Journal of Nutrition & Food Sciences
ISSN: 2155-9600

Watanabe and Yamasaki, J Nutr Food Sci 2017, 7:1
DOI: 10.4172/2155-9600.1000572

Research Article Open Access

Dynamics of Nitrite Content in Fresh Spinach Leaves: Evidence for Nitrite Formation Caused by Microbial Nitrate Reductase Activity

Naoko S Watanabe and Hideo Yamasaki*

Faculty of Science, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan

Abstract

Nitrite (NO_2^-) contained in dietary foods has long been recognized for its toxicity as the causative agent of methemoglobinemia and also as a source of mutagenic nitrosamines. Because of these potential toxicities, nitrite as well as nitrate contained in foods and drinks are strictly limited by regulations in many countries. Recent studies have offered us to update our recognition of nitrite; nitrite is an important precursor for Nitric Oxide (NO) that is required for fundamental physiological activities including vasorelaxation. Although it is well established that green vegetables contain high amounts of nitrate, there has been controversy regarding the source of nitrite accumulation in fresh green vegetables. In this study, we investigated the dynamics of nitrite and nitrate contents in spinach leaf extracts to verify the mechanisms of nitrite formation. The time course of nitrite production in leaf extracts showed a reciprocal relationship with nitrate degradation, suggesting a conversion from nitrate to nitrite. The reaction strongly depended on temperature and it was suppressed at a low temperature. Sodium tungstate, a nitrate reductase enzyme inhibitor, was effective to suppress the conversion. Pre-sterilization by autoclaving or filter sterilization completely prevented the formation of nitrite as well as degradation of nitrate. We suggest that previous reports of nitrite accumulation can be attributed to microbial nitrate reductase activities that occur during the degradation spinach leaves.

Keywords: Bacteria; Dietary food; Nitrite; Nitric oxide; Metabolic syndrome; Vegetable; Sodium tungstate

Abbreviations: AMO: Ammonia Monooxygenase; HAO: Hydroxylamine Oxidoreductase; NiR: Nitrite Reductase; NO: Nitric Oxide; NOS: Nitric Oxide Synthase; NR: Nitrate Reductase; ROS: Reactive Oxygen Species; RNS: Reactive Nitrogen Species

Introduction

A vegetable-based diet has been widely recognized effective in prevention of chronic diseases including metabolic syndrome. Nutritionally, dietary vegetable is an important source of vitamins, minerals and fibers [1]. Green leafy vegetables, in particular, are beneficial due to a high content of ascorbate (vitamin C), and colored vegetables include carotenoids (vitamin A precursor) and polyphenols (vitamin P), all of which act as strong antioxidants that detoxify Reactive Oxygen Species (ROS) formed under stress or disease conditions [2,3]. In addition to these aspects, recent progress in life science has made a new paradigm shift which impacts even on food sciences, i.e., physiology and biochemistry of Nitric Oxide (NO).

NO is a free radical gaseous molecule that was previously only recognized for its potential toxicity as an air pollutant [4]. After the discovery of a physiological function of NO in vasorelaxation mechanism, it has been revealed that NO exhibits a range of fundamental roles in mammalian physiology: regulating blood pressure [5], neurotransmission [6], regulation of immune responses through activation of macrophages [7] and penile erection [8]. Just as superoxide (O_2^-) is a primary source of ROS, NO and its reaction products are designated as Reactive Nitrogen Species (RNS) [9]. Thus, increase of NO availability through foodstuffs has attracted much attention from medical researchers in terms of chronic disease prevention [10].

In general, NO is produced in mammals through the enzymatic activity of Nitric Oxide Synthase (NOS) with the amino acid L-arginine as a substrate [5]. Since the NOS reaction requires O_2 to produce NO, it does not work in anoxia or anaerobic conditions where ischemia occurs [11,12]. Nitrite-dependent NO production mechanism does work even under such conditions [11,12], and is thereby considered to be an alternative backup mechanism for NO production in our body [11,12]. In the oral cavity, nitrite (NO_2^-) is produced from nitrate (NO_3^-) through symbiotic bacterial activities [13]. Of the nitrate absorbed from the intestine approximately one-quarter is returned to the upper gastrointestinal tract via the saliva, presumably to permit reduction of nitrate to nitrite by mouth bacterial flora [13-15].

In food science, nitrite contained in human diet has long been recognized for its toxicity as the causative agent of methemoglobinemia [12]. Due to the formation of mutagenic nitrosamines [16], it has also been presumed that nitrite is potentially carcinogenic [17]. Because of these two historical backgrounds, nitrite as well as nitrate contained in foods and drinks has been strictly limited by regulations in many countries [18-20]. However, extensive animal and epidemiological studies have not indicated that nitrite in foods leads to carcinogenesis [13].

