HYDROXYL RADICAL SCAVENGER ACTIVITY OF NATURAL SOD

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ABSTRACT

Introduction: Mammalian cells produce hydroxyl (·OH) radicals during normal and pathological metabolic processes. Due to their unpaired electron, ·OH radicals are able to degrade nearly any type of biomolecules including lipids, proteins and nucleic acids.

Objectives: As excessive ·OH radicals production leads to irreversible damage of cell structures, triggering and/or maintenance of pathological processes, using antioxidant supplements could have beneficial effects in reducing oxidative stress and maintaining a positive health status. The aim of this study was to evaluate the ·OH radical scavenger capacity of Natural SOD, green barley juice produced by Cantacuzino Institute.

Methods: By using Hydroxyl Radical Absorbance Capacity (HORAC) and Oxygen Radical Absorbance Capacity (ORAC) methods, we studied the antioxidant properties (hydroxyl and peroxyl radicals scavenging activities) of Natural SOD and its fractions (Natural SOD < 10 kDa and Natural SOD > 10 kDa).

Results: Our results showed that all Natural SOD samples had similar ·OH scavenger capacity, with non-significant time and temperature-dependent variations, while the peroxyl radical scavenging capacity significantly decreased after 11 months of storage at 4-8 ^{0}C or 20-25 ^{0}C.

Conclusion: The obtained data, correlated with our previous results, provide additional information on the mechanisms underlying antioxidant capacity of Natural SOD and offer premises for studying the role of Natural SOD in acute and chronic inflammatory disorders.

Keywords: hydroxyl radicals, green barley, antioxidant, scavenger, Natural SOD.

REZUMAT

Introducere: Celulele mamaliene produc radicali hidroxil (·OH) în procesele metabolice normale şi patologice. Datorită electronilor nepereche, aceşti radicali pot degrada aproape orice tip de biomolecule, inclusiv lipide, proteine și acizi nucleici.

Obiective: Producţia excesivă de radicali ·OH duce la deteriorarea ireversibilă a structurilor celulare. Utilizarea suplimentelor alimentare antioxidante ar putea avea efecte benefice în diminuarea stresului oxidativ şi menţinerea unei stări de sănătate corespunzătoare. Scopul acestui studiu a fost testarea capacitatii de epurare a radicalilor hidroxil de catre SOD Natural, suc de orz verde produs de Institutul Cantacuzino.

Metode: Utilizând metodele HORAC (Hydroxyl Radical Absorbance Capacity) și ORAC (Oxygen Radical Absorbance Capacity), am studiat proprietăţile antioxidante (de epurare a radicalilor hidroxil și peroxil) ale SOD Natural și ale fracțiunilor sale (SOD Natural < 10 kDa şi SOD Natural > 10 kDa).

Rezultate: Rezultatele obţinute au arătat că toate probele de SOD Natural prezintă capacităţi similare de epurare a radicalilor ·OH, cu variaţii nesemnificative în funcţie de timp şi temperatură; în schimb, capacitatea de epurare a radicalilor peroxil a scăzut semnificativ după 11 luni în care probele au fost depozitate la 4-8 ^{0}C sau 20-25 ^{0}C.

Concluzie: Datele obţinute, corelate cu rezultatele anterioare, aduc un plus de informaţie privind mecanismele ce stau la baza capacităţii antioxidante a SOD Natural şi oferă premisele pentru studierea rolului acestuia în disfuncţiile inflamatorii acute şi cronice.

Cuvinte-cheie: radicali hidroxil, orz verde, antioxidant, epurator, SOD Natural.

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INTRODUCTION

Hydroxyl radicals (·OH) are considered the most reactive oxygen species in biological systems [1]; they are produced continuously in oxygen-reducing reactions to water and the generated amounts become significant under hypoxia conditions [2].

Mammalian cells use several mechanisms to produce ·OH radicals (Fig. 1). In mitochondria, superoxide anion ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) can interact with transition metals such as iron or copper through a Haber-Weiss/ Fenton-type reaction leading to ·OH radical formation [3]:

$Metal^{3+} + \cdot O_2^- \rightarrow Metal^{2+} + O_2$

(Haber-Weiss reaction)

$Metal^{2+} + H_2O_2 \rightarrow Metal^{3+} + OH^- + \cdot OH$

(Fenton reaction)

Haber-Weiss/Fenton reactions were also described in lysosomal compartment and endoplasmic reticulum [4, 5].

H_2O_2 conversion to ·OH is of major physiological importance due to H_2O_2 ability to cross cell membranes and diffuse a considerable distance and promote free radical synthesis in cell compartments other than those already mentioned [6].

Moreover, in mitochondria, peroxynitrite conversion to ·OH (Beckman-Radi-Freeman pathway) represents an additional pro-oxidative mechanism [7].

Due to their high reactivity ($t_{1/2}$ = 1ns) OH radicals are able to degrade nearly any type of biomolecules, including lipids, RNA, DNA (both nuclear and mitochondrial) and proteins [7, 8, 9]. Hydroxyl radicals and their derivatives contribute to normal biological processes such as cell growth and differentiation, embryogenesis, neurotransmission, defence against microorganisms, biotransformation of xenobiotics, platelet aggregation etc [10, 11]. Similar to other reactive oxygen species, ·OH radicals are key players in complex responses to internal and environmental stimuli and adaptation processes.

As excessive free oxygen radicals (including ·OH radicals) production leads to irreversible damage of cellular components, aerobic organisms developed both enzymatic and non-enzymatic antioxidant systems able to reduce free radical production, to inhibit their action or to contribute to repair of altered cellular structures. Oxidative stress occurs when the balance between oxidant production and antioxidant defenses is disturbed in favor of oxidants.

