Bulgarian Chemical Communications, Volume 49, Number 1, (pp. 11 – 16) 2017

In vitro investigation of the antioxidant properties of *Cancer pagurus* hemocyanin

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Received May 17, 2016; Accepted June 2, 2016

Hemocyanins are copper-containing proteins in the hemolymph of many arthropods and mollusks, whose biological function is mainly related with the oxygen transport to the tissues. As components of marine food that is traditionally associated with a healthy diet, they are an interesting object for examination of their biological activity and pharmacological potential.

In the present study, the hemocyanin from the marine crab *Cancer pagurus* (CpH) was isolated and purified, and its antioxidant properties were *in vitro* investigated in prooxidant systems. Three radicals were used – superoxide, hydroxyl and 2,2-diphenyl-1-picrylhydrazyl (DPPH). The protection by CpH against oxidative damages of two model lipid membranes - rat liver supernatant and liposomal suspension in conditions of iron-induced lipid peroxidation was tested. CpH exhibited good DPPH and hydroxyl radicals scavenging activities in a concentration-dependent manner and a lower ability to capture superoxide radicals at a physiological value of pH. CpH showed very good capacity to inhibit Fe^{2+}-induced lipid peroxidation in the examined systems, and chelating activity toward iron ions.

This study reveals that CpH has the ability to act as an iron chelating protein, and may provide protection against oxidative stress and decline in this waythe risk of destruction of biomolecules, caused by initiation of harmful free radical-mediated chain reactions.

Keywords: *Cancer pagurus* hemocyanin, antioxidant properties, iron chelating activity

INTRODUCTION

In recent years researchers have investigated the potential of compounds in natural products, including sea food, that may possess bioactive properties, in particular, an antioxidant activity [1]. In conditions of rapid growth of the aquacultures industry, it is a challenge for scientists to utilize those valuable natural products and find novel substances, with leading biological function connected to their potential for antioxidant prevention and therapeutic applications. In particular, marine proteins have attracted a great deal of attention due to their potential effects in promoting health and reducing disease risk [1, 2]. A number of marine-derived compounds have been isolated and identified, and their therapeutic effects and pharmacological profiles characterized [3].

Hemocyanins (Hcs) are copper-containing proteins which act as oxygen carriers in the hemolymph of many molluscs and arthropods. In the latter, they exist as hexamers or multihexamers of subunits, e.g., 1×6, 2×6, 4×6, 6×6 or 8×6, depending on the species [4]. The subunits are ~75 kDa polypeptides containing a copper pair active site, capable of binding an O_2 molecule. The increasing interest in Hcs is due to their important functions in hemolymph connected with the oxygen transport, and with the possibility for practical application in immunology. There are data showing that Hcs have good ability to modulate immune defense [5-7]. Hcs have been used for therapy of superficial bladder cancer and murine melanoma models [8, 9]. Recently, a potential anti-cancer effect of Hcs on a murine model of colon carcinoma was demonstrated, suggesting their use for immunotherapy of different types of cancer [10]. Many physiological and pharmacological functions have been reported for a variety of Hcs, but little is known about their antioxidant activity. Quite often good immunomodulators exhibit good antiradical and antioxidant properties. Thus, it was suggested that the radical-scavenging properties of Hc from marine snails *Rapana thomasiana* are the basis of its radioprotective effect in gamma induced acute radiation syndrome [11]. Very recently, for the first time, a detailed investigation on the antioxidant activity of molluscan Hc, namely the Hc of terrestrial snails *Helix aspersa* maxima, was published [12].

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The only scientific report concerning the antioxidant activity of arthropodan Hc is that of Queinnec *et al*. [13]. 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.

The marine crab *Cancer pagurus* (Linnaeus, 1758) (Crustacea, Decapoda, Brachyura), commonly known as an edible crab or brown crab, is the commercially most important crab species in Western Europe. The molecule of Hc isolated from *Cancer pagurus* (CpH) is composed of four immunologically distinct ~75 kDa subunits forming 2-hexameric structures [14]. It has been reported that the CpH possesses intrinsic and inducible *o*-diphenoloxidase activity [15, 16].

In the present study, the Hc from the marine crab *Cancer pagurus*, originating from the Black Sea, was isolated and purified, and its antioxidant properties were *in vitro* investigated in some prooxidant model systems.