Nitrite ingested undergoes non-enzymatic acid-mediated reduction to NO in the stomach while the rest is absorbed into the bloodstream [13]. Figure 1 shows a schematic illustration for dietary source of NO. In general, natural foods such as leafy vegetables or seaweeds possess little nitrite but high levels of nitrate [13,21] a possible health benefit of the Japanese [22] and Mediterranean diets [23-25]. Direct supply of nitrite from diets may come from processed foods or curing meats.

***Corresponding author:** Hideo Yamasaki, Faculty of Science, University of the Ryukyus, Nishihara, Okinawa, Japan, Tel: 0988958595; E-mail: yamasaki@sci.u-ryukyu.ac.jp

Received November 21, 2016; **Accepted** December 12, 2016; **Published** December 20, 2016

Citation: Watanabe NS, Yamasaki H (2016) Dynamics of Nitrite Content in Fresh Spinach Leaves: Evidence for Nitrite Formation Caused by Microbial Nitrate Reductase Activity. J Nutr Food Sci 7: 572. doi: 10.4172/2155-9600.1000572

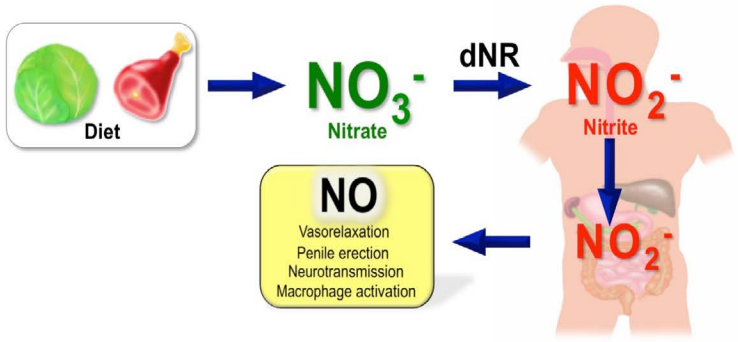

Figure 1: Exogenous sources of nitrite production in body. Dietary intakes of nitrate included in vegetables, meats and drinks lead to conversion of nitrate to nitrite by nitrate reductase of oral bacteria. Nitrite reaching the stomach through the digestive tract can be spontaneously converted to nitric oxide (NO) in the acidic conditions of the stomach. NO acts as an important factor in physiological functions, for example vasorelaxation, neurotransmission and activation of macrophages. dNR indicates dissimilatory nitrate reductase.

Therefore, bioavailable nitrite is mostly supplied exogenously as nitrate contained in foods and drinks (Figure 1).

Vegetables are major dietary sources of nitrate in general. More than 80% of the nitrate ingested can be attributed to vegetables [26]. Nitrate is an essential nutrient for plant growth [27] and its content varies widely between plant species [28,29] and even within the same tissue types of the same species, presumably due to differences in nitrate fertilization during cultivation [30]. Even in plant physiology, the conditions where leaves accumulate nitrite in the tissue are yet unclear [31]. Many physiological studies have suggested that plants produce nitrite only under stress conditions [32,33]. However, its production mechanism is still under debate [34].

There have been many reports on the nitrate and nitrite contents in retail green vegetables [28,35]. In respect to nitrite content, there are inconsistent and controversial results; some reports indicate the presence of nitrite [28,35-37] but others do not [38,39]. Hsu et al. suggested that nitrite detected in fresh vegetables may be due to bacterial activities [29]. To date, however, there has been no experimental evidence for the bacterial association with the nitrite formation. Here we demonstrate that the nitrite formation can be attributed to bacterial nitrate reductase activity which converts nitrate to nitrite in the process of spinach leaf decay.

Materials and Methods

Sample preparation

Spinach (*Spinacia oleracea* L.) was used as a representative green leaf vegetable due to a high amount of nitrate [40]. Fresh spinach leaves were obtained from local markets. After removing midrib, spinach leaves washed with tap water (300 g of fresh weight) were homogenized with distilled water (600 ml) at 0˚C for 30 s. The obtained homogenate was filtered through four-layers of gauze. The filtrate was centrifuged at 5,000 × g for 12 min and the supernatant obtained was used as the leaf extract for experiments.

The leaf extract (10 ml) was incubated in a gamma-sterilized Falcon tube at a constant temperature in darkness. During incubation, the tubes were continuously shaken at 120 rpm. Samples collected were frozen at –80˚C until analyzed.