Hydroxyl radical production is followed by cellular alterations - lipid peroxidation, mitochondrial dysfunction, exhausting enzyme activities, increase in intracellular free calcium and fibrin polymerization nuclear protein and DNA reactions - most often with pathological

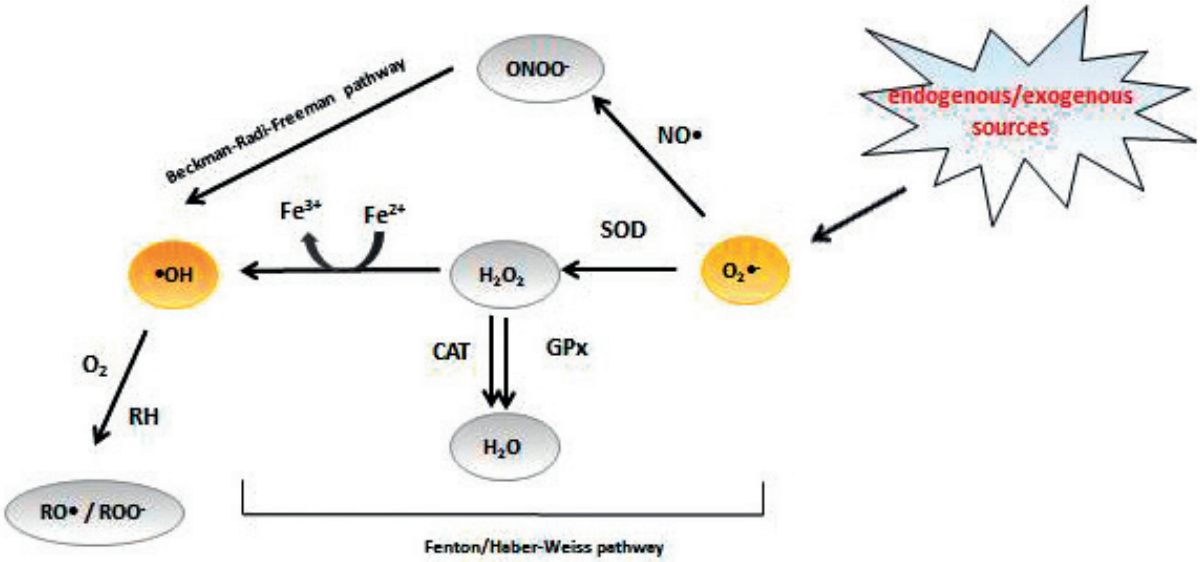

Fig. 1. Generation of free oxygen radicals in mammalian cells. $\cdot O_2^-$ superoxide anion; H_2O_2 hydrogen peroxide; OH hydroxyl radicals, RO· alkyl radicals; ROO^- peroxyl radicals; $ONOO^-$ peroxynitrite; NO· nitric oxide; SOD superoxide dismutase; GPx glutathione peroxidase; CAT catalase

consequences such as inflammation, altered immune functions including autoimmune processes, neurodegeneration, metabolic disorders, fetal growth restriction and malignant transformation [12, 13, 14, 15, 16, 17, 18].

Using antioxidant supplements could have beneficial effects in reducing oxidative stress and maintaining a positive health status. Recent studies have shown controversial effects of synthetic antioxidants. As plants are able to synthesize and to accumulate high amounts of free oxygen radical scavenging compounds [19], natural supplements with antioxidant properties represent a valuable option for reducing oxidative stress without adverse effects.

Because of its high content in antioxidant compounds (phenolics, aminoacids, biomolecules with scavenging properties, pigments, enzymes and minerals) barley *(Hordeum vulgare)* represents one of the most important raw materials for antioxidant supplements of vegetal origin [20, 21, 22]. *In vivo* studies have shown that green barley juice is able to inhibit percentages of lipid peroxidation in guinea pig brain (50% when compared with control group) [23]. In addition to a high content of biomolecules with SOD-like activity, barley crude extract is able to increase SOD activity in human (healthy donors) platelets [24].

Natural SOD is a green barley juice produced by Cantacuzino National Medico-Military Institute for Research and Development, with antioxidant properties (SOD-like, peroxidase-like and peroxyl radical scavenger activity) [25, 26], being recommended for people working under stress conditions (intense physical and mental effort), as well as environments with risk to health (e.g. ionizing radiations, toxic compounds).

Aiming for a deep investigation concerning the properties of Natural SOD, we obtained two fractions: < 10 kDa and > 10 kD. Series of green barley juice samples – Natural SOD (total), Natural SOD < 10 kDa and Natural SOD > 10 kDa - were stored at 4-8 ^{0}C (recommended storage temperature) or 20-25 ^{0}C (room temperature) for one year. Using HORAC (Hydroxyl Radical Absorbance Capacity) fluorimetric method all samples were tested monthly for hydroxyl radical scavenger capacity.

As pollution can affect crop soils, the accumulation of heavy metals in the soil could contaminate plants grown in those places. Heavy metals are able to interfere with photosynthetic processes in green barley, to induce oxidative stress and to inhibit plant growth [27]. Heavy metal intoxication in humans is associated with genotoxicity, carcinogenicity and neurotoxicity, all these pathological effects being mediated mainly through oxidative stress [3, 28, 29]. Thus, the possibility of heavy metal accumulation and the barley plant [30] could occur, which supports the analysis of the Natural SOD product and the content of heavy metals.

