Anti-Ice Nucleation Activities of Adenine and Poly-A Nucleotides

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Most of the ice nucleation activity inhibitor reported so far are compounds processing the hydroxyl group such as the polyphenolic derivative. After examining the anti-ice nucleation activity of the purine base, the highest compound is theophylline, and the activity showed 3.80±0.32°C at a final concentration of 0.1 mg/ml. We found that the activity of the adenine which was essential to genome information DNA was higher than that of guanine. After examining effect of adenine concentration, high activity showed 9.1±1.2°C and became approximately constant above 0.1 mg/ml. This active rise is a result of effect of concentration under alkaline condition. Therefore after examining effect of pH on the activity of adenine, this activity rose under an alkaline condition. The active rise predicts that an electric charge of adenine is a factor. Among four kinds of nucleotide of 6 bases, poly-A nucleotide was higher and showed 1.33±0.42°C at a final concentration of 0.1 mg/ml. This activity of poly-A were proportional to the number of the base. From these results, it was suggested that the poly-A and adenine could be able to be applied to the field to preserve the blood and tissue which differentiated in the generative medicine.

Key words: Anti-ice nucleation activity / Adenine / Poly-A.

INTRODUCTION

Various organisms that can avoid the freezing are some types of fishes, amphibians and insects (Margesin et al., 2007). Such species can supercool their bodies so they can weather the long and cold winters, with temperatures that can go as low as -45°C, and still survive. Other than the strategy of the maintenance of their supercooling state, some organisms can lower the freezing temperature by accumulating some sugars, polyol and polysaccharide in the intracellular space (Storey and Storey, 1986). The effect produced by these compounds is a depression of the freezing point. In order to activate the supercooled state, it is necessary to inhibit the formation of ice nuclei necessary for the freezing of water. Various compounds with anti-ice nucleation activity have been found in many plants. Eugenol from clove and hinokitiol, a compound found in the leaves of coniferous trees, have an anti-ice nucleation activity against P. fluorescens (Kawahara et al., 1996a; 2000). It has been reported that the deep supercooling xylem parenchyma cells of katsura trees (Cercidiphyllum japonicum) contain some flavonol glycosides exhibiting high anti-ice nucleation activity, that is, supercooling-facilitating (SCF) activity (Kasuga et al., 2008). Furthermore, various tissues of Rhododendron flower buds exhibited ice nucleation activity. This low ice nucleation activity in the florets helps them remain unfrozen during deep supercooling. Recently, although it is a polymer compound, Bredow et al. (2016) has reported that an annual species of brome grass, Brachypodium distachyon produces an ice binding protein with high ice-recrystallization inhibition activity and anti-ice nucleation activity against bacterial ice nucleation proteins.

Cell preservation is performed by cryopreservation or vitrification preservation, but preservation of differentiated tissues in regenerative medicine and regeneration of organs can not be frozen. Although these preservation must use non-toxic compounds, it has been reported that polyethylene glycol could protect primary hepatocytes during supercooling preservation (Puts et al., 2015). Practically, it is indispensable to use some...
compounds that can protect cells with highly safe compounds and preserve their supercooled state. Recently, Kawahara et al. (2017) found that hot water extract from coffee refuse exhibits high anti-ice nucleation activity. One of the active compounds was not polyphenol, but caffeine, the purine base. Many of the purine bases have a lot of components in the animal body fluids and blood. Purine bases with SCF activity can be expected to be applied to the preservation of cells, blood, and organs under supercooled states.

In this study, the anti-ice nucleation activities of some purine bases were examined. Among all purine bases, as adenine may use the cryopreservation of cell, tissue and organ and the impartation of crop freeze tolerance, the effect of concentration on SCF activity and the effect of polynucleotide formation were examined. From these results, we discuss the possibility of adenine-related compounds for preservation of cells and organs.