The frozen samples were thawed and denatured at 100˚C for 3 min to inactivate endogenous enzymatic activities. The heat-denatured samples were centrifuged at 15,000 × g for 10 min (4˚C) and the supernatant filtered with a syringe filter (0.45 μm mixed cellulose ester syringe filter, 25AS045AN, Advantec) to obtain a soluble fraction. We further passed the sample through a SPE removing chloride filter (IC-Ag, Altech) to remove Cl^- whose retention time was close to nitrite. A 0.2 μm PTFE syringe filter (13HP020CN, Advantec) was used before injection into the ion chromatography apparatus.

Ion chromatography

To quantify the nitrate and nitrite contents, we used HPLC ion chromatography with an anion column (Shim-pack IC-A3, Shimadzu) along with an electrical conductivity detector (CDD-10A_{VP}, Shimadzu). Experimental conditions were basically similar to the method reported by Ogata and coworkers [41]. A mobile phase liquid contained 3.2 mM Bis-tris, 8.0 mM 4-hydroxybenzoic acid and 50 mM boric acid (pH 4.5). The flow rate was 0.7 ml/min and the column temperature was kept at 40˚C. Calibration was carried out with an anion mixture standard solution (Wako, Saitama).

Time course experiments to monitor nitrite and nitrate contents

For the time-course experiments of changes in nitrate and nitrite contents, 200 ml of leaf extract in a 500 ml conical flask was used. Conical flasks were plugged with sponge silicone plugs which allow gas flow but prevent microbe penetration. The leaf extracts were incubated in darkness at 35, 25 and 15˚C for 48 h. During the incubation, conical flasks were rotated at a constant speed (60 rpm) for mixing and aeration. Aliquots (1 ml) of the leaf extract were taken and they were frozen at –20˚C until analyzed.

Experiments of inhibitors and sterilization

To verify microbial activities we sterilized the leaf extract with two distinct methods: autoclave sterilization and filter sterilization. Autoclaving was carried out at 120˚C for 5 min before the incubation. To sterilize without heat treatment, we used a filter sterilization method with a 0.22 μm PES filter cartridge (8020-500, IWAKI) before the incubation. In inhibitor experiments, allylthiourea, nitrapyrin and tungstate (1 mM for each) were added before incubation, and the leaf extracts (10 ml) were rotated at 120 rpm at 25˚C in darkness for 42 h. Aliquots of leaf extract (1 ml) were sampled and kept at –80˚C until analyzed.

Chemicals

Allylthiourea (1-allyl-2-thiourea) and nitrapyrin (2-chloro-6-

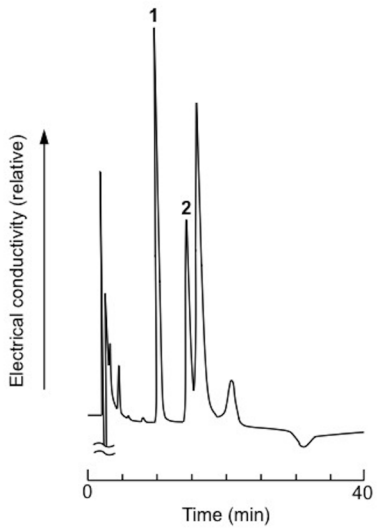

Figure 2: Ion chromatogram of spinach leaf extract. A typical chromatogram is shown. Peak 1, NO_2^-. Peak 2, NO_3^-. Each peak area was quantified with the anion mixture standard solution.

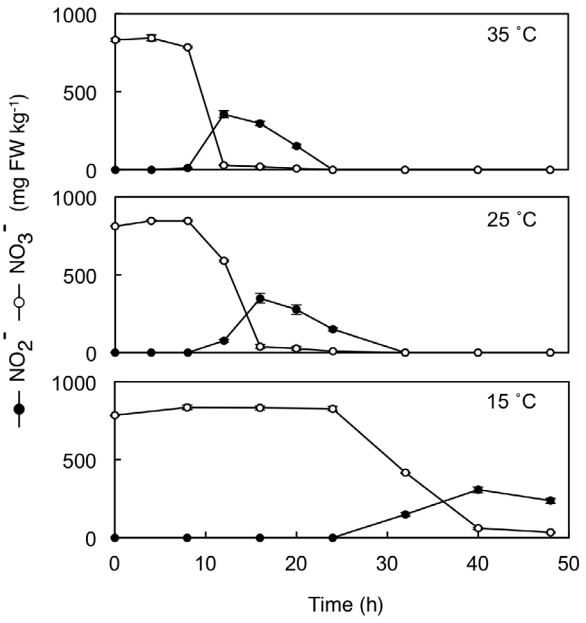

Figure 3: Dynamics of nitrate and nitrite. Closed circle, NO_2^-. Open circle, NO_3^-. Spinach leaf extracts were incubated at 35˚C, 25˚C and 15˚C in darkness. Each point represents the average with error bars (S.E., n=3).