MATERIALS AND METHODS

Samples:

"Natural SOD" samples from 8 different batches (2015, 2016) were used for centrifugal separation (Amicon Ultra 4 mL centrifugal filter NMWL 10 kDa, 4000 rpm, 15 min, 4 °C) of the SOD Natural > 10 kDa fractions (upper tube) and SOD Natural < 10kDa (on the bottom of the tube). Following aliquotation in hermetically sealed tubes under sterile hood to avoid atmospheric oxygen-induced decrease of antioxidant capacity and contamination, Natural SOD samples were kept at 4-8 ^{0}C and 20-25 ^{0}C respectively; the antioxidant capacity was tested monthly ($T_0 - T_{11}$).

HORAC (Hydroxyl Radical Absorbance Capacity) method is based on the degradation (hydrogen atom transfer (HAT) process) of fluorescein by the hydroxyl radicals generated by hydrogen peroxide (H_2O_2). The presence of an antioxidant compound reduces the degradation of fluorescein, a process evidenced by a slower decrease in fluorescence over time. The antioxidant capacity of the test products is quantified by reference to a gallic acid standard curve [31, 32]. The fluorescein degradation process was followed by fluorimetric measurements (kinetics, λex = 485 nm, λem = 523 nm, 50 cycles, Δt between readings = 1min). The results were expressed in gallic acid equivalents (Gallic Acid Equivalents = 1 μM gallic acid).

ORAC (Oxygen Radicals Absorbance Capacity) method is very similar with HORAC, designed to measure the scavenger activity of a compound or a product against peroxyl

radicals generated by 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH). We used a validated a hydrophylic ORAC technique, as described in a previous publication [25].

Aiming to identify and quantify some chemical and biochemical compounds (phosphorus, zinc, selenium and B1, B2, D3, E Vitamins respectively), as well as for testing the heavy metals content of Natural SOD, samples from the same batch were tested according to international standards (see Tables 1-3). The tests were made by Hamilton Romania. Given measurement uncertainty was estimated for the coefficient k = 2 and 95% confidence level.

Data analysis and statistical methods

All results are expressed as means +/- standard deviation of 8 batches – corresponding values per timepoint. Statistical significance was determined using two tails t student test. A p-value < 0.05 was considered significant.

RESULTS AND DISCUSSION

By studying the Natural SOD capacity to scavenge ·OH radicals, one cannot notice significant differences between samples kept at 4-8 ^{0}C and those kept at 20-25 ^{0}C (valid for all three types of samples - Natural SOD (total), Natural SOD > 10 kDa, Natural SOD < 10 kDa) (Fig 2). However, in all three cases, one can notice a decreasing trend of the ·OH radicals scavenging capacity for samples kept at room temperature. When comparing T_{11} timepoint (11 months) with T_0, we obtained significant variations of ·OH radical scaveger capacity for Natural SOD < 10 kDa and Natural SOD (total) samples kept at 20-25 ^{0}C (p = 0.03 and 0.01 respectively).

For the samples kept at 4-8 ^{0}C, apparently higher average values were obtained for Natural SOD > 10kDa. However the corresponding standard deviations are higher and the differences between samples are not significant.

Concerning the peroxyl radical scavenging capacity of Natural SOD, we noticed a significant decrease within 11 months for the three types of tested samples (p < 0.01): Natural SOD (total), Natural SOD < 10 kDa and Natural SOD < 10 kDa (Fig. 3).

The loss of scavenger activity was accelerated for Natural SOD (total) kept at 20-25 ^{0}C. These results, together with the data obtained for the other types of tests (SOD-like activity, peroxidase-like activity and HORAC), contribute to the establishment of the validity period for Natural SOD and recommend 4-8 ^{0}C as long-term storage temperature.

The scavenger capacities of Natural SOD (total) and Natural SOD < 10 kDa (for both ·OH and peroxyl radicals) showed similar time dependent variations, either for samples kept at 4-8 ^{0}C, or for samples kept at room temperature (20-25 ^{0}C).

Natural SOD samples (with already proven GPx-like activity) are added in HORAC reaction system before H_2O_2 to ·OH conversion to start. This makes difficult to consider if Natural SOD acts on 2 different routes - ·OH scavenger and GPx-like activity – or its effect is due to a direct action on H_2O_2 (by GPx-like activity), preventing H_2O_2 processing into ·OH (Fig. 4). Non significant time dependent

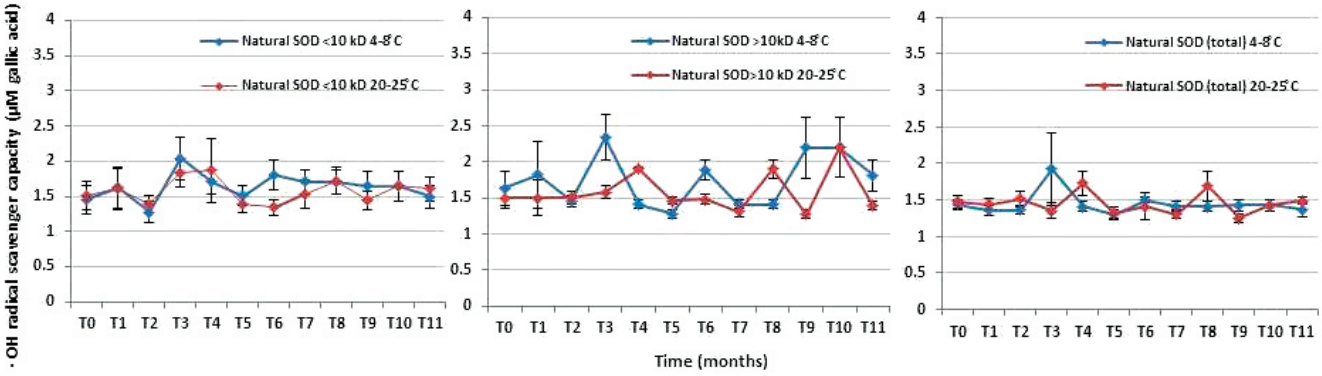

Fig. 2. The scavenging capacity of ·OH radicals for Natural SOD (total), Natural SOD > 10 kDa, Natural SOD < 10 kDa kept at 4-8 ^{0}C and 20-25 ^{0}C respectively. Each point represents the mean value for the 8 batches tested at the specified time and temperature

