



Mild Acid Hydrolysis-related Release of Water-soluble Sunscreen Pigments from the Exopolysaccharide Matrix of Edible Terrestrial Cyanobacteria

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ABSTRACT

Terrestrial cyanobacteria, *Nostoc flagelliforme* and *Nostoc commune*, are important natural resource for producing ultraviolet-absorbing mycosporine-like amino acids (MAAs), in addition to their edible value. MAAs can be used as sun-screening cosmetics, antioxidants and pharmaceuticals. Usually, water-soluble MAAs are extracted by pure or aqueous methanol after sample grinding. Development of new extraction technique without specific morphological destruction will contribute to adequately exploit the commercial values of both edible cyanobacteria. The N-acetylcysteine solution has been preliminarily mentioned to cause the release of MAAs from *N. flagelliforme* via mere incubation with the sample. In this study, we explored the potential mechanism underlying this influence and further applied it to extract MAAs from various samples. It was revealed that N-acetylcysteine solutions primarily played a mild acid hydrolysis role in causing the dissociation and release of MAAs from the exopolysaccharide matrix of samples. N-acetylcysteine solutions also exerted similar physiological or morphological effects on the samples as other acidic solutions, and the morphological integrity of the treated samples was not destroyed. Finally, we found that those samples with good vitality could achieve high MAA harvests. In general, means of incubation with acidic solution serves as a simple and relatively nondestructive technique for MAA extraction from the edible terrestrial cyanobacteria.

1. INTRODUCTION

Mycosporines and mycosporine-like amino acids (MAAs) are small (<400 Da), colorless and water-soluble molecules, with strong ultraviolet (UV) absorption maxima from 310 to 365 nm [1, 2]. More than 30 different compounds in the mycosporine family have been identified from natural sources, including cyanobacteria, fungi and marine algae [2, 3]. These UV-absorbing molecules (herein all referred to as MAAs) can dissipate the absorbed UV radiation as heat without the generation of reactive oxygen species [4]. They also exhibit antioxidant activity [5,6] and can serve as wound-healing reagents [7] and monoamine oxidase-B inhibitors [8]. Therefore, MAAs have a bright prospect in cosmetic and pharmaceutical industries.

Terrestrial cyanobacteria, *Nostoc flagelliforme* and *Nostoc commune*, are important edible resources [9, 10]. The former, which has been overexploited in the past years, is now in restoration in the vast arid or semi-arid steppes of China. The latter is a cosmopolitan species and most abundant in temperate regions. Typically MAAs are free intracellular compounds in marine algae, however they are generally oligosaccharide-linked in the exopolysaccharide (EPS) matrix of terrestrial cyanobacteria [11, 12, 13]. Because of the exposure to intense solar radiation and period desiccation, terrestrial cyanobacteria can be induced to produce rich UV-absorbing MAAs and scytonemin [14, 15]. Thus, edible terrestrial cyanobacteria are also important resources for obtaining MAAs.

The extraction of MAAs from cyanobacteria usually employs pure or aqueous methanol or even water [1, 5, 6, 13, 16, 17]. Samples are ground and destroyed by these extraction means. Recently, it was preliminarily mentioned that N-acetylcysteine (NAC) solution could cause the release of MAAs from *N. flagelliforme* via only incubation with the sample [12]. It has

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attracted our attention that the relevant technique may be developed for the purpose of deeply exploiting edible terrestrial cyanobacteria without specific morphological destruction. NAC functions as a precursor of glutathione in cells that can reduce the intracellular oxidant stress and protect the photosystem against UV radiation [18, 19, 20]. NAC can also markedly reduce the intrinsic viscosity of mucoprotein solutions [21, 22] and is used as a generic medication to treat acetaminophen overdose or to loosen thick mucus (The American Society of Health-System Pharmacists). However, due to its acidic property, we speculate that NAC solution may exert an acid hydrolysis-related role in the release of cyanobacterial MAAs. In this study, we investigated this potential mechanism and further applied it to extract MAAs in various *N. flagelliforme* and *N. commune* samples with different vitalities.