[trichloromethyl] pyridine) were obtained from Tokyo chemical. Tungstate (sodium tungstate dehydrate) was obtained from Nacalai tesque. Bis-tris was obtained from Dojindo. 4-Hydroxylbenzoic acid was obtained from Kishida chemical. Allylthiourea and tungstate were dissolved in distilled water (100 mM) and nitrapyrin was dissolved in DMSO (100 mM).

Results

Nitrate content in spinach leaves

As many studies have reported so far, the nitrate content in retail spinach leaves varies to a large extent: from a minimum of 110 mg/kg [37] and a maximum of 4,923 mg/kg [35]. We also found similar trend on the nitrate content in retail spinach leaves. In our analysis, the average nitrate content was 1,900 mg/kg ±194 (n=10; ±SD), with a 785 mg/kg as minimum and maximum of 2,544 mg/kg.

Although our results of nitrate content basically agreed with the previously reported values, we detected only negligible amounts of nitrite. As Phillips pointed out, some previously reported results might be accounted for by bacterial nitrite formation due to decay of vegetables [42]. We indeed found that nitrite was detected in one week artificially decayed spinach leaves (data not shown). Since experimental control of the decay of the leaves with good reproducibility was difficult and it was virtually impossible to apply chemicals to investigate the phenomenon, we decided to use a model system for analysis. In this study a crude leaf extract that includes a soluble fraction as well as small organelles and surface bacteria was used for analysis.

Time course of dynamics of nitrate and nitrite at different temperature

We first monitored the changes in nitrite and nitrate contents in spinach leaf extracts for 48 h, incubating at different temperatures. To quantify nitrate and nitrite, we applied an ion chromatography technique. Figure 2 shows a typical HPLC ion chromatogram. Nitrate (peak 1) and nitrite (peak 2) were successfully detected as distinct peaks (Figure 2). Nitrate and nitrite contents were determined with each peak area. Figure 3 illustrates changes in nitrate and nitrite contents incubated at 35˚C, 25˚C, and 15˚C. At all the temperatures tested, nitrate degradation followed by nitrite formation was observed during the incubation (Figure 3). The changes strongly depended on an incubation temperature; both changes (nitrate degradation and nitrite formation) went slower at a lower temperature. At 15˚C, no change was observed until 24 h. The maximum nitrite content was observed among the all incubation temperatures tested: 12 h (35˚C), 16 h (25˚C) and 40 h (15˚C). The maximum values of the formed nitrite at the peaks were 357 mg/kg, 350 mg/kg, and 307 mg/kg at 35˚C, 25˚C, and 15˚C, respectively. After reaching to the maximum, nitrite content decreased and eventually reached a negligible amount at all temperatures. It should be noted that an odd smell came from such leaf extracts.

Effects of inhibitors of bacterial nitrification and denitrification

To verify the involvement of biological activity in nitrite formation in the leaf extract, we investigated effects of inhibitors on nitrite formation. Inhibitors of nitrification (allylthiourea and nitrapyrin) and denitrification (tungstate) were tested.

Figure 4 shows suppressive effects of various treatments on nitrite formation in spinach leaf extract. As a negative control, we analyzed sterilized leaf-extract samples: autoclaved or filter sterilized. In both sterilization methods (with or without heat treatment), no nitrite formation was observed, indicating that the nitrite formation is attributable to microbial activities. Allylthiourea and nitrapyrin are known to inhibit the bacterial Ammonia Monooxygenase (AMO) which is a key enzyme for nitrification [43]. Allylthiourea showed no effect while nitrapyrin exhibited some (20% suppression). Tungstate is an inhibitor for nitrate reductase which is involved in denitrification. Interestingly, tungstate effectively prevented nitrite formation even in non-sterilized leaf extract sample (Figure 4).

The same trend was observed in the degradation of nitrate in the leaf-extract (Figure 5). As observed in nitrite formation, sterilization by autoclave and filter prevented the degradation of nitrate contained

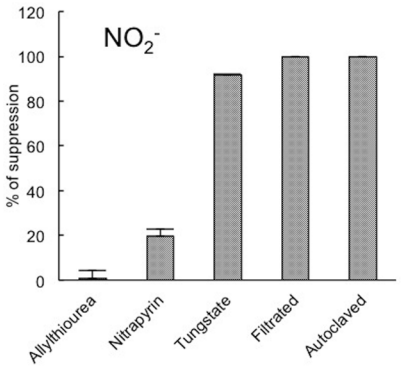

Figure 4: Suppression of nitrite formation. Inhibitory effects of enzyme inhibitors allylthiourea (AMO inhibitor), nitrapyrin (AMO inhibitor) and tungstate (nitrate reductase inhibitor) on NO_2^- formation activity. The final concentration was 1 mM for each. To confirm the association of microbial activities, the leaf extracts were treated by either filter or autoclave sterilization. Each bar represents the average with error bars (S.E., n=3).