MATERIALS AND METHODS

Materials
The DNA nucleotides were synthesized by FASMAC Co., Ltd. (Atsugi, Japan). All DNA nucleotides were dissolved in a TE buffer. All chemicals containing a purine base were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

Measurement of SCF activity (anti-ice nucleation activity)
The anti-ice nucleation activity was measured by modified a method of ice nucleating activity. The silver iodide suspension (1 mg/ml) of 90 µl and the sample or distilled water of 10 µl were mixed, and kept at room temperature for 30 min. Before measuring this activity each sample was passed through a 0.45-µm filter to remove small particles. The ice-nucleating temperature was measured with a freezing nucleus spectrometer (Thermoelectric cold plate model KM-1) (Vali 1971). That is, thirty drops, each of 10 µl, were placed on a temperature-controlled surface, and the temperature was lowered from 4°C to -20°C at a rate of 1.0°C per min. The temperature at which 50% of the drops froze was recorded (T_{50}). The anti-ice nucleation activity was defined as the difference between the ice-nucleating temperature (T_{50}) of sample and the distilled water.

Examination of SCF activities of all purine bases
Before examining the SCF activity, all purine bases were adjusted to each concentration with 0.1 M NaOH. In the examination of the pH effect on SCF activity, adenine alkali solution was prepared with 1.0 M NaOH, adenine neutral solution was prepared with 1.0 M HCl after dissolving with 0.1 M NaOH, and adenine acidic solution was prepared with 1.0 M HCl.

Statistical analysis
Data in the Figures and Tables were reported as the mean ± SE. Differences in P values that were <0.05 were considered significant.

RESULTS

Comparison of SCF activity from each purine base and purine related compounds
The SCF activity of the eight purine bases shown in Table 1 was examined. Among the eight compounds, caffeine, theobromine and theophylline, which are classified as a xanthine derivative, are plant-derived compounds and ingested from some foods. Caffeine and theophylline are bitter compounds in tea leaves. Also, theobromine is a kind of alkaloid resembling the structure of purine bases contained in cacao seeds. Theophylline and theobromine are both products of when caffeine is metabolized in the liver, and caffeine is converted to theophylline by 4%, and converted to theobromine by 10% (Oesterheld, 1998). Uric acid is synthesized by xanthine oxidase from oxypurine such as xanthine and hypoxanthine. In many mammalian species and other primates, uric acid is the oxidation end product of purine metabolism containing adenine and guanine.

Among eight purine bases and purine related compounds, we found that theophylline has the highest SCF activity. Its SCF activity is 3.80±0.32°C at a final concentration of 0.1 mg/ml. We found that the SCF activity of adenine, which is an essential component of genetic information DNA, was higher than that of guanine. Since caffeine with the highest number of methylations has the lowest SCF activity, the expression of this activity is predictable due to the electrons of the nitrogen atom.

Effect of adenine concentration on SCF activity
Effect of adenine concentration on SCF activity was shown in FIG.1. Similar to the effect of the concentration effect of caffeine, SCF activity did not increase at adenine concentrations of 0.1 mg/ml or more. SCF activity of adenine at a final concentration of 0.1 mg/ml was 9.1±1.2°C. This activity was the same as kaempferol-7-glucoside (9.0°C) and quercetin-7-glucoside (8.9°C) (Kasuga et al., 2010). As with adenine, guanine and uric acid also exhibited constant activity at a final concentration of 0.1 mg/ml or more. The reason why the SCF activity is kept constant by addition above a concentration of 0.1 mg/ml is because the surface area of ice nucleus of heterogeneous nucleation is constant and the site to be bound is saturated by the
TABLE 1. Anti-ice nucleation activity of each purine base

<table>
<thead>
<tr>
<th>Sample</th>
<th>Anti-ice nucleation activity (ºC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>0.70 ± 0.06</td>
</tr>
<tr>
<td>Theobromine</td>
<td>0.73 ± 0.48</td>
</tr>
<tr>
<td>Theophylline</td>
<td>3.80 ± 0.32</td>
</tr>
<tr>
<td>Uric acid</td>
<td>2.03 ± 0.26</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>1.36 ± 0.12</td>
</tr>
<tr>
<td>Xanthine</td>
<td>1.13 ± 0.17</td>
</tr>
<tr>
<td>Guanine</td>
<td>1.90 ± 0.35</td>
</tr>
<tr>
<td>Adenine</td>
<td>3.40 ± 0.26</td>
</tr>
</tbody>
</table>

Each compound at a final concentration of 0.1 mg/ml were examined by the assay system as described in Material and Methods.

FIG. 1. Effect of adenine concentration of anti-ice nucleating activity

Each activity indicates the mean value and standard deviation (n=3).