2. MATERIALS AND METHODS

2.1 Cyanobacterial samples

N. flagelliforme is distributed in arid or semiarid steppes of the west and west-northern parts of China [23]. Four *N. flagelliforme* samples sourced in 1999, 2004, 2013 and 2016 from Inner Mongolia and one sample sourced in 2016 from Ningxia province were used in this study. *N. commune* is distributed across the whole country. *N. commune* samples sourced at four different regions in 2013, 2014 and 2016 were used in this study. Air-dried samples were immersed in BG11₀ solution overnight (14–20 h) at 22°C and 20 μmol photons m⁻² s⁻¹ for physiological recovery as described by [24]. After the surface water was absorbed by filter paper, the rewetted samples were subjected to various experiments. Physiological recovery of samples after rehydration was indexed by the Photosystem II (PSII) activity parameter Fv/Fm [24,25], which was detected by a Handy PEA fluorometer according to the manufacturer's specification (Hansatech instruments Ltd., England). The *N. flagelliforme* sample (Inner Mongolia, 2016) was primarily used for the experiments, which had a well recovered Fv/Fm value of 0.44–0.54.

2.2 Incubation with NAC or other acidic solutions and MAA detection

Various solutions used in this study were prepared in BG11₀ solution, including NAC solutions (0.5–40 mM), sulfamic acid solutions (5–40 mM), aspartic acid solutions (5–40 mM) and 50 mM glycine-HCl buffers (pH 2.0, 3.0 and 4.0). The pH of each solution was determined by a pH meter (Mettler Toledo, Germany). The pH values of 0.5–40 mM NAC solutions range from 3.4 to 2.1. Rewetted samples of 1 g fresh weight (FW) were incubated with 10 or 20 ml solutions in 50-ml plastic centrifuge tubes. The tubes were placed in an air-conditioned chamber at 22°C with occasional shaking (1–2 h interval). After centrifugation at 6,000 rpm (Eppendorf Centrifuge 5810R, Germany) for 5 min, the supernatants were carefully collected and subjected to full- or fixed-wavelength spectroscopic analysis with a UV-2700 Ultraviolet-Visible Spectrophotometer (Shimadzu, Japan). The MAA compounds were characterized by their specific UV-

absorption spectrum around 312 nm in *N. flagelliforme* and *N. commune* [11, 12, 26].

2.3 Physiological and morphological analysis

The physiological inhibitions of acidic solutions on the samples were indexed by the Fv/Fm parameter as mentioned above. Briefly, physiologically fully recovered samples were immersed in the solutions for 1 h and then subjected to Fv/Fm detection. The morphological appearance was photographed after samples were incubated with acidic solutions for 3 h. Fresh weight/dry weight (FW/DW) ratio (fold) was calculated by comparing the final weights of rewetted samples (14–20 h immersion) to their original dry weights. For the elasticity analysis, *N. flagelliforme* filaments of 3–5 cm long were fixed at both ends and stretched until breakage. The stretching rate (fold) was calculated as the ratio of the extended length to the original length.

3. RESULTS AND DISCUSSION

3.1 Mild acid hydrolysis-related release of MAAs

Various concentrations of NAC solutions were first evaluated in causing the release of MAAs from *N. flagelliforme* (Fig. 1a). A short-time treatment (1 h) by 10 or 20 mM NAC solution could obviously lead to the release of MAAs, with an absorbance peak at 312 nm and a shoulder at around 335 nm as previously reported [12,26]. This characteristic spectrum around 312 nm represented a group of MAA compounds as indicated by HPLC analysis in *N. flagelliforme* [27]. The case was similar in *N. commune* [11, 13]. During the extended incubation (14 h), 10 or 20 mM NAC solution led to more release of MAAs and 5 mM NAC solution was also obviously observed to cause this release. In contrast, normal BG11₀ solution or lower concentrations of NAC solutions (0.5 or 1 mM) had no such effect after either short-time or extended incubation. NAC solutions are acidic and their acidities are correlated with NAC concentrations. When NAC solutions of different concentrations were buffered to be neutral, they lost this effect (Fig. 1b). Therefore, the role of NAC solution in causing the release of MAAs was closely related to its acidic property. Other acidic solutions were further used to examine this potential role (Fig. 2). Glycine-HCl buffers, sulfamic acid and aspartic acid solutions were used. It was noted that these acidic solutions as well as NAC solutions could be buffered by the EPS matrix to some extent, approaching neutral pH (e.g., as indicated for glycine-HCl buffers in Fig. 2a). The acid strength-related release of MAAs was still observed for the three groups of acidic solutions. These results implied that acid hydrolysis promoted the dissociation of oligosaccharide-linked MAAs from the EPS matrix. The different molecular effects also existed, to some extent, among these reagents, since the absorbance at 312 nm of equivalent amounts of MAAs in these acidic solutions varied at a range of 0–20% (data not shown). In addition, the solution acidity of above pH 5.0 after the EPS buffering seemed not to cause the release of MAAs (Fig. 2). The original pH 3.2 of 1 mM NAC