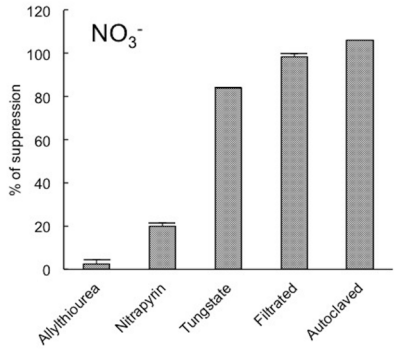

Figure 5: Suppression of nitrate degradation. Inhibitory effects of enzyme inhibitors allylthiourea (AMO inhibitor), nitrapyrin (AMO inhibitor) and tungstate (nitrate reductase inhibitor) on degradation activity of NO_3^-. The final concentration was 1 mM for each. To confirm the association of microbial activities, the leaf extracts were treated by either filter or autoclave sterilization. Each bar represents the average with error bars (S.E., n=3).

in the leaf-extract (Figure 5). Tungstate effectively prevented the degradation of nitrate, whereas nitrapyrin and allylthiourea showed weaker effects. Overall, the effects of the treatments were identical between nitrite formation and nitrate degradation, suggesting that bacterial conversion of nitrate to nitrite occurred in the leaf extract as has been suggested.

Discussion

Fresh spinach leaves do not accumulate nitrite

Our results have provided experimental evidence to confirm that nitrite accumulation merely occurs in fresh intact vegetables; bacterial activity mediates nitrite formation through nitrate reducing activity in decayed leaves. This nitrite formation can be suppressed at a low temperature, implying that nitrite should not be accumulated as long as fresh intact spinach is properly stored in a refrigerator at a non-frozen temperature. Therefore, it can be concluded that previous reports of high nitrite content reflected bacterial activity due to physical damage or degradation of spinach leaves as discussed by Phillips [42].

Nitrite accumulation mechanism in spinach leaves

As mentioned in the introduction, plant leaves do not accumulate nitrite under favorable conditions; nitrite can be detected in leaves when plants are exposed to stress conditions. Nitrite exists as an intermediate metabolite in nitrate assimilation. Plants absorb nitrate mainly from soil to synthesize amino acids. Using solar light energy, absorbed nitrate from the soil is reduced to nitrite by Nitrate Reductase (NR) in the cytosol [4]. Nitrite translocated into the chloroplasts is converted to ammonium ion (NH_4^+) by nitrite reductase (NiR) located in the chloroplasts [4] and then ammonium ion is assimilated into amino acids [4]. The process can be disturbed by biotic (infectious or herbivoric) as well as abiotic (environmental) stresses. It has been found that root of tomato (*Lycopersicon esculentum* cv. Rondello) forms nitrite under anoxic conditions [33]. In rice seedling (*Oryza sativa* L. cv. Akitakomachi) Suzuki et al. reported that exposure of the tissues (root and stem) to different temperatures resulted in accumulation of nitrite in the leaves after light/dark transition [32]. It should be emphasized that accumulated nitrite can be reduced to NO by NR and the gas will be released into the air [4,44]. At an early stage of this study, we hypothesized that retail spinach leaves stored at a refrigerator temperature under light for display might accumulate nitrite because ROS is overproduced under such condition [45]. We did detect a small and transient NO emission from leaves but nitrite content was negligible (data not shown). As long as we examined, there was no clear indication for the accumulation of nitrite in spinach leaves by stress treatment that is presumed to occur in a retail process. The only case we detected nitrite was following physical damage of the leaves, which facilitated bacterial degradation.

It has been known that processed vegetable foods sometimes include high nitrite. Chetty and Prasad; and Phillips reported that baby foods made from vegetable included nitrite [42,46]. Moreover, some studies reported nitrite in canned and frozen vegetables [37,42,47]. It is highly likely that vegetables were already spoiled, and sterilization was not enough to prevent microbial growth in the processing vegetables. Fermentation in pickling vegetable can result in high accumulations of nitrite. Ji et al. reported that nitrite was formed and nitrate was degraded in pickled Chinese cabbage (*Brassica campestris* L.) during the fermentation [48]. Yan et al. found that nitrite in cabbage (*Brassica oleracea* var. capitata) increased during fermentation for Chinese paocai [49].

