was performed. Consequently, the methylation to monomethylarsonic acid (MMAA) and that to dimethylarsinic acid (DMAA) could be confirmed; however, the production of AB could not be confirmed because of low conversion efficiency to TMAO.

A recent study identified \textit{arsM} as the gene encoding As(III) \textit{S}-adenosyl-L-methionine (SAM) methyltransferase, which catalyzes the transfer of a methyl group from SAM to As(III)\textsuperscript{19}. To improve the conversion efficiency to TMAO, we decided to extract arsenic methyltransferase (ArsM), which catalyzes the methylation in the cells, from arsenic-methylating bacteria and directly evaluate the methylation \textit{in vitro}. However, this method was limited in that the yield of ArsM is low in wild-type strains. To increase the yield of ArsM, the \textit{arsM} gene was expressed in \textit{E. coli} using gene recombination technology. In this study, ArsM was overexpressed in recombinant \textit{E. coli} strains and the resultant crude enzymes were examined for methylation efficiency. Moreover, to ensure continuous methylation while maintaining enzyme activity, enzyme-immobilized microcapsules (MCs) were developed and tested for their performance in arsenic methylation.

2. Experiments

2.1 Preparation of recombinant \textit{E. coli}

To express \textit{arsM} from \textit{Cellulomonas} sp. K63 and \textit{B. petrii} KC42 in \textit{E. coli} cells, its open reading frame from the genomic DNA of each strain was amplified using polymerase chain reaction (PCR) using the corresponding primer set (F-primer; ATGAAGCTTATGAAAGAAGTG/ R-primer; TTAGGTACCATTTGTGCCG and F-primer; ATGAAGCTTATGAAAGAAGTG/ R-primer; TTAGGTACCATTTGTGCCG). The PCR product from each strain was cloned into the pFLAG-CTC expression vector (SIGMA-ALDRICH) to produce pFLAG-CTC-\textit{arsM}. The resultant vector was introduced into the \textit{E. coli} strain DH5α (TOYOBO). The recombinant \textit{E. coli} strain carrying the \textit{arsM} gene of \textit{Cellulomonas} sp. K63 or \textit{B. petrii} KC42 was selected as single colony on a Luria–Bertani (LB) plate [triptone 1.0% (w/v), yeast extract 0.5% (w/v), and sodium chloride 1.0% (w/v)] comprising glucose 0.4% (w/v), 100 mg/mL ampicillin 0.05% (v/v) and agar 2.0% (w/v), and \textit{E. coli} strain K63 or KC42, respectively.

2.2 Culture methods

The recombinant \textit{E. coli} strains were precultured at 37°C for 16 h with shaking at 140 strokes/min in LB media. For ArsM expression in the main culture, the LB medium was inoculated with 1.0% (v/v) of pre-culture and 100 mmol/L isopropyl \textit{β}-D-1-thiogalactopyranoside (IPTG) 0.5% (v/v). The main culture was incubated under shaking conditions at 140 strokes/min for 6 h at 37°C.

2.3 Preparation of crude enzyme solution

The recombinant \textit{E. coli} cells were collected from the main culture by centrifugation (14,000 rpm, 10 min) and then freeze-dried. The dried cells were suspended in purified water, disrupted using ultrasonication, and separated from the supernatant by centrifugation (14,000 rpm, 15 min). The resultant supernatant was used as the crude enzyme solution.

2.4 Preparation of enzyme-immobilized MC

To prepare an enzyme-immobilized MC, a water/oil (W/O) emulsion was initially prepared using an internal aqueous phase containing the enzyme and an organic phase containing a monomer that formed a supporting layer for the MC. Furthermore, the resultant emulsion was dispersed in the external aqueous phase to prepare a water/oil/water (W/O/W) emulsion. The resultant emulsion was exposed to light, and the monomers in the organic phase were polymerized by a photopolymerization reaction to prepare MC.

The detailed method is shown below. An internal aqueous phase was prepared by mixing the crude enzyme solution (1.0 mL) and 16.0 mL of McIlvaine buffer (pH 6.5). The solution was then mixed with 0.55 g of surfactant 818sx, 26.65 g of divinylbenzene, and 2.84 g of toluene and stirred overnight. Subsequently, 1.41 g of terephthalic acid chloride, 0.22 g of 4-(dimethylamino) ethyl benzoate, and 0.19 g of camphorquinone were added to form an organic phase. Surfactant Q12s
(3.15 g) was added to 410 mL of distilled water, which was heated to 80°C and stirred, after which 9.0 g of polyvinyl alcohol and 1.04 g of Na₂CO₃ were added and stirred overnight. Finally, L-lysine (1.14 g) was added to form an external aqueous phase.