solution was buffered to pH 5.5, which solution also had no this effect (in Fig. 1a). Thus, an acidity threshold or enough acid strength was equally critical for the dissociation of MAAs from the EPS matrix. Stronger acids such as hydrofluoric, sulphuric and trifluoroacetic acids are usually adopted to hydrolyze polysaccharides under high temperature for composition analysis [28,29] or hydrolyze the glycosylated MAAs for sugar determination [13]. However, no monosaccharide or oligosaccharide was released in our acid treatment experiments. Therefore, NAC solutions as well as other three acidic solutions in this study exerted a mild acid hydrolysis in causing the dissociation and release of MAAs from the samples.

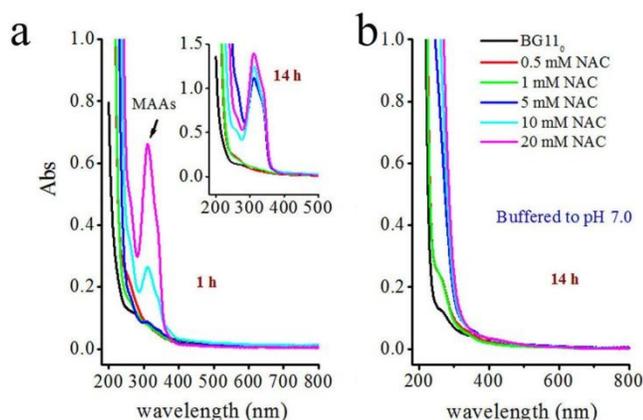


Fig. 1: The spectroscopic scanning of the released MAAs from *N. flagelliforme* samples. a, aliquots of 1 g FW rewetted samples were incubated in 10 ml NAC solutions for 1 and 14 h, respectively. b, the rewetted samples were similarly incubated for 14 h in the NAC solutions (pH 7.0) buffered by 20 mM HEPES. The arrow indicates the characteristic absorption peak of MAA compounds at 312 nm.

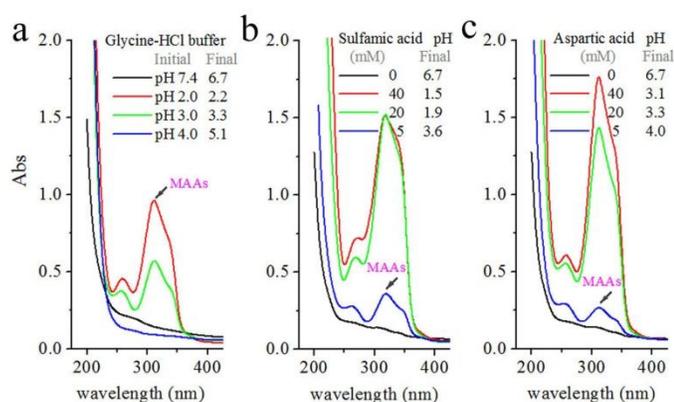


Fig. 2: The release of MAAs from *N. flagelliforme* samples following incubation in various acidic solutions. a, glycine-HCl buffers with pH 2.0, 3.0 and 4.0, respectively. b, sulfamic acid solutions of 5, 20 and 40 mM, respectively. c, aspartic acid solutions of 5, 20 and 40 mM, respectively. The rewetted samples of 1 g FW were incubated with 10 ml of solutions for 3 h. Due to the pH buffering of the EPS matrix, the initial and/or final solution pH was indicated.

