Comparison of anti-hyaluronidase activities and sugar compositions of extracts from four edible species of *Nostoc* (cyanobacteria)

Yuji Yamaguchi\(^1\)\(^2\), Toshio Sakamoto\(^3\) and Mamoru Koketsu\(^4\)*

**Abstract**: The edible species of *Nostoc* are unique cyanobacteria because they can grow in some extreme environments, and some of these species are rare. Anti-hyaluronidase activity and sugar composition of the extracts were compared among five edible *Nostoc* strains which were cultured using 180 liter large scale reactor. The IC\(_{50}\) against hyaluronidase of the extracts of *Nostoc sphaericum*, *Nostoc flagelliforme* and *Nostoc verrucosum* were 14.4, 46.5 and 56.2 µg mL\(^{-1}\), respectively. These anti-hyaluronidase activities were higher than disodium cromoglycate which is known as an anti-allergic drug and used as a positive control substance. The ratio of glucuronic acid in constituent sugar of *N. sphaericum* extract was 13.3%, it was the highest among the five strains tested. The extract of *N. sphaericum* had the highest yield and highest anti-hyaluronidase activity when compared with the other four *Nostoc* strains.

**Keywords**: edible cyanobacteria, hyaluronidase inhibitor, *Nostoc*, polysaccharide, sugar composition

**Introduction**

Cyanobacteria are prokaryotic microalgae, and some of them make macroscopic colonies by forming trichome or extracellular polysaccharide. Some kinds of cyanobacteria have been eaten in the world for a long time. In Japan, *Nostoc commune*, *Nostoc verrucosum* and *Aphanotoche sacrum* are known as edible cyanobacteria. Their Japanese names are Ishikurage, Ashitsuki and Suizenjinori (Takenaka and Yamaguchi 2010, 2012), respectively.

*Nostoc commune* is a terrestrial cyanobacterium, therefore, it is not easy to wash this alga until a level suitable for eating. The dietary habits of *N. commune* are not inherited except Okinawa. *Nostoc verrucosum* (Oku et al. 2014) and *A. sacrum* (Kabata et al. 2007) are designated as endangered species, so it is hard to get these wild colonies. But *A. sacrum* is cultivated in artificial river using the spring (Yoshida 2012). In China, a terrestrial *Nostoc flagelliforme*, the Chinese name is Facai, is an edible cyanobacterium, and it inhabits the arid region. This is also an endangered species (Aruga 2012). In Peru, *Nostoc shaericum*, the Peruvian name is Cushuro (Masuda 1980), is also an edible cyanobacteria, and grows spontaneously in lakes at high altitude. In this way, it is not easy to use this wild species of *Nostoc* as foods. However, these edible cyanobacteria have several bioavailabilities (Yamaguchi et al. 2009; Takenaka and Yamaguchi 2010; Oku et al. 2014; Kanekiya et al. 2005; Knübel et al. 1990). We need to culture

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these edible cyanobacteria which have a high bioavailability for effective utilization as foods, but the commercial scale culture of edible species of *Nostoc* is yet to succeed.

The ethanol insoluble fraction of edible cyanobacterium *Spirulina platensis* has anti-hyaluronidase activity (Fujitani et al. 2001). Edible cyanobacterium *Nostochopsis lobatus* produces a lot of polysaccharides having strong anti-hyaluronidase activity (Sakaki et al. 2012; Yamaguchi and Koketsu 2016). Therefore, other cyanobacteria may also have an anti-hyaluronidase activity.

Hyaluronidases are a class of enzymes which predominantly hydrolyze hyaluronic acid and play an important role in physiological processes such as angiogenesis (Liu et al. 1996), carcinogenesis (Menzel et al. 1998; Lokeshwar et al. 2008), inflammation (Edelstam et al. 1992; McKee et al. 1997) and type I allergy (Marandy et al. 1997). The measure of anti-hyaluronidase activity is used for screening anti-allergy substances (Sakamoto et al. 1980).