Table 1. Vitamin content of Natural SOD Product

Test	Result	Method	Recommended daily allowance (RDA) for adults
Vitamin B1 (thiamine)	0.03 mg/100 ml	PN-A-79011-4;1998	1.4 mg
Vitamin B2 (riboflavin)	0.03 mg/100 ml	PN-A-79011-4;1998	1.6 mg
Vitamin B9 (folic acid)	2.92ug/100 g	PB-327 ed I / 30.11.2015	0.4 mg (optimum 1 mg) [46]
Vitamin C (ascorbic acid)	5.7 mg/100 g	PB-135/HPLC, ed II / 15.09.2015	75 mg – women; 90 mg – men [47]
Vitamin E (α-tocopherol)	< 0.1 mg/100 ml	PB-40/HPLC ed. III / 28.02.2009	15 mg
Vitamin D_3 (cholecalciferol)	< 0.25 µg/100 g	PN-EN 12821:2009	20 mg

variations of the ·OH radical scavenging capacity for all types of Natural SOD samples (Natural SOD (total), Natural SOD > 10 kDa, Natural SOD < 10 kDa), together with the significant time - dependent decrease of GPx-like activity (data not shown) sustain the hypothesis that the ·OH decrease shall be on the basis of Natural SOD GPx-like activity and not on the basis of a scavenging capacity itself for the mentioned radicals.

Oxidative stress is often associated with an accumulation of iron in the cell. Studies conducted in our laboratory have demonstrated that Natural SOD has non significant capacity to reduce Fe^{3+} ions (FRAP - Ferric Reducing Antioxidant Power – method) and to neutralize ONOO- radicals (non-cellular Griess method) (data not shown).

To summarize, the antioxidant capacity of Natural SOD is due to SOD and GPx- like activity, as well as scavenging activity against ·OH and $ROO^{\cdot}$ radicals (Fig. 4). The measured antioxidant effects could be due to enzymes, pigments (e.g. chlorophyll), hydrosoluble vitamins, or to other compounds not yet identified [33]. Natural SOD contains the assembly of existing amino acids within the green barley juice, including serine, glycine, histidine, arginine, threonine, alanine, tyrosine, leucine and tryptophan (data not shown). *In vivo* studies demonstrated that the vast majority of the listed amino acids were involved in reducing oxidative stress [34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45].

Natural SOD contains antioxidant vitamins such as B1 (thiamine), B2 (riboflavin), B9 (folic acid) and C (ascorbic acid) (Table 1). The contents of Vitamins E and D3 in the Natural SOD (green barley juice) are under the detection limits; these results can be explained by the fact that both of them are liposoluble vitamins.

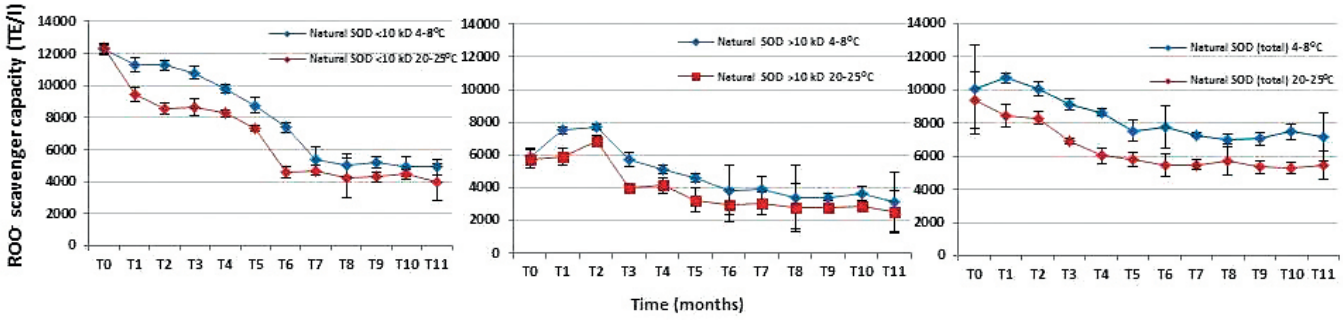

Fig 3. The $ROO^{\cdot}$ radicals scavenging capacity for Natural SOD (total), Natural SOD < 10 kDa, Natural SOD > 10 kDa samples, kept at 4-8 °C and 20-25 °C respectively. Each point represents the mean value for 8 tested batches at the specified time and temperature. TE = Trolox Equivalent (1TE = 1 µM Trolox)