3.2 Physiological or morphological effects of acid treatments

Physiological or morphological effects of NAC solutions on *N. flagelliforme* samples were evaluated, as compared with glycine-HCl buffers (Fig. 3). *N. flagelliforme* and *N. commune* are

sensitive to acidic conditions, which lead to physiological inhibition [23,30]. As indexed by PSII activity parameter Fv/Fm, NAC solution and glycine-HCl buffer both resulted in physiological reduction, associated with their concentrations or acidities (Fig. 3a). Moreover, the treated *N. flagelliforme* filaments by both kinds of solutions showed an increased elasticity (Fig. 3b), implying an obvious softening effect on the EPS matrix. Finally, the morphological appearance of the treated samples seemed not to be particularly affected except the chlorosis at higher acidic conditions (Fig. 3c). The cases were similar for *N. commune* samples (data not shown). These results further demonstrated that NAC solutions exerted a typical acidic effect on cyanobacterial samples. More importantly, acid treatments did not destroy the morphological integrity of the samples. High temperature has a remarkable influence on the stability of MAAs [3]; these compounds will be lost or destroyed in the edible cyanobacteria during the cooking process. Therefore, mere incubation with NAC solution or other acidic solutions contributed to the MAA exploitation from edible cyanobacteria with little influence on their reuse in food business.

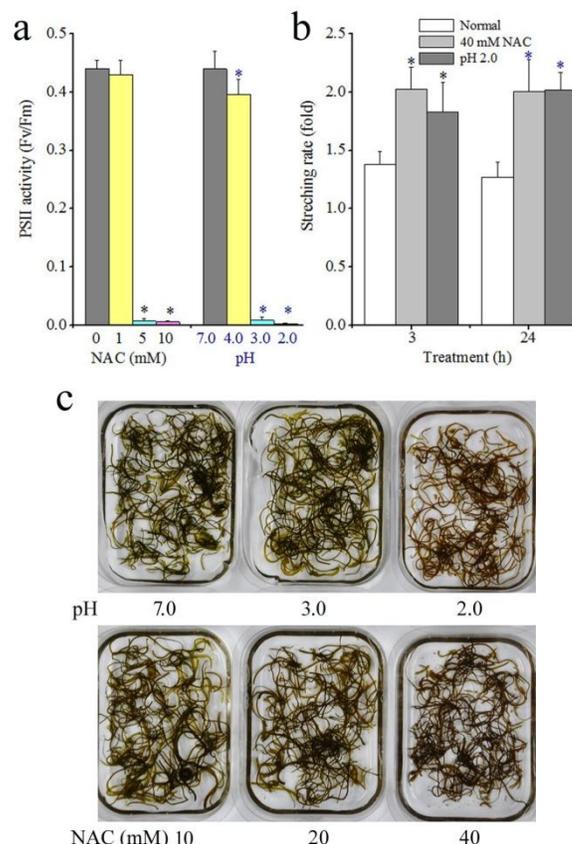


Figure 3 The physiological and morphological effects of acid treatments on *N. flagelliforme* samples. NAC solutions and glycine-HCl buffers were compared. a, PSII activity (in terms of Fv/Fm) changes of the rewetted samples after 1-h incubation in the solutions. Data shown are the means \pm SD (n=5). b, elastic changes of the treated *N. flagelliforme* filaments. Control samples are incubated in normal BG11₀ solution. Data shown are the means \pm SD (n=12). * indicate significant difference (P<0.05) with respect to the respective control (student's t-test). c, the morphological appearance of the treated samples after incubation for 3 h.

Table 1: The released MAA contents by NAC solution treatment in various *N. flagelliforme* (*Nf*) and *N. commune* (*Nc*) samples.