Effect of adenine nucleotide chain on SCF activity

The majority of adenine was one of the constituents of the nucleoside of DNA or RNA backbone and the constituent of ATP. Generally, nucleotides such as poly-A were used in TE buffer (pH 8.0). Therefore, the SCF activity of adenine (0.1 mg/ml) in TE buffer was 1.5ºC. This activity was almost the same as the activity of the adenine solution (pH 8.10) (FIG.2). The SCF activity of four species of nucleotide polymers with 6 bases was examined. As shown in Table 2, poly-A with 6 bases (1.33ºC) had high SCF activity among four polymers. As SCF activity of poly-C with 6 bases was 0.53ºC, amino group was predicted to play an important role in the expression of SCF activity. Since the DNA double helix rotated once by 10 bases, the effect of chain length of poly-A on the SCF activity was examined. When the SCF activity was examined up to 12 bases, although no significant difference in activity increase could be confirmed, this activity tended to increase with increasing chain length (FIG.3). The poly-A polymer with 12 bases was most active because its helical structure may surround silver iodide as an ice nucleus.

DISCUSSION

The results of our previous study on anti-ice nucleation activity from various natural sources suggested that the most effective compound showing SCF activity might be an aromatic compound containing polyphenol having some hydroxyl groups (Kawahara et al., 1996a; 2000). In this study, we found that purine bases and...
purine base-related compounds also had SCF activity (Table 1). Other than the anti-ice nucleation activity from high molecular weight material, we have also discovered a bacterial strain with anti–ice nucleation activity (Kawahara et al., 1996b); this strain, KINI-1, was identified as *Acinetobacter calcoaceticus*. This protein, which is a hydrophobic protein, can strongly bind to a foreign substance, and the action of controlling the surface charge of foreign matter caused by the binding is a factor of the expression of SCF activity (Kawahara et al., 1996b). Furthermore, one strain of *Bacillus thuringiensis* produces an anti-ice-nucleating polysaccharide with a molecular mass of 130,000 (Yamashita et al. 2002). Since this polysaccharide contains chitosan as a constituent, this SCF activity was identified as having a similarity to ice crystal lattice, and the anti–ice nucleation activity of chitosan was the same as that of silver iodide by carboxyl group. Franks reported that the dominant factors for the formation of ice nuclei by materials included the following three conditions: similarity to ice crystal lattice, the paucity of the surface charge in water, and high level of hydrophobicity of ice nuclei (Franks, 1985). The crystal structure of silver iodide is a hexagonal crystal type similar to ice crystals. Similar to the effects of the bacterial protein from *A. calcoaceticus* KINI-1 and the chitosan-containing polysaccharide, the SCF activity of adenine can be predicted to be attributable to the amino group of adenine for controlling the charge of foreign material. As shown in Table 2, the SCF activities of poly-A and poly-G with 6 bases were higher than those of poly-C and poly-T. This result supported that the presence or absence of electric charge of the amino group was a cause of the expression of the activity. In a comparison between cytosine and thymine having a pyrimidine ring, the SCF activity of poly-C was higher than that of poly-T (Table 2). It can be judged that this difference is the presence or absence of an amino group.

As the length of the poly-A chain increased, the SCF activity increased (FIG. 3). In this comparison, poly-A was measured at a final concentration of 0.1 mg/ml. Under this condition, poly-A having different chain lengths had the same number of moles. From this result, it can be concluded that this increase in SCF activity may be an influence of the poly-A conformation. The ice nucleus in this study, silver iodide was of the same hexagonal type as ice and its bonding distance is 0.28 nm. In addition, the interchain length of the helical structure of DNA strand is 0.2 nm. In the DNA, the amino group of adenosine is hydrogen bonded to the oxygen of the carbonyl group of thymine. Since there is no hydrogen bond in poly-A, the SCF activity of poly-A is considered to be developed. The activity of poly-A with 12 bases is about 0.3°C higher than that of poly-A with 6 bases (FIG. 3). The reason of this is that poly-A with 12 bases is forming a helical structure, which may be sterically stabilized.

In this research, it could be predicted that the adenine, uric acid and poly-A could be expected to be applied to preservation solutions of biological components such as blood and differentiated tissues of regenerative medicine. In future research, it is necessary to confirm the SCF activity of poly-A with longer than 12-bases.

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**REFERENCES**


**FIG. 3.** Effect of base number in poly A on anti-ice nucleation activity
Each activity indicates the mean value and standard deviation (n=3).
SFC activity was examined at a final concentration of 0.1 mg/ml.