EXPERIMENTAL

Reagents

Sephacryl S-300 was purchased from Fluka AG. Superoxide dismutase (SOD) from bovine erythrocytes and nitro blue tetrazolium (NBT) were supplied by Sigma–Aldrich Chemie GmbH. All other chemicals used were of analytical grade.

Isolation and purification ofhemocyanin

The CpH was isolated according to the procedure described in [15]. Briefly, the native CpH was obtained from the hemolymph, collected from the species *Cancer pagurus* by ultracentrifugation at 180 000 g (ultracentrifuge Beckman LM-80, rotor Ti 45) for 4 h at 4 ºC. The pellets were resuspended in stabilized buffer (50 mM Tris-HCl, containing 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 100 mM NaCl, pH 7.2) and CpH was purified by gel filtration chromatography on a Sephacryl S-300 column (80 × 1.6 cm), equilibrated and eluted with the stabilized buffer. The purity of the isolated CpH was controlled by 7.5 % SDS-PAGE. The concentration of protein was determined spectrophotometrically using the specific absorption coefficient $A_{278}^{0.1\%}$ = 1.265 $ml.mg^{-1}.cm^{-1}$ for CpH.

Extraction of liposomes and preparation of liver supernatant

Liposomal suspension was obtained from phospholipids of egg yolk as described in [17]. After evaporation under vacuum, the chloroform fraction was dissolved in 50 mM K-Na phosphate buffer, pH 7.4, to a final concentration of 2 mg $lipid.ml^{-1}$.

Wistar rats (180-200 g) were used for preparation of the liver supernatant. Livers were washed *in situ* with ice-cold 1.15% KCl. After homogenization in PBS at a ratio tissue: PBS = 1:3 (w/v) and centrifugation (10 min, 5000 rpm) the supernatant was used as a working medium, containing 2 $mg.ml^{-1}$ protein.

Estimation of antioxidant activity

Antioxidant activity in the liposomal suspension and in the liver supernatant was determined by formation of endogenous lipid peroxidation products, reacting with 2-thiobarbituric acid (TBARS), and detected spectrophotometrically (λmax = 532 nm) according to [18]. The induction of lipid peroxidation was initiated by adding 50 µl Fe^{2+} to a final concentration of 1 mmol. l^{-1}. Each sample contained 1 ml supernatant and 0.8 ml PBS to a final concentration of 2 mg. l^{-1} protein (or 1.8 ml liposomal suspension with concentration of 1 mg lipid. l^{-1}), 50 µl of 2.1 mmol. l^{-1} ascorbate and 100 µl solution of CpH to achieve concentrations of 10^{-3} – 10^{-5} mol. l^{-1}. The amount of TBARS generated in the system was determined after incubation for 30 min at 37 ºC. The activity of CpH was compared to the activity of Trolox as a hydrosoluble analogue of tocopherol, known as chain breaking antioxidant, at the same concentrations. The ratio of the absorption at 560 nm for the sample containing the tested substances in different concentrations and the same absorption for the controls (without CpH or Trolox) in percentage is called antioxidant activity (AOA, %). The experiments were performed in triplicate.

Superoxide - scavenging assay

The generation of reactive oxygen species (ROS) in the model system xanthine-xanthine oxidase (XO) and the changes occurring upon the CpH were investigated photometrically by the NBT test. The detailed procedure has been described elsewhere [18]. Briefly, spectrophotometric registration of $O_2^{\bullet -}$ was carried out by measuring the amount of formazan which is generated upon $O_2^{\bullet -}$ induced reduction of NBT. The decrease of absorbance in presence of antioxidants indicates the consumption of superoxide anion in the reaction mixture. Data are calculated in percentage as spectrophotometric scavenger index (SpSI) - the ratio of the absorption at 560 nm for the sample with CpH, and the same absorption for the control (without CpH).

DPPH - scavenging assay

The antioxidant activity using the DPPH assay was measured by modifying the method of Blois [19]. An ethanolic solution of DPPH (100 mM) was

incubated with CpH in the applied concentrations, and the optical density (OD) was monitored spectrophotometrically at λ517 nm, after 30 min of incubation. Inhibition of DPPH in percentage (I, %) was calculated as given below:

I, % = [(OD control – OD sample) / (OD control)] ×100

Hydroxyl radical scavenging assay

Scavenging of the hydroxyl radicals (HO•) generated by the ascorbic acid/Fe^{3+}/H_2O_2 system was used. The reaction mixture contained 0.3 mM 2-deoxy D-ribose, 0.5 mM H_2O_2, 50 μM Fe^{3+} and 52 μM EDTA without or with CpH in PBS, pH 7.4. The reaction was triggered by the addition of 50 μM ascorbate and the mixture was incubated at 37 °C for 30 min. Solutions of $FeCl_3$, ascorbate and H_2O_2 were made up in ultra pure water immediately before use. The extent of deoxyribose degradation by HO• was measured with the TBA test [20]. Percentage inhibition of deoxyribose degradation was calculated as DI, %.