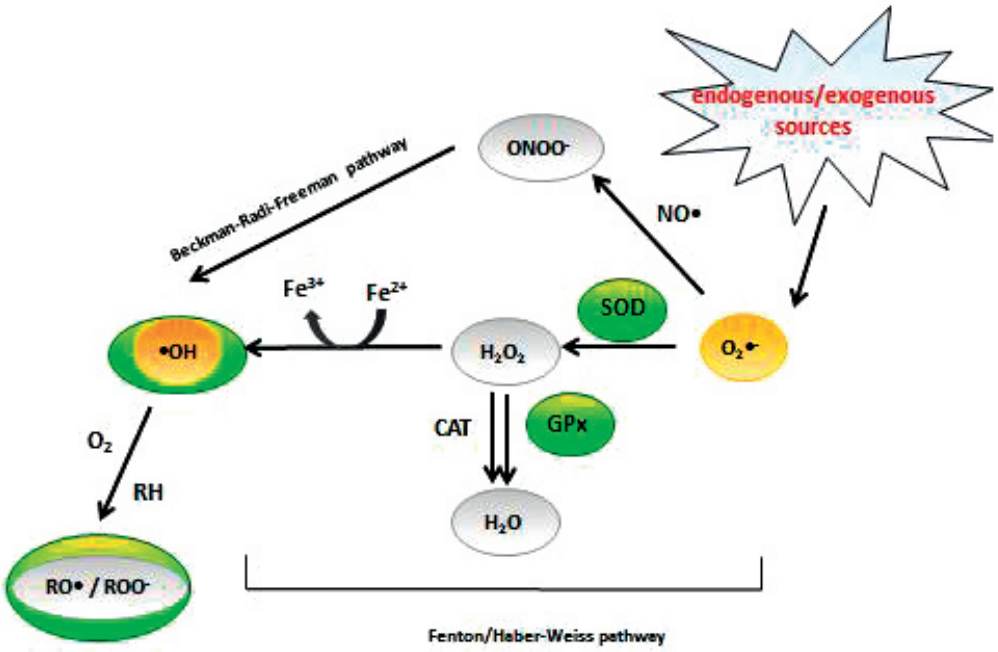

Fig. 4. The main reactive oxygen species with biological significance (superoxide, $O_2^{\cdot-}$, hydroxyl radical, ·OH, peroxyl, ROO·, nitric oxide and peroxynitrite, $ONOO^-$) and the key points where Natural SOD exerts its antioxidant activity (green)

Our results also point out the fact that the vitamin content is under the recommended daily allowance (RDA), sustaining the fact that Natural SOD could be used as a food supplement without any overdosing risk concerning these biochemical compounds. Moreover, the natural forms of vitamins are preferred, being absorbed with higher efficiency compared with synthetic vitamins.

The chemical and biochemical content of Natural SOD (Table 2) explains the beneficial effect of the product in promoting healthy state: maintaining electrolyte balance and good functioning of the nervous system (sodium, potassium) [48, 49, 50, 51], cardiovascular system (folic acid, vitamin C, potassium) [48, 49, 50, 51, 52, 53, 54, 55] of the bone system (calcium, phosphorus) [56, 57, 58], adequate energy metabolism (phosphorus, carbohydrates) [59], antioxidant processes and immune function (vitamin C) [55, 60] (vitamin C, proteins) [55] etc.

Vitamin C (ascorbic acid) is important in maintaining iron and copper in reduced state at the active sites of many oxidases and peroxidases [61].

Table 2. Chemical and biochemical composition of Natural SOD

Test	Result	Method	Recommended daily allowance (RDA) for adults
Proteins	0.56 %	SR EN 25663:2000	
Carbohydrates	2.5 %	PB-287 ed I, 27.09.2014	
Phosphorus	2 mg/liter	SR EN ISO 10304-1:2009	800-1200 mg [62]
Potassium	4170 mg/kg	SR EN ISO 15587-2:2003	40-100 mEq [63]
Calcium	295 mg/kg	SR EN ISO 11885:2009	1000-1200 mg [64]
Sodium	239 mg/kg		2300 mg [65]
Magnesium	128 mg/kg		320 mg – women; 420 mg – men [66]
Manganese	1.25 mg/kg		8-10 mg [67]
Gluten	Not detected	PB-139 ed IV, 13.05.2013	Not recommended in case of gluten intolerance

Table 3. The concentration of heavy metals in Natural SOD product

Test	Natural SOD corresponding value	Method	Toxicity limit
Cadmium	0.0022 mg/L	PN-EN 15763:2010	≤ 0.005 mg/L
Nickel	0.044 mg/L	PB-223/ICP, ed. II from 12.01.2015	< 1 mg
Lead	0.022 mg/L	PN-EN 15763:2010	There is no known level of lead exposure that is considered safe.
Mercury	< 0.0006 mg/L	PB-30/CVAAS ed. V from 18.09.2012	< 0.002 mg/L
Arsenic	< 0.01 mg/L	PN-EN 15763:2010	0.01 mg/L

Phosphorus (as phosphates) is an essential nutrient involved in the body's energy cycle (through ATP molecules), regulation of acid-base balance, cell signaling, mineralization of bones and teeth (as a component part of hydroxyapatite). Phosphorus plays an important structural role in membrane phospholipids and genetic material (siRNA, DNA). The level of phosphorus in the normal diet is not dangerous, especially due to the proper intake of calcium and Vitamin D [68]. Most dietary supplements do not contain significant doses of phosphorus, maintaining the low intake of this nutrient [69]. Phosphorus supplementation could play a role in calcium efficacy by reducing the risk of osteoporosis [70, 71].

Similar to whole plants (comestible parts), the green barley juice confirms the "food synergy" concept [72], the action of the matrix on human organisms being greater than the summed actions of the individual components that could be isolated during technological process.

The analysis of the heavy metal composition of SOD Natural revealed the following results (Table 3).

Our results show that none of the tested heavy metals exceeded the toxicity limit, which supports both the safe use of the Natural SOD product and the lack of these elements in the soil where barley culture is cultivated.

Fractions with the scavenging capacity for ·OH radicals have greater antioxidant activity than Natural SOD (total). However, this finding does not indicate the need to introduce an intermediate step of product splitting. It is possible and preferable that these biochemical compounds with antioxidant capacity should be released from macromolecular complexes inside the organism, as a result of gut digestion and absorbtion.

Comparative testing of single batches from different years is required to identify the variation range for HORAC values associated with Natural SOD. In addition, our results demonstrate that the HORAC method is suitable for testing the scavenger capacity of ·OH radicals for the Natural SOD product, both for its better characterization and as a quality control method for the batches of the current production.