We have cultured five strains of edible species of *Nostoc* such as *N. commune* (two strains), *N. flagelliforme*, *N. verrucosum* and *N. sphaericum*. The anti-hyaluronidase activities of these cultured *Nostoc* species have not been reported yet. In the present study, we have compared anti-hyaluronidase activity and sugar compositions among the extracts of five strains of cultured *Nostoc*.

### Materials and Methods

**Strains and culture conditions**

*Nostoc commune* was purchased as food ingredient from China. *Nostoc sphaericum* was purchased a small quantity at a market in Lima, Peru. *Nostoc commune* strain YK-04, *Nostoc flagelliforme* strain NXU and *Nostoc verrucosum* strain KU005 (Sakamoto et al. 2011) were supplied from Dr. Hiroshi Katoh (Mie Univ.), Prof. Jianyu Su (Ningxia Univ., China) and Associate Prof. Toshio Sakamoto (Kanazawa Univ.), respectively. After grind of the purchased *N. commune* and *N. sphaericum*, respectively, cellular filaments were isolated from them. The isolated cellular filaments (*N. commune* strain #31 and *N. sphaericum* strain MAC0910PER) were cultured in nitrogen-free BG11 medium (Stanier et al. 1971) under fluorescent light at 20–25°C. After the isolated cellular filaments formed macroscopic colonies, they were grown with air-bubbling in culture flask. Nitrogen-free BG11 culture medium was used for *N. verrucosum* and *N. sphaericum* while *N. commune* and *N. flagelliforme* were grown in a nitrogen-free modified BG11. The concentration of reagents were modified as follows, Na₂SiO₃·7H₂O 60 mg L⁻¹, FeSO₄·7H₂O 4.8 mg L⁻¹ and EDTA·2 Na 1 mg L⁻¹. Large scale cultures of these algae were carried out in 180 liter cylindrical acrylic tanks with air-bubbling at 20–25°C in continuous illumination of 300 μmol m⁻² s⁻¹ under fluorescent light. These cultured algae were harvested by filtration after sedimentation, and were dehydrated by lyophilization.

**Extraction from cultured Nostoc**

Each lyophilized *Nostoc* (5 g) was extracted with 90°C water (1,000 mL) for 3 hour. The extract was filtered by glass filter (GA-100, Advantec, Japan) after centrifugation, and concentrated in vacuo. The concentrated extract was added into 4 volumes of ethanol with stirring and kept at 7°C overnight. The precipitate was then dissolved in deionized water with stirring and kept at 7°C overnight. The precipitate was then dissolved in deionized water and dialyzed in tap water overnight followed by deionized water for 24 hrs. The dialyzed sample was lyophilized and weighed to determine the extracted product yield.

**Determination of anti-hyaluronidase activity**

Anti-hyaluronidase activities of the extracts were determined by methods previously described (Sakaki et al. 2012; Yamaguchi and Koketsu 2016). Fifty microliters of 4000 unit mL⁻¹ hyaluronidase (Sigma Chemical Co., USA), pre-incubated in advance with 50 μL of test sample at 37°C for 20 min, were incubated with 0.1 mL of mixture of 0.5 mg mL⁻¹ compound 48/80 (Sigma Chemical Co., USA), 12.5 mM CaCl₂ and 0.75 N NaCl at 37°C for 20 min. After then, 0.25 mL of 0.8 mg mL⁻¹ hyaluronic acid sodium salt (Wako Pure Chemical Industries, Japan) was added and the reaction mixture was further incubated at 37°C for 40 min, and then the
reaction was terminated by adding 0.1 mL of 0.4 N NaOH. The inhibitory effect was determined by a modified Morgan-Elson method (Reissig et al. 1955). Test samples were replaced by the buffer solution for the control, while the enzyme solution was replaced by buffer solution for the blank. Disodium cromoglycate (DSCG, Enzo Life Sciences, Switzerland) was used as a positive control substance.

Percent of inhibition was calculated as follows:

\[
\text{Inhibition (\%)} = \left( \frac{(A-B)-(C-D)}{(A-B)} \right) \times 100,
\]

where A: OD_{585} without sample, B: OD_{585} without sample and hyaluronidase, C: OD_{585} sample, and D: OD_{585} without hyaluronidase sample.

