Հայաստանի Գիտությունների Ազգային Ակադեմիա
Национальная Академия Наук Армении
National Academy of Sciences of Armenia

Հայաստանի Կենսաբանական Հանդես
Биологический Журнал Армении
Biological Journal of Armenia

• Փորձարարական և տեսական հոդվածներ • Экспериментальные и теоретические статьи • • Experimental and theoretical articles •

Biolog. Journal of Armenia, 2 (74), 2022

DOI:10.54503/0366-5119-2022.74.2-46

SUPEROXIDE-PRODUCING THERMOSTABLE COMPLEX FROM PLANT FOODS: ISOLATION, PURIFICATION AND PROPERTIES

R.M. SIMONYAN[1], G.M. SIMONYAN[1], A.S. ALEXANYAN[2], M.A. BABAYAN[1], S.S. ALEXANYAN[2], M.A. SIMONYAN[1]

[1]H. Buniatyan Institute of Biochemistry, NAS RA, Yerevan
[2]M. Nalbandyan State Ped. University, Gyumry
mbabayan@gmail.com

From Armenian plant food – tomato (*Solanum lycopersicum esculentum)*, carrot (*Daucus carota subsp. sativus*), green bean (*Phaseolus vulgaris*) and potato (*Solanum tuberosum*), the isoforms of superoxide (O_2^-) producing complexes between NADPH containing protein component (NPC) and Fe(III) – NPC-Fe(III), were isolated and purified for the first time. At the same time, the fractionation of these complexes at pH 9,5 and 4,8 were carried out [1]. The isoforms of NPC-Nox are high thermostable biosystems (up to 100^{o}C). The immediately mechanism of the production of O_2^- by these complexes, due to the transfer of the electron from NPC to the Fe(III), then to O_2 for its reduction up to O_2^- was conditioned. As a substrate for NPC-Fe(III) is not free NADPH, but NADPH is connected with the protein component (PC). The forms of optical absorption spectra of these complexes in visible region essentially were differed, although, in UV region the characteristic for the proteins maximal optical absorption at 260-280 nm were observed. The higher specific content (mg/g) for complexes from tomato, than from carrot, green bean and potato were observed. The direct proportional dependence between the content of the NADPH in the composition of NPC and stationary concentration of produced O_2^- in homogenous phase (in solution) and gas phase were presented. The isolated NPC from indicated above complexes, at the expense of NADPH, only the reductive (antioxidant) effect was indicated and was activated the NADPH oxidase (Nox) *in vitro*, in particular from erythrocytes membranes (EM) and leukocytes membranes (LM). By the blowing of the slightly opalescent aqueous solutions of these complexes with oxygen the gas phase O_2^- were produced, which were transferred oxygen through the silicone or glass tubes.

Thus, from tomato, carrot, green bean and potato the new prooxidant systems – the isoforms of thermostable O_2^--producing complex NPC-Fe(III), were isolated and purified for the first time. These complexes were produced O_2^- in solution and gas phase after blowing with oxygen. The stimulating effect of NPC on the Nox of immune cells (erythrocytes and leukocytes) by the forming of hybrid associates with these Nox (hNCP-Nox) were determined *in vitro*.

Superoxide radical – complex – plant food

Առաջին անգամ բուսական ծագման մթերքներից (լոլիկ, գազար, կանաչ լոբի և կարտոֆիլ) անջատվել և մաքրվել են սուպերօքսիդ (O_2^-)-գոյացնող կոմպլեքսների իզոձևեր՝ NADPH, պարունակող սպիտակուցային բաղադրամասի (ՆՍԲ) և Fe(III)-ի միջև՝ ՆՍԲ-Fe(III): Ընդ որում իրականացվել է այդ կոմպլեքսների ֆրակցիոնացում pH 9,5-ում և pH 4,8-ում [1]. ՆՍԲ-Fe(III)-ի իզոձևերը համարվում են ջերմակայուն (մինչև 100°C) կենսահամակարգեր: Որոշված է այդ կոմպլեքսներով O_2^- գոյացման անմիջական մեխանիզմ, ինչը պայմանավորված է էլեկտրոնի