Another reason for inconsistency in nitrite content in vegetables may be attributed to artefactual factors due to the methods used. Many earlier studies used the Griess method (a colormetric method) for nitrite quantification of vegetables. The tissues of plants or vegetables contain abundant antioxidants or reductants such as ascorbate and polyphenols which may affect certain assays [3]. Additionally, photosynthetic pigments may act as photosensitizers to produce reactive oxidants such as ROS under light [4]. These technical factors might confound the quantification of nitrite.

Source of bacteria

In principle, nitrite is formed through two distinct dissimilation metabolisms, namely, nitrification and denitrification [31,50]. In nitrification, two enzymes, Ammonium Monooxygenase (AMO) and Hydroxylamine Oxidoreductase (HAO), are involved in nitrite formation [31,50]. Ammonium ion is oxidized to hydroxylamine by AMO. Subsequently, HAO converts hydroxylamine to nitrite [31,50]. In denitrification, nitrite is formed from nitrate by nitrate reductase similar to plants [31,50]. Allylthiourea specifically inhibits AMO by binding to copper in its active center [43,51], whereas nitrapyrin inhibits AMO by acting as an alternative substrate [43]. Protein modification by the oxidized product irreversibly inactivates not only AMO but other

proteins in the cell as well [43,52]. Tungstate competes with molybdate in molybdate-dependent enzymes including NR, resulting inactivation of these enzymes [53,54]. In this study, we examined the effects of inhibitors of nitrification or denitrification on nitrite formation and nitrate degradation. As shown in Figures 4 and 5, nitrite formation and nitrate degradation were strongly inhibited by tungstate, whereas that allylthiourea and nitrapyrin had little effects. Based on these results, it is reasonable to conclude that nitrite formation was due to denitrification by NR but not nitrification by AMO and HAO. It should be noted that bacterial populations colonize in spermosphere as well as phyllosphere of spinach (*Spinacia oleracea*) seedlings and plants [55]. The nitrifying bacteria that produce nitrite would be included in such spermosphere and phyllosphere microbiota.

Prospects

Nitrite has long been considered as a toxic agent in human diet. Since inorganic nitrate is not an essential nutrient for humans, intake of nitrate and nitrite has been thought to be nonbeneficial. However, recent studies have suggested that dietary nitrate and nitrite are important for preventing metabolic syndromes [10]. In fact, there are less cardiovascular diseases in regions where consumption of vegetables is high [56] whereas there is no strong evidence that nitrate and nitrite intake from foods results in methemoglobinemia [57]. This is probably because, unlike infants, adults can reduce methemoglobin by the activity of NADH-cytochrome b5 reductase [58]. Also, vegetables contain lots of antioxidants as well as molecules that can suppress nitrosative stress. We suggest that nitrate contained in vegetables is beneficial for human health and that nitrite is not contained at physiologically effective concentrations in leafy vegetables as long as they are properly stored.

Acknowledgements

This work was supported by the supported by JSPS KAKENHI (15H02843) Grant-in-Aid for Scientific Research (B) to H. Y. from Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. We thank Dr. Michael Cohen for his critical reading of the manuscript.