Sample no. (year, source)	Recovered Fv/Fm ^a	FW/DW ratio (fold)	Absorbance at 312 nm ^b	MAA content (% of DW) ^c
<i>Nf</i> -1 2016, Inner Mongolia	0.53 ± 0.04	16.15	0.548 ± 0.011	0.14
<i>Nf</i> -2 2016, Yinchuan, Ningxia	0.52 ± 0.02	15.56	0.283 ± 0.019	0.07
<i>Nf</i> -3 2013, Inner Mongolia	0.10 ± 0.02	15.96	0.086 ± 0.004	0.02
<i>Nf</i> -4 2004, Inner Mongolia	0	13.16	0	/
<i>Nf</i> -5 1999, Inner Mongolia	0	15.86	0	/
<i>Nc</i> -1 2016, Jingmen, Hubei	0.44 ± 0.07	22.15	0.921 ± 0.092	0.31
<i>Nc</i> -2 2014, Wuhan, Hubei	0.25 ± 0.08	9.20	0.282 ± 0.008	0.04
<i>Nc</i> -3 2013, Shandan, Gansu	0	20.22	0	/
<i>Nc</i> -4 2013, Ninghai, Zhejiang	0	9.46	0	/

^a The physiological recovery was indexed by Fv/Fm after the samples were rehydrated in BG11₀ solution for 24 h; data shown are the means ± SD (n=6). FW/DW, the fresh weight of the rewetted sample over its original dry weight. ^b Rewetted samples of 1 g FW were incubated with 20 ml of 40 mM NAC solution for 4 h; data shown are the means ± SD (n=3). ^c Calculated based on the estimated average extinction coefficient of 120 liters g⁻¹ cm⁻¹ [1] with a slight modification, namely 131 liters g⁻¹ cm⁻¹, due to the absorbance difference in different solvents.

3.3 MAA extraction in various samples with different vitalities

The extraction efficiency of MAAs can be affected by several factors such as reagents, temperature, extraction duration or even sample states (ground or not) [1,3,6]. No significant difference in the extraction effect was observed at temperature range of 20–40°C or during 2–6 h incubation (data not shown). Thus, the MAA extraction was still performed at 22°C in various *N. flagelliforme* and *N. commune* samples but the incubation was extended to 4 h. To combat the pH buffering from the EPS matrix, an increased liquid-to-solid ratio was adopted by incubating 1 g of rewetted samples with 20 ml of 40 mM NAC solution. The released amounts of MAAs in five *N. flagelliforme* and four *N. commune* samples were compared (Table 1). These samples had different vitality (in terms of the recovered extent of Fv/Fm) and moisture absorption capability (FW/DW ratio). Usually, newly collected or well kept *N. flagelliforme* and *N. commune* samples have a good Fv/Fm recovery of above 0.3. As shown in table 1, the newly collected samples (in 2016) showed overall high MAA contents, ranging from 0.07–0.31%. The big difference in MAA content between two *N. flagelliforme* samples in 2016 possibly implies different environmental influence on its synthesis (e.g., UV intensity and duration of exposure). However, MAAs could not be detected in either *N. flagelliforme* or *N. commune* samples with no photosynthetic physiological recovery. The long-term storage can lead to the reduction or even loss of the vitality of samples [24]. Unlike scytonemin, MAAs are generally instable molecules [3]. Therefore, these results suggested that the timely extraction of MAAs from newly collected samples is beneficial to achieving a relatively high harvest.

4. CONCLUSION

In this study, it was revealed that NAC solutions primarily play a mild acid hydrolysis role in causing the dissociation and release of MAAs from the edible terrestrial cyanobacteria. Similar physiological or morphological effects between NAC solutions and other acidic solutions provided further evidence. The pH buffering role of EPS matrix was also found. However, more importantly, acid treatments *via* mere incubation did not destroy the morphological integrity of samples, in addition

to rapid extraction of MAAs at a temperate condition. Therefore, acid hydrolysis-related MAA extraction serves as a simple and efficient technique for achieving the purpose of deeply exploiting edible terrestrial cyanobacteria. Additionally, it was found that the newly collected samples are an important source for MAA harvest.

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