փոխանցմամբ ՆՍԲ-ից Fe(III)-ին, ապա O_2-ին, վերականգնելով այն մինչև O_2^-: Որպես ՆՍԲ-Fe(III)-ի սուբստրատ համարվում է ոչ թե ազատ, այլ սպիտակուցային բաղադրամասի (ՍԲ) հետ կապված NADPH-ը: Այս կոմպլեքսների օպտիկական կլանման սպեկտրների ձևերը տեսանելի մարզում էականորեն տարբերվում են, չնայած ՈւՄ մարզում առկա են սպիտակուցներին բնորոշ մաքսիմալ օպտիկական կլանումներ 260-280 նմ-ում: Այդ կոմպլեքսների տեսակարար պարունակությունը (մգ/գ) բարձր է լոլիկի, ապա գազարի, կանաչ լոբու և կարտոֆիլի մեջ: Դիտվում է ուղիղ համեմատական կախվածություն ՆՍԲ-ի կազմում գտնվող NADPH-ի և գոյացող O_2^--ի ստացիոնար կոնցենտրացիայի միջև, հեղուկ (լուծույթում) ֆազում և գազ ֆազում: Վերոհիշյալ կոմպլեքսներից առանձնացված լեյկոցիտների ՆՍԲ-ն, շնորհիվ NADPH-ի, ցուցաբերում է վերականգնիչ (հակաօքսիդանտային) ազդեցություն և ակտիվացնում NADPH-օքսիդազը (Nox), մասնավորապես անջատված էրիթրոցիտների թաղանթներից (ԷԹ և ԼԹ) *in vitro*: Այդ կոմպլեքսների թույլ օպալեսցենցվող ջրային լուծույթի մեջ թթվածին փչելիս գոյանում են գազ ֆազային O_2^--ներ, որոնք մոլեկուլային թթվածնի հետ տեղափոխվում են սիլիկոնային կամ ապակյա խողովակով:

Այսպիսով, առաջին անգամ լոլիկից, գազարից, կանաչ լոբուց և կարտոֆիլից անջատվել և մաքրվել են նոր պրոօքսիդանտային համակարգեր՝ ՆՍԲ-Fe(III) ջերմակայուն կոմպլեքսի իզոձևեր: Այդ կոմպլեքսները գոյացնում են O_2^- ինչպես հեղուկ ֆազում, այնպես էլ գազ ֆազում: Որոշված է իմունային բջիջների (էրիթրոցիտների և լեյկոցիտների) Nox-երի ստիմուլյացիայի էֆեկտը առանձնացված ՆՍԲ-ով *in vitro*՝ հիբրիդային ասոցիատի (hNCP-Nox) կազմավորման ճանապարհով:

Սուպերօքսիդ ռադիկալ – կոմպլեքս – բուսական սննդամթերք

Впервые из пищевых продуктов растительного происхождения (помидоры, морковь, зеленая фасоль и картофель) выделены и очищены изоформы супероксид (O_2^-)-продуцирующего комплекса между NADPH содержащим белковым компонентом (НБК) и Fe(III) – НБК-Fe(III). При этом было осуществлено фракционирование этих комплексов при pH 9,5 и pH 4,8 [1]. Изоформы НБК-Fe(III) являются термостабильными (до 100^0C) биосистемами.

Определен непосредственный механизм продуцирования O_2^- этими комплексами, который обусловлен передачей электрона от НБК к Fe(III), далее к O_2, восстанавливая его до O_2^-. Как субстрат фермента НБК-Fe(III) является не свободная, а связанная с белковым компонентом (БК) NADPH. Формы оптических спектров поглощения этих комплексов в видимой области существенно отличаются, хотя в УФ области имеются характерные для белков максимальные оптические поглощения при 260-280 нм. Удельное содержание (мг/г) этих комплексов выше в помидорах, далее в моркови, зеленой фасоли и картофеле. Наблюдается прямопропорциональная зависимость между содержанием NADPH в составе НБК и стационарной концентрацией продуцируемых O_2^- в жидкой фазе (в растворе) и газ фазе. Отделенный от приведенных комплексов НБК, за счет NADPH, оказывает только восстановительный (антиоксидантный) эффект и активирует NADPH оксидазу (Nox) *in vitro*, в частности, выделенные из мембран эритроцитов (ЕМ) и лейкоцитов (ЛМ). При продувании слабоопалесцирующего водного раствора этих комплексов кислородом продуцируются газ фазные O_2^-, которые транспортируются с молекулярным кислородом посредством силиконовой или стеклянной трубок.

