ACTA MICROBIOLOGICA BULGARICA

Antioxidant Activity of *Helix aspersa maxima* (Gastropod) Hemocyanin

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Abstract

Hemocyanins (Hcs) are oligomeric copper-containing proteins that function as oxygen carriers in the hemolymph of several mollusks and arthropods. In this study we present an investigation on the antioxidant activity of the Hc isolated from snails *Helix aspersa* maxima (HaH), using various experimental models.

The free radical scavenging activity was determined against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] free radical cation (ABTS). Cupric ion reducing antioxidant capacity (CUPRAC) and Ferric-reducing antioxidant power (FRAP) assay also were carried out. The results obtained show that HaH exhibits good radical scavenging activity by 59.54% reduction of the DPPH and 62.31% inhibition of ABTS radicals. Furthermore, HaH demonstrated a strong chelating effect on copper ions measured through CUPRAC method.

In conclusion, the present study revealed for the first time the antioxidant properties of molluscan Hc. The antioxidative activity of HaH could probably involve quenching of reactive oxygen species and metal ion chelation, thereby reducing the potential of prooxidants to attack cellular components.
Keywords: hemocyanin, *Helix aspersa*, mollusc, antioxidant activity

Резюме

Хемоцианините са олигомерни мед-съдържащи протеини, които функционират като кислородни носители в хемолимфата на повечето мекотели и ракообразни организми. Представено е изследване на антиоксидантната активност на хемоцианин, изолиран от охлюви *Helix aspersa* maxima (HaH), при използване на различни експериментални модели.

Радикал улавящата активност на хемоцианина е определена спрямо свободните радикали 1,1-diphenyl-2-picrylhydrazyl (DPPH) и [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] (ABTS). Антиоксидантната активност на хемоцианина е анализирана и с методите CUPRAC и FRAP, които отчитат степента на електронен трансфер на антиоксиданта към радикала. Получените резултати показват, че HaH притежава добра радикал улавяща активност, изразена в 59,54% намаление на DPPH и 62,31% инхибиране на ABTS радикалите. Освен това, анализите с метода CUPRAC демонстрират, че HaH притежава силна способност да хелатира медни йони.

В заключение, настоящото изследване демонстрира за първи път антиоксидантните свойства на хемоцианин от молюски. Вероятно, антиоксидантната активност на HaH включва, както деактивиране на реактивни форми на кислорода, така и хелатиране на метални йони, като по този начин намалява потенциала на прооксидантите да атакуват клетъчни компоненти.

Introduction

Reactive oxygen species (ROS) are involved in normal and pathogenic oxygen metabolism. That is why, oxidative stress occurs when ROS ($O_2^{\cdot}$, $OH^{\cdot}$, H_2O_2 and $RO_2^{\cdot}$) exceed the organism's protective capacity to remove or quench them, thus leading to cellular lipids, proteins and DNA damage resulting in aging and carcinogenesis. Therefore, it is of critical importance for the organism to possess well working mechanisms for protection against foreign

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and self-produced toxic substances, such as redox systems. Many investigations have proved the positive response to the oxidative stress of catalase, superoxide dismutase, glutathione/glutaredoxin and thioredoxine systems, devoting their activity to the presence of sulphydryl (-SH) groups (Aispuro-Hernandez *et al*., 2008) or transition metals like copper, nickel and zinc (Shobana *et al*., 2013). Copper is also a substantial catalytic co-factor in biological systems for a variety of metabolic reactions, electron transfer and oxygen transport proteins such as azurin, plastocyanin, laccase and hemocyanin. Copper acts as a reductant in the enzyme superoxide dismutase, cytochrome oxidase, lysyl oxidase, dopamine hydroxylase and several other oxidases which reduce molecular oxygen (Mistry *et al*., 2003).