**Analysis of sugar composition**

The analysis of sugar composition was evaluated using the previously reported methods (Akiyama et al. 2011; Yamaguchi and Koketsu 2016). The extract (10 mg) dissolved in water (0.85 mL) was hydrolyzed with trifluoroacetic acid (TFA) (0.15 mL) at 105 °C for 3, 6 or 12 h in screw cap tube filled with nitrogen gas. Hydrolyzed sample (0.2 mL) was dehydrated by vacuum drying, and 0.1 mL of ethanethiol-TFA (2:1) was added and allowed to stand for 10 min at room temperature. The sample was added with 0.25 mL of pyridine, 0.5 mL of hexamethyldisilazane and 0.15 mL of TFA and allowed to stand again for 1 h at room temperature. The solvent was removed by nitrogen gas. Two milliliter of hexane and 0.25 mL of water were added. One microliter of hexane layer was analyzed for sugar composition by GC-MS (GC: Agilent 6890, MS: JEOL GC mate II) using DB-5MS column (30 m × φ0.25 mm, J&W) at temperature condition of 165 °C for 2 min followed by 165→235 °C (2 °C min⁻¹). The sugar composition of the polysaccharides was identified by retention time and mass spectrum of GC-MS. The molar ratio of sugar composition was calculated by calibration curve using the concentration of standard sugars.

**Results and Discussion**

All *Nostoc* strains (*N. commune* strain YK-04, *N. commune* strain #31, *N. flagelliforme*, *N. sphaericum* and *N. verrucosum*) were cultured under the same conditions except for the culture medium. The growth curves of these *Nostoc* were not indicated because the accurate cell concentrations could not be measured by their aggregates.

The yields of extracts of *N. commune* strain YK-04, *N. commune* strain #31, *N. flagelliforme*, *N. sphaericum* and *N. verrucosum* were 14.5, 8.3, 9.1, 35.8 and 14.5% for dry algae, respectively.

**Table 1. Yields and anti-hyaluronidase activities of the extracts of cultured edible *Nostoc***

<table>
<thead>
<tr>
<th>Strains</th>
<th>Yield (%)</th>
<th>IC₅₀ (µg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nostoc commune</em> YK-04</td>
<td>14.5</td>
<td>---</td>
</tr>
<tr>
<td><em>Nostoc commune</em> #31</td>
<td>8.3</td>
<td>---</td>
</tr>
<tr>
<td><em>Nostoc flagelliforme</em> NXU</td>
<td>9.1</td>
<td>46.5</td>
</tr>
<tr>
<td><em>Nostoc sphaericum</em> MAC0904PER</td>
<td>35.8</td>
<td>14.4</td>
</tr>
<tr>
<td><em>Nostoc verrucosum</em> KU005</td>
<td>14.5</td>
<td>56.2</td>
</tr>
<tr>
<td>DSCG</td>
<td>105.6</td>
<td></td>
</tr>
</tbody>
</table>

a: The yields were indicated as % for dry algae
b: The anti-hyaluronidase activities were indicated as IC₅₀
c: IC₅₀ of two strains of *N. commune* were not calculated because the inhibition rates of them were less than 20%
d: DSCG, disodium cromoglycate
The yield of *N. sphaericum* extract was the highest and was more than twice those of the other four strains.

The inhibitory rates against hyaluronidase of *Nostoc* extracts were showed in Fig. 1. The maximum inhibition rate of *N. flagelliforme* was 59.6%, and the inhibition rate decreased along with the increase of the concentration.