Таким образом, впервые из помидоров, моркови, зеленой фасоли и картофеля выделены и очищены новые прооксидантные системы – изоформы комплекса термостабильного НБК-Fe(III). Эти комплексы продуцируют O_2^- как в жидкой фазе, так и в газ фазе. Определен эффект стимуляции изоформ Nox иммунных клеток (эритроцитов и лейкоцитов) изолированным НБК, путем формирования гибридного ассоциата (hNCP-Nox) *in vitro*.

Супероксидный радикал – комплекс – пищевой продукт

In tomato, carrot, green bean and potato the corresponding antioxidative status were presented, which are due to presence of lycopene, ascorbic acid, phenolics, flavonoids, vitamin E, beta-carotene, linoleic acid, carotenoids. However, in the plant food the physiological balance between anti- and prooxidative systems must by observed. On the other hand, the isoforms of superoxide producing complex were isolated and purified from various fruits [2-5].

The aim of this work is to isolate, purify and investigate the new prooxidative system (the superoxide-producing complex) from tomato, carrot, green bean and potato.

Materials and methods. Isolation *and purification of NPC-Fe(III) complex from plant food (tomato, carrot, green bean and potato).*

Using the universal method [1], the isoforms of NPC-Fe(III) from Armenian plant food were isolated and purified. In particular, after homogenization of plant food (50g) in water (200ml), the latter were incubated at pH 9,5 in 37^{o}C for 1,5 hours. After its centrifugation at 5800×g for 10 min, 0,1 M HCI up to pH 4,8 was added to the supernatants. After centrifugation the obtained precipitates were homogenized in water (1:100 v/v) at pH 9,5 and after centrifugation, the supernatants underwent ion exchange chromatography in separate column of cellulose DE-52, also at pH 9,5. The eluates (complexes) undergo thermotreatment in boiling water during 10-12 min. After its centrifugation the supernatants (complexes) were concentrated and underwent gel-filtration on separate columns with Sephadex G-100 at pH 9.5. Primary fractions eluted with symmetric elution chart were collected.

Isolation of NPC from NPC-Fe(III) complex

After incubation of 5mg/ml NPC-Fe(III) complex with EDTA (0,005 M) at 37^{o} C for 25-30 minutes, the hatching mixture undergo ion-exchange chromatography on DE-52 cellulose, equilibrated with water at pH 9,5. Under these conditions EDTA joins to Fe(III) and remains in the column, whereas NPC is eluted without delay.

Determination of the Fe(III) in the NPC-Nox.

Using the orthophenantroline optical spectral method [6], the content of Fe(III) in these complexes was determined only after separation of the Fe(III) by EDTA and its reduction by sodium dithionite.

NPC-Fe(III) and NPC contents were determined by weighing after their desalting and vacuum lyophilization.

The formation of hybrid associate between NCP from NCP-Fe(III) complex and isoform of Nox (hNCP-Nox).

To the water solution of total fraction of the Nox1+Nox2 (2,5 mg/ml) from donor blood erythrocytes membranes (EM) and leukocytes membranes (LM) [7] was added NPC (10 mg/ml) and incubated at 37^{o}C, at pH 9,5 during 15-20 min. Then, after delution of this solution with water (up to 5 times), its ion-exchanging chromatography on the cellulose DE-52, again at pH 9,5 was carried out. The formed hNPC-Nox associate free eluted from this DE-52 column, and the excessive Nox remained on the column and eluted by 0,2 M potassium phosphate buffer, pH 7,4 (PPB).

Determination of the stationary concentration of O_2^-, produced by isoforms of NPC-Fe(III) complexes and hybrid associate hNPC-Nox .

The stationary concentration of O_2^-, produced by the isoforms of complex NPC-Fe(III) and hybrid associate hNPC-Nox, was determined by adrenaline method, by measuring of the maximal optical absorbance of adrenochrome (at 500 nm), which is formed during oxidation of adrenaline by produced O_2^- [8]. The stationary concentration (M) of produced O_2^- is equivalent to the concentration of formed adrenochrome, with the molar extinction (E) up to 750 $M^{-1}см^{-1}$. By the determine of the value of A_{500}/E, the stationary concentration (M) of O_2^-, produced by the isoforms of NPC-Fe(III) complex and associate hNPC-Nox, was determined in homogeneous phase (in solution) and in gas phase (during blowing by 0,1 atm oxygen at 10 min, at room temperature). As a control, the optical absorbance of adrenochrome, which is forming during oxidation of the solution of adrenaline only by the oxygen in similar conditions, was used.