The internal aqueous phase was added to the organic phase and homogenized for 5 min at 3,000 rpm. An additional ultrasonication was performed for 3 min to prepare a W/O emulsion, which was then added to the external aqueous phase and stirred at 500 rpm for 5 min to prepare a W/O/W emulsion. This W/O/W emulsion was stirred at 250 rpm for 6 h while being illuminated with light-emitting diode light to prepare MCs.

### 2.5 Arsenic methylation

To monitor the arsenic methylation, 0.25 mL of phosphate buffer (25 mmol/L, either pH 6.5 or 7.0), 0.05 mL of As(III) solution (10.0 mg As/L), 0.05 mL of SAM (20 mmol/L), and 0.05 mL of glutathione solution (GSH, 70 mmol/L) were added to the crude enzyme solution of 0.10 mL. The reaction mixture was then incubated at 35°C for 2 h. Finally, the concentrations of the arsenic compounds produced in the mixture were measured using an atomic absorption spectrophotometer (AAS).

To monitor the arsenic methylation using MCs, 0.70 mL of phosphate buffer (25 mmol/L; either pH 6.5 or 7.0), 0.10 mL of As(III) solution (10.0 mg As/L), 0.10 mL of SAM (20 mmol/L), and 0.10 mL of GSH (70 mmol/L) was added to 2.0 g of MCs. The reaction mixture was rotated at 40 rpm and 35°C for 2 h. The supernatant was collected using centrifugation (4°C, 14,000 rpm, 5 min) to measure the concentrations of the arsenic compounds.

### 2.6 Analyses

Quantitative analysis of arsenic species was performed using an AAS (Shimadzu AA6650) with an arsenic speciation pretreatment system (Shimadzu ASA-2sp). For this measurement method, arsenic vaporized with a reducing agent was recovered in a trap tube once cooled with liquid nitrogen, and then as the trap tube returned to room temperature, the arsenic species (arsine, monomethylarsine, dimethylarsine, trimethylarsine) were separated as per the differences in boiling points. All tests were performed in triplicates and each sample was measured in duplicates. The data presented in this study are presented as the means of measured values.
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8.3% and 10.9% in *E. coli* strains K63 and KC42, respectively.

Compared with the microorganisms reported to date,[14,15] these results showed extremely high conversion efficiency of inorganic arsenic to methylated organic arsenic compounds, almost the same as those of both crude enzymes extracted from the wild-type strains. In particular, the conversion efficiency of TMAC was high. Therefore, if large quantities of the enzyme can be prepared using the recombinant *E. coli* strains, the efficiency of arsenic methylation can be increased and the productivity of TMAO can be improved.

### 3.3 Arsenic methylation using MCs

As the enzyme-immobilized MCs, a polymerized MC containing an enzyme solution prepared from recombinant *E. coli* strains was produced. MCs with approximately 100-μm particle diameters were prepared; 71.0 g for K63 and 91.8 g for KC42. The inside of the resultant MCs had a porous structure with connected spherical holes.

Table 1 summarizes the results of arsenic methylation using the prepared MCs. The total yield of methylated organic arsenic compounds was 12.6% in *E. coli* strain K63 and 5.7% in *E. coli* strain KC42. Furthermore, the TMAC yield was 5.3% in *E. coli* strain K63 and 0.9% in *E. coli* strain KC42. If all enzyme molecules are immobi-
lized while retaining their enzymatic activity, the theoretical yield of methylated organic arsenic compounds is 23.8% in *E. coli* strain K63 and 11.6% in *E. coli* strain KC42. Compared with the actual results, the residual enzymatic activity is calculated to be 53.1% in *E. coli* strain K63 and 48.8% in *E. coli* strain KC42. By optimizing preparation conditions for enzyme immobilization using MCs, the residual enzymatic activity could be enhanced. When the arsenic methylation was performed using MCs 10 days after preparation, there was almost no change in the proportion of the methylated organic arsenic compound. Therefore, continuous methylation can be performed while maintaining enzyme activity using the prepared MCs.

4. Conclusion

In this study, arsM was overexpressed in recombinant *E. coli* strains and the resultant crude enzymes were examined for the efficiency of arsenic methylation. Compared with the microorganisms reported to date, these enzymes showed extremely high conversion efficiency, which was the same as that of crude enzymes obtained from wild-type strains. These results suggest that large quantities of the enzyme can be prepared from recombinant *E. coli* strains, and the efficiency of arsenic methylation can be increased and the productivity of TMAO can be improved.

Moreover, enzyme-immobilized MCs using the crude enzyme solution prepared using the recombinant *E. coli* strains were developed and tested for their role in arsenic methylation. Consequently, under optimized preparation conditions for enzyme immobilization in MCs, the residual enzymatic activity was enhanced. It was thus confirmed that methylation can be continuously performed while maintaining enzyme activity.

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