Hemocyanins (Hcs) are respiratory proteins of high molecular mass that use copper binding sites to bind and transport oxygen in the hemolymph of many arthropods and mollusks (van Holde and Miller, 1982). Molluscan Hcs are potent natural immunostimulants. When inoculated in mammals, they enhance the innate and adaptive immune response with beneficial clinical applications (Harris and Markl, 1999; Tchorbanov *et al*., 2008; Arancibia *et al*., 2012; Gesheva *et al*., 2015). Hcs have been used for therapy of superficial bladder cancer and murine melanoma models (Jurincic *et al*., 1988; Rizvi *et al*., 2007). Very recently, we demonstrated a potential anti-cancer effect of Hcs on a murine model of colon carcinoma suggesting their use for immunotherapy of different types of cancer (Gesheva *et al*., 2014). Quite often good immunomodulators exhibit good antiradical and antioxidant properties. Till now, there has not been enough information about the antioxidant activity of Hcs. The only scientific report concerning an antioxidant activity of Hc is the one of Queinnec *et al*., 1999. In order to determine the antioxidant activity of Hc, isolated from scorpion *Androctonus australis*, the authors have investigated the kinetics of superoxide anion decays using pulse radiolysis. Recently, a radioprotective effect of *Rapana thomasiana* hemocyanin (RtH) in gamma induced acute radiation syndrome has been established (Kindekov *et al*., 2014). It was suggested that the radical-scavenging properties of this Hc are at the basis of the mitigation of radiation injuries.

In this study, we present for a first time a detailed investigation on the antioxidant activity of a molluscan Hc, namely the hemocyanin isolated from garden snails *Helix aspersa* maxima (HaH), using various experimental models.

Material and Methods

Hemocyanin preparation

HaH was isolated from the hemolymph of garden snails *Helix aspersa* maxima and purified additionally by gel filtration chromatography as described in Raynova *et al*., 2013. The protein concentration was determined spectrophotometrically using the specific absorption coefficient $A_{278}^{0.1\%}$ = 1.42 mg^{-1} ml^{-1} cm^{-1} (20°C).

Antioxidant activity determination

DPPH assay

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Sigma) scavenging activity assay was performed according to the procedure described by Thaipong et al., 2006, with slight modifications. A solution of 0.1 mmol DPPH in methanol was prepared and 2.85 mL of the solution were mixed with a 0.15 mL sample. A blank sample was prepared in the same way, but replacing the sample with the same amount of water. The reaction mixture was incubated at 37°C in darkness and the decrease in the absorbance was measured spectrophotometrically after 15 min at 517 nm against methanol. The antioxidant activity was expressed as mmol Trolox equivalents (TE) per gram protein, using calibration curve (absorption vs. concentration) of Trolox dissolved in methanol at different concentrations (0.1; 0.2; 0.3; 0.4; 0.5 mmol).

ABTS assay

The procedure was previously described by Re *et al*., 1999, and some modifications were applied. A stable (for 2-3days) ABTS radical was generated after 16 h at ambient temperature in darkness by mixing in equal quantities of two stock solutions: 7.0 mmol 2,2'azinobis (3)-ethylbenzthiazoline-6-sulfonic acid (Sigma) in dd H_2O and 2.45 mmol $K_2S_2O_8$ (Sigma) in dd H_2O. Prior the analysis, 2.0 mL of the $ABTS^{\cdot+}$ solution were diluted to 60 mL with methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm. 0.15 mL of the samples were allowed to react with 2.85 mL freshly prepared $ABTS^{\cdot+}$ solution and after 15 min at 37°C the decolorization of $ABTS^{\cdot+}$ was recorded at 734 nm against methanol. A blank sample was prepared in the same way, but replacing the sample with the same amount of water and measured against methanol as well. Results were expressed in terms of Trolox equivalent antioxidant capacity (mmol Trolox equivalents per gram protein), using calibration curve (absorption vs. concentration) of Trolox dissolved in methanol at different concen-

trations (0.1; 0.2; 0.3; 0.4; 0.5 mmol).