The specific content of NPC-Fe(III) and hNPC-Nox was determined by its weighting, after deionization and vacuum lyophilization of its solutions and conveyed by mg in 1g food (mg/g).

Determination of NADPH in NPC-Fe(III) complex or in NPC.

The spectrofluorimetric intensity "F" in relative units of the NADPH group in the NPC-Fe(III) complex or NPC was determined by spectrofluorimetric method [9]. The emission peak of NADPH group as part of NPC was recorded at 430 nm with 370 nm excitation length [9].

Electrophoresis of the obtained NPC was realized in 10% polyacrylamide gel (PAAG) for acidic and basic character proteins.

During the invwstigation the spectrophotometer "Cary 60 UV/VIS" (USA), spectrofluorimeter "Perkin Elmer" (USA), centrifuges K-24 and K-70 ("Janetzki", Germany), as well as the cellulose DE-52 ("Whatman" England) and Sephadex G-100 ("Pharmacia" Sweeden), adrenaline ("Sigma") were used.

Results and Discussion. During ion exchange chromatography of fractions of the isoforms of NPC-Fe(III) from tomato, carrot, green bean and potato on the column of cellulose DE-52 at pH 9,5, the isoforms of NPC-Fe(III) doesn't linger in the column and easily eluted. After thermal treatment, concentration and gel-filtration of these complexes of NPC-Fe(III) in the column of Sephadex G-100 at pH 9,5, the primary fractions of NPC-Fe(III) was collected with symmetrical elution chart. After joining of these fractions together, desalting and vacuum lyophilization its weight was determined. As a result of the thermal treatment, the possible traces of proteins (antioxidant proteins) were removed by centrifugation.

During electrophoresis the above mentioned complexes NPC-Fe(III) didn't pass through the tube with PAAG, they were aggregated at the entrance of this gel. However, after staining PAAG for water soluble acidic and basic proteins traces, accompanying to NPC-Fe(III), were not detected. Thus, on the base of symmetrical elution chart of NPC-Fe(III) through G-100, absence of coloring bands for acidic and basic water-soluble proteins on PAAG were not changed of the optical spectral purity value (A_{280}/A_{430}), we can indirectly speak about the purify of these complexes.

After purification by the above mentioned method, the forms of optical absorption spectra of the weak opalescence aqueous solutions at pH 9,5 of the isoforms of NPC-Fe(III) complex from tomato, carrot, green bean and potato in visible region were distinguished significantly (fig. 1).

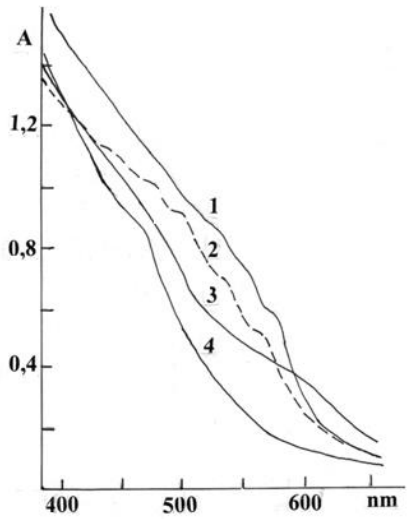

Fig. 1. Optical absorption spectra of the weakly opalescent aqueous solutions of the isoforms NPC-Fe(III) complex from carrot (1) tomato (2), green bean (3) and potato (4) at pH 9,5. In the UV region there are the optical absorbance maximumes, which are characteristic for proteins at 260-280 nm.

Some quantitative characteristics of indicated above O_2^--producing complexes from tomato, carrot, green bean and potato were determined (tab. 1).

As presented in tab. 1, the correlation between specific contents of indicated complexes, Fe(III) and fluorescence intensity ("F"), stationary concentration of produced O_2^- in homogeneous phase (in solution) and in gas phase were observed.

Electrophoretically homogenous total fraction of the isoforms of Nox1+Nox2 from EM and LM were isolated and purified in our laboratory by licensed method, using the recently discovered phenomenon of unstable complex formation of ferrihemoglobin with isoforms of Nox and their release from biomembranes into a soluble phase [7].