Table 1 showed the yields and IC₅₀ against hyaluronidase of extracts of each *Nostoc*. The IC₅₀ of *N. commune* 2 strains could not be calculated, because the maximum inhibition rates were less than 20%. The IC₅₀ of *N. flagelliforme*, *N. sphaericum* and *N. verrucosum* were 46.5, 14.4 and 56.2 µg mL⁻¹, respectively. The IC₅₀ against hyaluronidase of *N. sphaericum* extract was the highest of all the *Nostoc* extracts, and it was 7.5 times higher than disodium cromoglycate (DSCG, IC₅₀: 105.6 µg mL⁻¹) which is known as an anti-allergic drug and used as a positive control substance. We reported that the 80% ethanol-insoluble extract of *Nostochopsis lobatus* was 39.5% yield for dried alga and IC₅₀ against hyaluronidase inhibitory activity of the extract was 12.3 µg mL⁻¹ (Yamaguchi and Koketsu 2016). *Nostoc sphaericum* had approximately the same yield and hyaluronidase inhibitory activity with *N. lobatus*.

The sugar compositions of *Nostoc* extracts was analyzed by using GC-MS (Table 2). All *Nostoc* extracts contained glucose, galactose, mannose, xylose and glucuronic acid. Fucose and rhhamnose were not contained in *N. sphaericum*. The percentage of glucuronic acid in constituent sugar of *N. sphaericum* extract was 13.3%, and it was the highest among the five strains. We (Yamaguchi and Koketsu 2016) and Sawabe et al. (1992) observed that the amount of uronic acid in the polysaccharide (pectic acid) correlated with hyaluronidase inhibitory activity. However, significant relation between the glucuronic acid content and hyaluronidase inhibitory activity was not observed in the present study. Other factors besides glucuronic acid content may be involved in the inhibition of hyaluronidase activity. Tioda et al. (1999) reported that hyaluronidase inhibition was correlated with the degree of O-sulfonation in O-sulfonated glycosaminoglycans. Furthermore, polysaccharides from soy sauce have hyaluronidase inhibitory activity, and the high molecular weight fraction (over 12 kDa) had a higher activity than low molecular weight fraction (Kobayashi et al. 2004). The hyaluronidase inhibitory activity of polysaccharide may have the correlations with also sulfate group or molecular weight not only glucuronic acid content. Further study is necessary to characterize the mechanism of hyaluronidase activity inhibition, while the present results suggested that *N. sphaericum* can produce a large amount of polysaccharides having strong hyaluronidase inhibitory properties.

### Acknowledgements

The authors gratefully thank Dr. Hiroshi Katoh and Prof. Jianyu Su for supply of the strains, and Prof. Satoshi Yoshida of Gifu University for GC-MS analysis. We also owe our gratitude to Dr. Hiroyuki Takenaka and the member of MAC Gifu Research Institute for their support. We wish to thank The Koshiyama Research Grant for financial support.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Gle</th>
<th>Gal</th>
<th>Fuc</th>
<th>Man</th>
<th>Rha</th>
<th>Xyl</th>
<th>Gle UA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nostoc commune</em> YK-04</td>
<td>39.5</td>
<td>15.8</td>
<td>tr</td>
<td>19.4</td>
<td>4.2</td>
<td>5.6</td>
<td>9.9</td>
</tr>
<tr>
<td><em>Nostoc commune</em> #31</td>
<td>36.4</td>
<td>15.8</td>
<td>2.9</td>
<td>20.2</td>
<td>11.6</td>
<td>11.8</td>
<td>1.2</td>
</tr>
<tr>
<td><em>Nostoc flagelliforme</em> NXU</td>
<td>51.9</td>
<td>1.1</td>
<td>tr</td>
<td>25.5</td>
<td>3.5</td>
<td>18.0</td>
<td>tr</td>
</tr>
<tr>
<td><em>Nostoc sphaericum</em> MAC9904PER</td>
<td>28.9</td>
<td>25.5</td>
<td>nd</td>
<td>20.1</td>
<td>nd</td>
<td>12.1</td>
<td>13.3</td>
</tr>
<tr>
<td><em>Nostoc verrucosum</em> KU005</td>
<td>27.8</td>
<td>24.8</td>
<td>4.5</td>
<td>14.4</td>
<td>11.5</td>
<td>8.8</td>
<td>8.3</td>
</tr>
</tbody>
</table>

nd: not detected, tr: trace amount.
References


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