Ferric reducing antioxidant power (FRAP)

FRAP assay was conducted by following the modified method of Benzie and Strain (Benzie and Strain, 1996). Fresh FRAP reagent was prepared by mixing the following stock solutions: 10 parts of 300 mmol sodium acetate buffer with pH 3.6; 1 part of 10 mmol 2,4,6-tripyridyl-s-triazine (TPTZ) (Sigma) solution in 40 mmol HCl and 1 part of 20 mmol $FeCl_3x6H_2O$ (Sigma) solution in dd H_2O. The working sample was prepared by mixing 3.0 mL of FRAP reagent with 0.1 mL of the investigated sample. The blank sample was prepared in the same way, but the sample was replaced with water. After 4 min at 37°C, the absorption was read against the blank sample at 593 nm. The results were expressed as mmol Trolox equivalents per gram protein, using calibration curve (absorption vs. concentration) of Trolox dissolved in methanol at different concentrations (0.1; 0.2; 0.3; 0.4; 0.5 mmol).

Cupric reducing antioxidant capacity (CUPRAC) assay

The method was adapted according to Apak *et al*., 2004. Stock solutions of 10 mmol $CuCl_2x2H_2O$ (Sigma) in dd H_2O; 1.0 M ammolonium acetate buffer in dd H_2O with pH 7.0; 7.5 mmol neocuproine (Sigma) in 96% ethanol were prepared. The reaction was performed in the following order: 1.0 mL 10 mmol $CuCl_2x2H_2O$ + 1.0 mL 7.5 mmol neocuproine + 1.0 mL 1.0 M ammolonium acetate buffer + 0.1 mL of the investigated sample + 1.0 mL methanol. A blank sample was prepared in the same way, but using water instead. The reaction mixture was heated to 50°C for 20 min and the absorbance was measured at 450 nm against the blank sample. Results were expressed as mmol Trolox equivalents per gram protein, using calibration curve (absorption vs. concentration) of Trolox dissolved in methanol at different concentrations (0.1; 0.2; 0.3; 0.4; 0.5 mmol).

Statistics

The antioxidant activity of HaH was analyzed according to the above mentioned methods in triplicates. The presented values are means (n=3) with the corresponding standard deviations (±SD).

Results and Discussions

Until now there has not been a unified method that ensures full assessment of the antioxidant properties of a given compound against all radicals. The antioxidant activity of hemocyanin from marine snails *Rapana thomasiana* was tested in an experimental liposomal system (Kindekov *et al*., 2014). The results about Fe^{2+} - induced oxidation of an aqueous emulsion system of egg liposomes showed about 30 % less antioxidant activity of RtH compared to a common antioxidant butyl hydroxytoluene (BHT). An enhanced chemiluminescence-based assay has been used to measure the antioxidant capacity of albumin and other proteins (Medina-Navarro *et al.,* 2010).

For the purpose of a more complete characterization of Hc from snails *Helix aspersa* maxima, in the present study the antioxidant activity was determined according to four complementary methods that rely on different reaction mechanisms. DPPH and ABTS utilize predominantly Hydrogen Atom Transfer reaction mechanism (HAT), while FRAP and CUPRAC methods are based on Single Electron Transfer mechanism (SET). This is the first report for evaluation of antioxidant activity of Hc through the aforementioned methods. The data are presented on Table 1.

DPPH is based on the measurement of the scavenging ability of antioxidants towards the stable radical 1,1-diphenyl-2-picrylhydrazyl. The antioxidant activity of HaH according to this method is 13.86 mM Trolox equivalents/g protein and 59.54 % inhibition of the DPPH radical.

The ABTS assay is based on the decolorization of the ABTS radical. The antioxidant activity here is higher than that measured according to DPPH - 93.94 mM Trolox equivalents/g protein and 62.31 % inhibition of the ABTS radical. This higher activity here could be explained by the structural configuration of the DPPH radical which hinders high molecular antioxidants from interacting

Table 1. Antioxidant activity of *Helix aspersa* maxima (garden snail) hemocyanin, analyzed by different methods and radical inhibition.