CONCLUSIONS

Our results indicate that Natural SOD is able to neutralize ·OH radicals. All tested samples, Natural SOD (total), Natural SOD > 10 kDa, Natural SOD < 10 kDa, show a similar ·OH scavenger capacity, with nonsignificant temperature dependent variations. However, a decreasing trend of the ·OH radicals scavenging capacity was observed for samples kept at room temperature (20-25 ^{0}C).

The free oxygen radical scavenger properties of Natural SOD seem to be based on peroxyl radicals scavenging capacity (higher for Natural SOD < 10 kDa fraction). Neutralizing peroxyl radicals is highly dependent on the temperature (significant decreased values for all samples kept at room temperature). These data, correlated with our previous results, indicate 4-8 ^{0}C as the optimal storage temperature for Natural SOD.

The biomolecules responsible for the antioxidant capacity of Natural SOD, as well as the corresponding reaction mechanisms, are not yet identified and fully understood. Future studies could clarify the biochemistry of Natural SOD and could help identifying new beneficial effects for human health.

In most cases, oxidative stress contributes to the onset and development of inflammatory processes. Our results could sustain *in vitro* and *in vivo* studies aiming to investigate the capacity of Natural SOD to act as an adjuvant in treating acute and chronic inflammatory processes.

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Conflict of interests: Authors declare no confict of interests.

REFERENCES

1. Gill SS, Tuteja N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry. 2010;909-930.
2. Valko M, Leibfrit D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. The International Journal of Biochemistry & Cell Biology. 2007;39:44–84.
3. Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative Stress and Antioxidant Defense, World Allergy Organ J. 2012; 5(1): 9–19.
4. Liu Q, Berchner-Pfannschmidt U, Moller U, Brecht M, Wotzlaw C, Acker H, Jungermann K, Kietzmann T. A Fenton reaction at the endoplasmic reticulum is involved in the redox control of hypoxia-inducible gene expression. Proc Natl Acad Sci USA. 2004;101(12):4302-7.
5. Kurz T, Eaton JW, Brunk UT. Redox activity within the lysosomal compartment: implications for aging and apoptosis. Antioxid Redox Signal. 2010;13(4):511-23.
6. Kurutas EB. The importance of antioxidants which play the role in cellular response against oxidative / nitrosative stress: current state. Nutrition Journal. 2016;15:71.
7. Valdez LB, Lores AS, Bustamante J, Alvarez S, Costa LE, Boveris A. Free radical chemistry in biological systems. Biol Res. 2017;33(2): 65-70.
8. Toyokuni S. Iron and thiols as two major players in carcinogenesis: friends or foes? Front Pharmacol. 2014; 5: 200, PMCID: PMC4147246, PMID: 25221514
9. Kong Q, Lin C-I G, Oxidative damage to RNA – mechanisms, consequences and diseases, Cell Mol Life Sci, 2010;67(11):1817-1829.
10. Pavelescu LA. On Reactive Oxygen Species Measurement in Living Systems, Journal of Medicine and Life, 2015; 8: 38–42.
11. Klotz LO, Steinbrenner H. Cellular Adaptation to Xenobiotics: Interplay between Xenosensors, Reactive Oxygen Species and FOXO Transcription Factors, Redox Biology. 2017;13:646–654.
12. Stohs SJ, Bagchi D. Oxidative mechanisms in the toxicity of metal ions..Free Radic Biol Med. 1995; 18:321–336.
13. Pham-Huy LA, He H, Pham-Huy C. Free Radicals, Antioxidants in Disease and Health. International Journal of Biomedical Science. 2008;4(2):89–96.
14. Khalil M, Teunissen C, Langkammer C. Iron and Neurodegeneration in Multiple Sclerosis, Multiple Sclerosis International. 2011; Article ID 606807. PubMed PMID: 22096640.
15. Pham-Huy LA, He H, Pham-Huy C. Free Radicals, Antioxidants in Disease and Health, Int J Biomed Sci. 2008;4(2):89-96.
16. Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative Stress and Neurodegenerative Diseases: A Review of Upstream and Downstream Antioxidant Therapeutic Options, Neuropharmacology. 2009;7:65-74.
17. Lipinski B. Hydroxyl Radical and Its Scavengers in Health and Disease, Oxidative Medicine and Cellular Longevity. 2011; Article ID 809696, Available from: http://dx.doi.org/10.1155/2011/809696.
18. Rahal A, Kumar A, Singh V, Yadav B, Tiwari R, Chakraborty S, Dhama K. Oxidative Stress, Prooxidants and Antioxidants: The Interplay. Biomed Research International. 2014; Article ID 761264, Available from: http://dx.doi.org/10.1155/2014/761264.
19. Kasote DM, Katyare SS, Hegde MV, Bae H. Significance of Antioxidant Potential in Plants and its Relevance to Therapeutic Applications, Int J Biol Sci. 2015;11:982-991.
20. Maillard MN, Soum MH, Boivin P, Berset C. Antioxidant Activity of Barley and Malt: Relationship with Phenolic Content. LWT - Food Science and Technology. 1996;29(3):238-244.
21. Omwamba M, Hu Q. Antioxidant activity in