Table 1. The arithmetic means of the specific amount of O_2^--producing complexes from Armenian plant food and Fe(III), fluorescence intensity in relative units (F) of these complexes and stationary concentration of produced O_2^- by these complexes in solution and gas phase (n=6)

Sources	Specific amount (mg/g)	Fe(III) mkg/mg	"F" relative units	mkM O_2^- in solution	mkM O_2^- in gas phase
Tomato	15,5	2,1	34,3	3,2	2,8
Carrot	11,2	1,7	28,1	2,1	1,7
Green bean	4,4	1,5	23,7	1,6	1,3
Potato	2,7	1,1	10,1	1,1	1,1

Optical absorption spectra of total fraction of Nox1+Nox2 isoforms from EM in oxidized and reduced states with absorption typical for Nox at 558 are shown in fig.2.

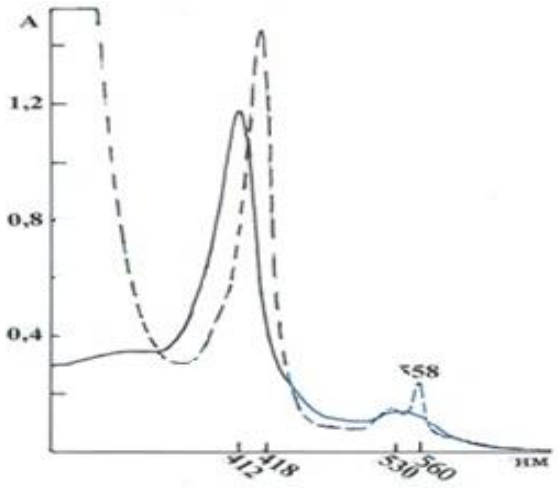

Fig. 2. Optical absorption spectra of total fraction of Nox1+Nox2 from EM (___) of donor blood at pH 7,4. After reduction of Nox with sodium dithionite, the acute absorption, typical for Nox at 558nm (- - -) is observed.

TAfter separation of Fe(III) from NPC-Fe(III) complexes, the obtained isoforms of NPC were formed the hybrid associate, in particular, with the Nox from EM (hNCP-Nox). Using the Fe(III) of hem group of the isoforms of Nox, (as an electronic bridge) the hNCP-Nox transfer the electron from NADPH to O_2 reducing it up to O_2^- in solution and gas phase, after blowing of the solution with oxygen. In fact, the isoforms of these complex NPC-Fe(III) from plant food and hNCP-Nox were «electronic automatons», which continuously were fired electrons only to oxygen target, reducing its up to O_2^-.

However, the stationary concentration of produced O_2^- by the isoforms of hNPC-Nox in solution and in gas phase were similar to the analogical indicies of the isoforms of NPC-Fe(III).

These isoforms of NPC-Fe(III) complexes and of hNPC-Nox associate were high thermostabile biosystems (after heating in boiling water during 10-12 min, the decrease of the stationary concentration of produced O_2^- and the losing of its nativity practically doesn`t observed (up to 5-6%)).

Thus, there are some perspectives for using NPC-Fe(III) complex from plant food (tomato, carrot, green bean and potato) and hNPC-Nox as energetic, natural and relatively stable and new biosystems for production of O_2^-, as natural bactericidal and antiviral agents [10]. On the other hand, NPC from the mentioned sources can be used as natural agents for stimulation of the decreased O_2^- producing activity of Nox isoforms from EM and LM at decreased immune activity (immune-deficiency) of mammals [11] in experiment and, in perspective, in clinics. Additionally, these NPC reduce $KMnO_4$ and suppress the oxidation of adrenaline up to adrenochrom. Thus, these NPC are possessed antioxidative activity.

As we have already mentioned above, these NPC-Fe(III) complexes in lyophilized state, especially in the nitrogen atmosphere, practically don't lose O_2^--producing activity when kept at -10-12^oC throughout the year. It serves as an opportunity to introduce NPC into animal`s blood. We should remember that an analogous agent (suprol) from serum of the placental blood didn't cause any adverse unfavorable effects after injection of suprol into white rats in much more amount than in norm, moreover, the introduced suprol has an antitumor effect [12,13].