References

1. Slavin JL, Lloyd B (2012) Health benefits of fruits and vegetables. Adv Nutr 3: 506-516.
2. Stahl W, Sies H (2003) Antioxidant activity of carotenoids. Mol Aspects Med 24: 345-351.
3. Sakihama Y, Cohen MF, Grace SC, Yamasaki H (2002) Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants. Toxicology 177: 67-80.
4. Yamasaki H (2000) Nitrite-dependent nitric oxide production pathway: implications for involvement of active nitrogen species in photoinhibition *in vivo*. Philos Trans R Soc Lond B Biol Sci 355: 1477-1488.
5. Alderton WK, Cooper CE, Knowles RG (2001) Nitric oxide synthases: structure, function and inhibition. Biochem J 357: 593-615.
6. Garthwaite J, Boulton CL (1995) Nitric-oxide signaling in the central-nervous-system. Ann Rev Physiol 57: 683-706.
7. MacMicking J, Xie QW, Nathan C (1997) Nitric oxide and macrophage function. Ann Rev Immunol 15: 323-350.
8. Andersson KE (2001) Pharmacology of penile erection. Pharmacol Rev 53: 417-450
9. Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, et al. (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39: 44-84.
10. Carlstrom M, Larsen FJ, Nystrom T, Hezel M, Borniquel S, et al. (2010) Dietary inorganic nitrate reverses features of metabolic syndrome in endothelial nitric oxide synthase-deficient mice. Proc Natl Acad Sci USA 107: 17716-17720.
11. Yamasaki H (2005) The NO world for plants: achieving balance in an open system. Plant Cell Environ 28: 78-84.
12. Yamasaki H, Watanabe NS, Fukuto J, Cohen MF (2014) Nitrite-dependent nitric oxide production pathway: diversity of NO production systems. Studies on pediatric disorders, Springer, pp: 35-54.
13. Lundberg JO, Weitzberg E, Gladwin MT (2008) The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. Nat Rev Drug Discov 7: 156-167.
14. Lundberg JO (2012) Nitrate transport in salivary glands with implications for NO homeostasis. Proc Natl Acad Sci USA 109: 13144-13145.
15. Lundberg JO, Weitzberg E, Cole JA, Benjamin N (2004) Opinion - Nitrate, bacteria and human health. Nat Rev Microbiol 2: 593-602.
16. Wolff IA, Wasserman AE (1972) Nitrates, nitrites and nitrosamines. Science 177: 15-19.
17. Huang YG, Ji JD, Hou QN (1996) A study on carcinogenesis of endogenous nitrite and nitrosamine, and prevention of cancer. Mutat Res-Fund Mol M 358: 7-14.
18. http://www.fsai.ie/uploadedFiles/Reg1258_2011.pdf
19. http://www.epa.gov/sites/production/files/2015-11/documents/howeparegulates_mcl_0.pdf
20. http://www.nhmrc.gov.au/_files_nhmrc/publications/attachments/eh34_adwg_11_06.pdf
21. Martinez B, Rico JM (2002) Seasonal variation of P content and major N pools in *Palmaria palmata* (Rhodophyta). J Phycol 38: 1082-1089.
22. Sobko T, Marcus C, Govoni M, Kamiya S (2010) Dietary nitrate in Japanese traditional foods lowers diastolic blood pressure in healthy volunteers. Nitric Oxide 22: 136-140.
23. Lundberg JO, Feelisch M, Bjorne H, Jansson EA, Weitzberg E (2006) Cardioprotective effects of vegetables: Is nitrate the answer? Nitric Oxide 15: 359-362.
24. Nadtochiy SM, Redman EK (2011) Mediterranean diet and cardioprotection: The role of nitrite, polyunsaturated fatty acids, and polyphenols. Nutrition 27: 733-744.
25. Shiva S, Sack MN, Greer JJ, Duranski M, Ringwood LA, et al. (2007) Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. J Exp Med 204: 2089-2102.
26. Lundberg JO, Carlstrom M, Larsen FJ, Weitzberg E (2011) Roles of dietary inorganic nitrate in cardiovascular health and disease. Cardiovasc Res 89: 525-532.
27. Yamasaki H, Cohen MF (2016) Biological consilience of hydrogen sulfide and nitric oxide in plants: Gases of primordial earth linking plant, microbial and animal physiologies. Nitric Oxide 55-56: 91-100.
28. Hord NG, Tang YP, Bryan NS (2009) Food sources of nitrates and nitrites: the physiologic context for potential health benefits. Am J Clin Nutr 90: 1-10.
29. Hsu J, Arcot J, Lee NA (2009) Nitrate and nitrite quantification from cured meat and vegetables and their estimated dietary intake in Australians. Food Chem 115: 334-339.
30. Zhen R, Leigh R (1990) Nitrate accumulation by wheat (*Triticum aestivum*) in relation to growth and tissue N concentrations: Plant nutrition—Physiology and applications, Springer, pp: 17-20.
31. Cohen MF, Mazzola M, Yamasaki H (2006) Nitric oxide research in agriculture: bridging the plant and bacterial realms: Abiotic stress tolerance in plants. Dordrecht, Springer, pp: 71-90.
32. Suzuki K, Ohmori Y, Nagao M (2013) Accumulation of nitrate and nitrite in chilled leaves of rice seedlings is induced by high root temperature. Plant Cell Physiol 54: 1769-1779.
33. Morard P, Silvestre J, Lacoste L, Caumes E, Lamaze T (2004) Nitrate uptake and nitrite release by tomato roots in response to anoxia. J Plant Physiol 161: 855-865.
34. Yamasaki H, Itoh RD, Bouchard JN, Dghim AA, Hossain KK, et al. (2010) Nitric oxide synthase-like activities in plants. Annual Plant Reviews Volume 42: Nitrogen Metabolism in Plants in the Post-Genomic Era, pp: 103-125.
35. de González MTN, Osburn WN, Hardin MD, Longnecker M, Garg HK, et al. (2015) A Survey of nitrate and nitrite concentrations in conventional and organic-labeled raw vegetables at retail. J Food Sci 80: C942-C949.
36. Hunt J, Turner M (1994) A survey of nitrite concentrations in retail fresh vegetables. Food Addit Contam 11: 327-332.