barley (Hordeum Vulgare L.) grains roasted in a microwave oven under conditions optimized using response surface methodology, J Food Sci. 2010; 75(1):C66-73, doi: 10.1111/j.1750-3841.2009.01426.x., PubMed PMID:20492152.
22. Dvořáková M, Douanier M, Jurková M, Kellner V, Dostálek P. Comparison of Antioxidant Activity of Barley (Hordeum vulgare L.) and Malt Extracts with the Content of Free Phenolic Compounds Measured by High Performance Liquid Chromatography Coupled with CoulArray Detector. Journal of the Institute of Brewing. 2008;114:150–159.
23. Popescu M, Danciu T, Danciu E, Ivopol G, Manea S. Natural Antioxidants, Free-radical scavengers and Minerals in fresh juices and Vegetables, Rev Chim (Bucharest), 2011, 62:8.
24. Gul S, Ahmend N, Kifl N, Uddin QT, Tahir NB, Hussain A et al. Multiple pathways are responsible for Anti-inflammatory and Cardiovascular Activities of Hordeum vulgare, Journal of Translational Medicine. 2014;12:316.
25. Lupu AR, Cremer L, Ionescu G, Szegli G, Herold A, Cristea M. The peroxyl scavenger activity of the vegetal product NATURAL SOD measured using ORAC method, Romanian Biotechnological Letters. 2013;18(4):8511-8520.
26. Lupu AR, Cremer L. Study to establish the acceptance range for peroxyl radical scavenger capacity of Natural SOD. Roum Arch Microbiol Immunol. 2015;74(3-4):91-6.
27. Juknys R, Vitkauskaitė G, Račaitė M,Venclovienė J. The impacts of heavy metals on oxidative stress and growth of spring barley. Central European Journal of Biology. 2012;7(2): 299–306.
28. Valko M, Morris H, Cronin MT. Metals, toxicity and oxidative stress. Curr Med Chem. 2005; 12(10):1161-208.
29. Flora SJ, Mittal M, Mehta A. Heavy metal induced oxidative stress and its possible reversal by chelation therapy. Indian J Med Res. 2008; 128(4):501-23.
30. Stanisic Stojic SM, Ignjatovic LM, Popov S, Skrivanj S, Dordevic AR, Stojic A. Heavy metal accumulation in wheat and barley: the effects of soil presence and liquid manure amendment, Plant biosystems. 2016;150,1:104-110.
31. OxiSelect™ Hydroxyl Radical Antioxidant Capacity (HORAC) Activity Assay, Product Manual, Cell BiolabsInc, Available from: http://www.cellbiolabs.com/sites/default/files/STA-346-horac-assay-kit.pdf.
32. Fathi Z, Keshmirizadeh E, Use of Fenton Reagent as Advanced Oxidative Process for Removal of Basic and Acid Red Dyes from Aqueous Solutions, Journal of Applied Chemical Research. 2015;9(3):7-19.
33. MG Ferruzzi, V Bohm, PD Courtney, SJ Schwartz, Antioxidant and antimutagenic activity of dietary chlorophyll derivatives determined by radical scavenging and bacterial reverse mutagenesis assay, JFS: Food Chemistry and Toxicology. 2002;67(7):2589-2595.
34. R Sivakumar, PV Babu, CS Shyamaladevi. Aspartate and glutamate prevents isoproterenol-induced cardiac toxicity by alleviating oxidative stress in rats, Exp Toxicol Pathol. 2011; 63(1-2):137-42.
35. M Wu, H Xiao, W Ren, J Yin, B Tan, G Liu. Therapeutic effects of glutamic acid in piglets challenged with deoxynivalenol. PLoS One. 2014; 9(7):e100591, PubMed PMID: 24984001
36. Matilla B, Mauriz JL, Culebras JM, González-Gallego J, González P. Glycine: a cell-protecting anti-oxidant nutrient, US National Library of Medicine National Institutes of Health Nutr Hosp. 2002;17(1):2-9.
37. Wade AM, Tucker HN. Antioxidant characteristics of L-histidine. J Nutr Biochem. 1998; 9:308-315.
38. Zheng P, Yu B, He J, Tian G, Luo Y , Mao X, et al. Protective effects of dietary arginine supplementation against oxidative stress in weaned piglets, British Journal of Nutrition. 2013; 109:2253–2260.
39. Kishnan N, Dickman MB, Becker DF, Proline modulates the intracellular redox environment and protects mammalian cells against oxidative stress., Free Radic Biol Med, 2008;44(4):671-81.
40. L-Tyrosine, Alternative Medicine Review. 2007; 12(4): 364-368. Available from: http://www.altmedrev.com/archive/publications/12/4/364.pdf.
41. Cojocaru E, Filip N, Ungureanu C, Filip C, Danciu M. Effects of Valine and Leucine on Some Antioxidant Enzymes in Hypercholesterolemic Rats. Health. 2014;6:2313-2321.
42. Zhao J, Liu Y, Jiang J, Wu P, Jiang W, Li S et al. Effects of dietary isoleucine on the immune response, antioxidant status and gene expression in the head kidney of juvenile Jian carp (Cyprinus carpio var. Jian)., Fish Shellfish Immunol. 2013; 35(2):572-80.
43. Dong Y, Bai Y, Liu G, Wang Z, Cao J, Chen Y, et al. The immunologic and antioxidant effects of L-phenylalanine on the uterine implantation of mice embryos during early pregnancy, Histol Histopathol. 2014, 29(10):1335-42.
44. Li XY, Liu Y, Jiang WD, Jiang J, Wu P, Zhao J et

al. Co- and Post-Treatment with Lysine Protects Primary Fish Enterocytes against Cu-Induced Oxidative Damage. PLoS ONE. 2016. 11(1): e0147408. doi:10.1371/journal.pone.0147408. PubMed PMID: 26812682, PubMed Central PMCID: PMC4727818