The high thermostability of the isoforms of NPC-Fe(III) complex can be conditioned with nanosecond high pulsative temperature (280^oC) metabolic processes [14]. The stabilizing effect of molecular oxygen on the gas phase O_2^- was observed, also. In perspective the produced gas phase monocomponent O_2^- by the oxygen mask can by used at lung inflammation diseases as a antibacterial and antiviral agent in experiment and in perspective in clinics, also.

It is concluded, that the isoforms of O_2^- producing complexes NPC-Fe(III), isolated from Armenian plant food (tomato, carrot, green bean and potato) for the first time, are a new natural, continuously O_2^- producing (prooxidant) agents in solution and gas phases, and NPC can be as stimulating for the Nox immune cells *in vitro.*

REFERENCES

1. *Simonyan R.M., Simonyan M.A.* Method of the preparation of superoxide producing themostable systems from biomembranes and biofluids. License of invention AM 20210030, Yerevan, Armenia, 2021.
2. *Charanjeet Kaur. Binoy George. N Deepa. Balraj Singh.* Antioxidant status of fresh and processed tomato - A review Journal of Food Science and Technology Mysore, *41*, p. 479-486, 2004.
3. *Stephen T. Talcott, Luke Howard, Carmen Hernández-Brenes.* Antioxidant Changes and Sensory Properties of Carrot Puree Processed with and without Periderm Tissue. Journal of Agricultural and Food Chemistry, *48*, 4, p.1315-1321, 2000.
4. *Keyvan Dastmalchi. Isabel Wang, Ruth E.* Stark Potato Wound-Healing Tissues: A Rich Source of Natural Antioxidant Molecules with Potential for Food Preservation. Food Chemistry, *210*, 3, pp. 2016. DOI:10.1016/j.foodchem, 04.123.

5. *Jelena Dumanović, Eugenie Nepovimova,* Maja Natić , Kamil Kuča, Vesna Jaćević. The Significance of Reactive Oxygen Species and Antioxidant Defense System in Plants: A Concise Overview/ Front. Plant Sci., 06 January, 2021, https://doi.org/10.3389/fpls, 552969, 2020.
6. *Umesh C. Gupta*. Phenanthroline method for determining iron in plant materials *Plant and Soil*, 28, p. 298-305, 1968.
7. *Simonyan R.M., Simonyan G.M., Simonyan M.A.* Method for isolating isoforms of NADPH oxidase (Nox) from biosystem. Individual Property Agency Invention License. RA N2818 A, Yerevan, 2014.
8. *Misra H.P., Fridovich I.* The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem., *247,* 10, p. 3170-3175, 1972.
9. *Симонян М.А., Карапетян А.В., Бабаян М.А., Симонян Р.М.* NADPH-содержащая супероксид-продуцирующая липопротеиновая фракция из сыворотки крови, выделение, очистка, краткие характеристики и механизм действия. Биохимия, *61*, 5, p. 932-938, 1996.
10. *Ernst Peterhans.* Reactive oxygen species and nitric oxide in viral diseases. *Biological Trace Element Research*, *56*, p.107-116, 1997.
11. *Chen T.P., Roberts R.L., Wu K.G., Ank B.J., Stiehm E.R.* Decreased superoxide anion and hydrogen peroxide production by neutrophils and monocytes in human immunodeficiency virus-infected children and adults. Pediatr. Res. *34*, 4, p. 544-550, 1993.
12. *Синонян М.А., Карапетян А.В., Галстян Д.А., Симонян Р.М., Бабаян М.А.* Супероксид-продуцирующий липопротеин как фактор подавления роста опухолей, повышения числа лейкоцитов, ускорения деления клеток в кулхтуре. Биохимия, *61*, 9, p. 1578-1583, 1996.
13. *Алексанян М.К., Симонян Г.М., Симонян Р.М., Аракелян Л.Н., Алексанян С.С., Симонян М.А.* Противоопухолевый и антистрессорный эффект супрола, введенного белым крысам при сарлкоме-45. Мед.Наука.Армении, *LII*, 3, p. 23-35, 2012.
14. *Bradley C. Steel, David R. McKenzie, Marcela M.M. Bilek, Neil J. Nosworthy, Cristobal G. dos Remedios.* Nanosecond Responses of Proteins to Ultra-High Temperature Pulses. Biophys J., *91*, 6, p. L66-L68, 2006.

Received on 02.03.2022