DPPH		ABTS		FRAP	CUPRAC
mM Trolox equivalents/g protein	Inhibition of DPPH radical, (%)	mM Trolox equivalents/g protein	Inhibition of ABTS radical, (%)	mM Trolox equivalents/g protein	mM Trolox equivalents/g protein
13,86±0,18	59,54±1,29	93,94±2,25	62,31±1,52	3,68±1,00	412,65±6,35

with it (Sánchez-Moreno *et al*., 2002). Elias *et al*., 2008, have noticed that the protein's overall antioxidant activity can be increased by disruption of its tertiary structure to increase the solvent accessibility of amino acid residues that can scavenge free radicals and chelate prooxidative metals.

The reducing power of FRAP is based on SET mechanism and cannot detect antioxidants that act by radical quenching (H transfer), especially thiols and proteins. The method is based on the reduction of Fe (III) to Fe (II) and was carried out at pH 3.6 to maintain its solubility. Originally, the method was developed to measure the reducing power in plasma and later adapted to assay other antioxidants (Prior *et al*., 2005). But obviously these conditions are not suitable for HaH and here the lowest antioxidant values of 3.68 mM Trolox equivalents/g protein are detected.

CUPRAC is a method also based on SET mechanism like FRAP. But the method is carried out at different conditions, pH 7.0 and is based on the reduction of Cu (II) to Cu (I) (Prior *et al*., 2005). Here, the highest values for an antioxidant activity of HaH - 412.65 mM Trolox equivalents/g protein are detected. This pH is more favourable for the redox potential, which facilitates the electron transfer and makes it suitable for hydrophilic and lipophilic antioxidants (Prior *et al*., 2005). Nevertheless, the ability to reduce transition metal ions as Cu (II) is considered to be a potent pro-antioxidant activity of the antioxidants (Mistry *et al*., 2003; Prior *et al*., 2005). The physiological value of pH (7.0) is in the pH-stability region of the protein, so it is able to reveal its antioxidant potential in a better way.

The reduction of iron and copper and the formation of Cu(I) complexes is of critical importance, since when they are in "free" form they can catalyze the production of highly toxic hydroxyl radicals. When they are kept at minimum concentrations by sequestering the metals in complexes, they are unable to take part in redox reactions with activated oxygen species (Brouwer *et al.,* 1998; Levy *et al.,* 2001). In a review, Roche *et al*., 2008, show that the antioxidant activity of serum albumin is mainly due to its ligand-binding capacity, especially the binding of free transition metals, so the protein is able to limit the damage caused by hydroxyl radicals produced from Fenton reaction between iron/copper and H_2O_2.

The high copper reduction capacity in our study suggests that hemocyanin could be the first line of defence system against copper toxicity and serve as a copper chelator by sequestering the metal in a non-redox-active form. This mechanism of the antioxidant activity is beneficial to living organisms due to the preventive oxidative damage of the cellular membranes and is essential for cell survival.

Conclusion

In conclusion, for the first time the antioxidant activity of Hc from garden snails *Helix aspersa* maxima was determined by DPPH, ABTS, FRAP and CUPRAC methods. These methods are suitable to demonstrate the ability of this Hc to scavenge different radicals and to reduce or chelate different ions as Fe (II) and Cu (II). The highest antioxidant activity was recorded by CUPRAC method, which is evidence of the high pro-antioxidant activity of the protein and that these conditions are the most suitable for revealing its antioxidant potential. According to our investigations, Hc might perform an essential detoxification function against copper and iron as a constituent of the antioxidant defence network in the garden snail *Helix aspersa* maxima. This function would be beneficial for maintaining the metal homeostasis and protect the function of cellular structures against the damaging effects of reactive oxygen species.

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