37. Jaworska G (2005) Nitrates, nitrites, and oxalates in products of spinach and New Zealand spinach - Effect of technological measures and storage time on the level of nitrates, nitrites, and oxalates in frozen and canned products of spinach and New Zealand spinach. Food Chem 93: 395-401.

38. Hertz J, Baltensperger U (1984) Determination of nitrate and other inorganic anions (NO_2^-, PO_4^{3-}, Cl^-, SO_4^{2-}) in salad and vegetables by ion chromatography. Fresen Z Anal Chem 318: 121-123.

39. Merusi C, Corradini C, Cavazza A, Borromei C, Salvadeo P (2010) Determination of nitrates, nitrites and oxalates in food products by capillary electrophoresis with pH-dependent electroosmotic flow reversal. Food Chem 120: 615-620.

40. Santamaria P, Elia A, Serio F, Todaro E (1999) A survey of nitrate and oxalate content in fresh vegetables. J Sci Food Agr 79: 1882-1888.

41. Ogata F, Tominaga H, Ueda A, Tanaka Y, Iwata Y, et al. (2013) Application of activated carbons from coal and coconut shell for removing free residual chlorine. J Oleo Sci 62: 241-244.

42. Phillips WEJ (1968) Changes in the nitrate and nitrite contents of fresh and processed spinach during storage. J Agr Food Chem 16: 88-91.

43. Iizumi T, Mizumoto M, Nakamura K (1998) A bioluminescence assay using *Nitrosomonas europaea* for rapid and sensitive detection of nitrification inhibitors. Appl Environ Microbiol 64: 3656-3662.

44. Yamasaki H, Sakihama Y, Takahashi S (1999) An alternative pathway for nitric oxide production in plants: new features of an old enzyme. Trend Plant Sci 4: 128-129.

45. Chang AC, Yang TY, Riskowski GL (2013) Ascorbic acid, nitrate, and nitrite concentration relationship to the 24 hour light/dark cycle for spinach grown in different conditions. Food Chem 138: 382-388.

46. Chetty AA, Prasad S (2016) Flow injection analysis of nitrate and nitrite in commercial baby foods. Food Chem 197: 503-508.

47. Siciliano J, Krulick S, Heisler EG, Schwartz JH, White JW (1975) Nitrate and nitrite content of some fresh and processed market vegetables. J Agric Food Chem 23: 461-464.

48. Ji F, Ji B, Li B, Lu F (2009) Effect of fermentation on nitrate, nitrite and organic acid contents in traditional pickled Chinese cabbage. J Food Process Preserv 33: 175-186.

49. Yan PM, Xue WT, Tan SS, Zhang H, Chang XH (2008) Effect of inoculating lactic acid bacteria starter cultures on the nitrite concentration of fermenting Chinese paocai. Food Control 19: 50-55.

50. Cohen MF, Lamattina L, Yamasaki H (2010) Nitric oxide signaling by plant-associated bacteria. Nitric Oxide Plant physiology, Weinheim, Wiley-VCH,, pp: 161-172.

51. Hooper AB, Terry KR (1973) Specific inhibitors of ammonia oxidation in *Nitrosomonas*. J Bacteriol 115: 480-485.

52. Vannelli T, Hooper AB (1992) Oxidation of nitrapyrin to 6-chloropicolinic acid by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. Appl Environ Microbiol 58: 2321-2325.

53. Xiong J, Fu GF, Yang YJ, Zhu C, Tao LX (2012) Tungstate: is it really a specific nitrate reductase inhibitor in plant nitric oxide research? J Exp Bot 63: 33-41.

54. Medina-Andres R, Solano-Peralta A, Saucedo-Vazquez JP, Napsucialy-Mendivil S, Pimentel-Cabrera JA, et al. (2015) The nitric oxide production in the moss physcomitrella patens Is mediated by nitrate reductase. PLoS ONE 10: e0119400.

55. Lopez-Velasco G, Carder PA, Welbaum GE, Ponder MA (2013) Diversity of the spinach (*Spinacia oleracea*) spermosphere and phyllosphere bacterial communities. FEMS Microbiol Lett 346: 146-154.

56. Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, et al. (2004) Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. Lancet 364: 937-952.

57. Bryan NS, van Grinsven H (2013) The role of nitrate in human health. Advances in agronomy 119: 153-182.

58. Umbreit J (2007) Methemoglobin - It's not just blue: A concise review. Am J Hematol 82: 134-144.