45. Mao X, Lv M, Yu B, He J, Zheng P, Yu J, et al. The effect of dietary tryptophan levels on oxidative stress of liver induced by diquat in weaned piglets. Journal of Animal Science and Biotechnology. 2014;5:49.
46. Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington. DC: National Academy Press, 1998, ISBN-10: 0-309-06411-2, ISBN-13: 978-0-309-06411-8. Bookshelf ID: NBK114310 PubMed PMID: 23193625 DOI: 10.17226/6015.
47. National Institutes of Health (NIH), Office of Dietary Supplements. 2013. Vitamin C. Dietary Supplement Fact Sheet. Available from http://ods.od.nih.gov/factsheets/VitaminC-HealthProfessional/.
48. Institute of Medicine (IOM). 2005. Dietary Reference Intakes for Water, Potassium, Sodium Chloride, and Sulfate. Washington. DC: National Academy Press, p.186-397, ISBN 978-0-309-13335-7. Available from: https://www.nap.edu/read/10925/chapter/1.
49. Vaseghi M, Shivkumar K. The Role of the Autonomic Nervous System in Sudden Cardiac Death. Prog Cardiovasc Dis. 2008. 50(6):404–419.
50. Gordan R, Gwathmey JK, Xie LH. Autonomic and endocrine control of cardiovascular function, World J Cardiol. 2015; 7(4):204-214.
51. Sica DA, Wilson DK. Sodium, Potassium, the Sympathetic Nervous System, and the Renin-Angiotensin System. Impact on the Circadian Variability in Blood Pressure. In: White William B, editor. Blood Pressure Monitoring in Cardiovascular Medicine and Therapeutics, Part of the series Clinical Hypertension and Vascular Diseases. 2007; p. 203-223.
52. Boushey CJ, Beresford SA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: probable benefits of increasing folic acid intakes. JAMA. 1995;274:1049–1057.
53. den Heijer MD, Koster T, Blom HJ et al. Hyperhomocysteinemia as a risk factor for deep-vein thrombosis. N Engl J Med. 1996;334:759–762.
54. Malinow MR. Plasma homocyst(e)ine: a risk factor for arterial occlusive diseases. J Nutr. 1996; 126:1238S–1243S.
55. National Institutes of Health, Office of Dietary Supplements, Vitamin C. Fact Sheet for Consumers, Available from: https://ods.od.nih.gov/factsheets/VitaminC-Consumer/ .
56. Dietary Reference Intakes for Calcium and Vitamin D. Institute of Medicine (US) Committee to Review Dietary Reference Intakes for Vitamin D and Calcium; Editors: A Catharine Ross, Christine L Taylor, Ann L Yaktine, and Heather B Del Valle. Washington (DC): National Academies Press (US); 2011. Bookshelf ID: NBK56070PMID: 21796828DOI: 10.17226/13050 Available from: https://www.ncbi.nlm.nih.gov/books/NBK56070.
57. Food and Drug Administration (FDA), Food labeling: health claims; calcium and osteoporosis. Federal Register. 1994; 58:2665–2681.
58. Arnaud CD, Sanchez SD. Calcium and phosphorus. In: Ziegler EE, Filer LJ, editors. Present Knowledge of Nutrition. 7th ed. Washington, DC: ILSI Press. 1996. p. 245–255.
59. Alam MN, Bristi NJ, Rafiquzzaman M. Review on in vivo and in vitro methods evaluation of antioxidant activity. Saudi Pharmaceutical Journal. 2013; 21(2):143-152.
60. Tricker AR, Preussmann R. Carcinogenic N-nitrosamines in the diet: occurrence, formation, mechanisms and carcinogenic potential. Mutat Res.1991;259:277-289.
61. Johnson C, Steinberg F, Rucker RB. Ascorbic acid. In: Zempleni J, Suttie J, Gregory JF, Stover PJ, editors. Handbook of Vitamins, 5th Edition, CRC Press, Boca Raton F2;.2013. p.515-549.
62. Mayo Clinic, Available from: http://www.mayoclinic.org/drugs-supplements/phosphate-supplement-oral-route-parenteral-route/description/drg-20070193.
63. Astle SM. Restoring electrolyte balance. RN. 2005 May;68(5):34-9. quiz 40. PubMed PMID:15931929
64. Dietary Reference Intakes for Calcium and Vitamin D. Institute of Medicine (US) Committee to Review Dietary Reference Intakes for Vitamin D and Calcium; Editors: A Catharine Ross, Christine L Taylor, Ann L Yaktine, and Heather B Del Valle. Washington (DC): National Academies Press (US); 2011:35-74.
65. Mayo Clinic, Available from: http://www.mayoclinic.org/healthy-lifestyle/nutrition-and-

healthy-eating/in-depth/sodium/art-20045479.
66. National Institutes of Health (NIH), Office of Dietary Supplements. Magnesium. Available from: https://ods.od.nih.gov/factsheets/Magnesium-HealthProfessional.
67. WHO (World Health Organization). Trace Elements in Human Nutrition: Manganese. Report of a WHO Expert Committee. Technical Report Service, 532, WHO, Geneva, Switzerland. 1973; p. 34-36.
68. Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride. Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. Washington (DC): National Academies Press (US); 1997; p.146-189. ISBN-10: 0-309-06350-7ISBN-10: 0-309-06403-1.
69. Bailey RL, Fulgoni VL III, Keast DR, Dwyer JT. Dietary supplement use is associated with higher intakes of minerals from food sources. Am J Clin Nutr. 2011;94:1376–1381.
70. Heaney RP, Nordin BEC. Calcium effects on phosphorus absorption: implications for the prevention and co-therapy of osteoporosis. J Am Coll Nutr. 2002;21:239–244.
71. Heaney RP, Weaver CM. Calcium and vitamin D. Endocrinol Metab Clin North Am. 2003;32:181–189.
72. Jacobs Jr DR, Gross MD, Tapsell LC, Food synergy – an operational concept for understanding nutrition. Am J Clin Nutr. 2009;89 Suppl:1543s-8s.