Metal chelation ability

Iron chelation ability was determined by adding 200 μM of $FeCl_2$ to CpH in PBS, pH 7.4 (protein concentration 5 mg/ml). After incubation at room temperature for 10 min, absorption spectra were recorded. The chelation of iron ions by CpH was evaluated by monitoring the spectral shift after incubation [21].

Statistics

The results were expressed as mean ± SE. Data were analyzed by the program Statistica for Windows and using Student's t-test. The results were accepted as statistically significant when $p < 0.05$.

RESULTS

The isolation and purification of Hc from the hemolymph of *Cancer pagurus* yielded a pure protein preparation, as assessed by gel filtration chromatography and SDS-PAGE (Fig. 1A). SDS-PAGE confirmed the presence of Hc subunits with a molecular mass of ~75 kDa (Fig. 1B). The ratio OD 340 nm/OD 280 nm = 0.2, found for the peak fractions of CpH is typical for fully oxygenated Hc and indicates that the protein is isolated with preserved active sites.

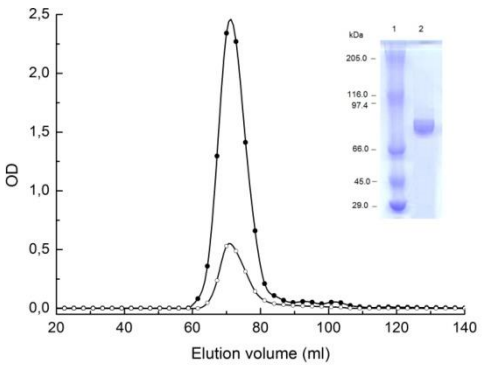

Fig. 1. A. Gel filtration chromatography of CpH, isolated from the hemolymph of *Cancer pagurus*, on a Sephacryl S-300 column (80 × 1.6 cm), equilibrated and eluted with the stabilized buffer, at a flow rate of 0.3 ml/min. (-•-) OD at 280 nm, (-o-) OD at 345 nm. B. SDS-PAGE on 7.5% running gel: lane 1, protein markers (from the top): myosin (205.0 kDa), β-galactosidase (116.0 kDa), phosphorylase b (97.4 kDa), albumin (66.0kDa), ovalbumin (45.0 kDa), carbonic anhydrase (29.0 kDa) (Merck); lane 2, purified CpH (subunit).

The effect of CpH on Fe^{2+}-induced lipid peroxidation in liposomal system, as well as in liver supernatant, was measured by the TBARS test. Results are expressed as percentage of inhibition of the oxidation process in comparison to the control sample (without CpH or Trolox) in which the oxidation is assumed to proceeded as much as possible. Lower AOA value means higher AOA. Figure 2A shows the results of Fe^{2+}-induced oxidation of an aqueous emulsion system of egg liposomes as an AOA test. On Figure 2B the results of the AOA of CpH in terms of Fe^{2+}-induced oxidation, in a system of mice liver supernatant, are shown. In both systems CpH showed an efficient dose-dependent inhibition of lipid peroxidation. Control experiments indicated that CpH did not affect the level of TBARS without creating of prooxidant conditions (data not shown). The results obtained reveal that the CpH has a potential to inhibit the oxidative processes in a slightly lower degree than Trolox.

Metal-mediated formation of free radicals may cause various modifications to DNA bases, as well as enhance lipid peroxidation. Because elemental species, such as the ferrous ion (Fe^{2+}), can facilitate the production of ROS, the ability of substances to chelate iron can be a valuable antioxidant property. Bound to CpH, iron is less susceptible to participation in the Fenton reaction. Our results demonstrated that CpH possesses ability to chelate iron ions. The absorption spectrum of CpH showed characteristic for Hcs bands at 280 and 340 nm, corresponding to aromatic residues and Cu^{II}-O_2^{2-}-

Cu^{II} complexes at the active sites, respectively. Metal ions caused a spectral shift and absorbance change at 340 nm (Fig. 3). Data shown on Fig. 3 reveal that the ability of CpH to act as a peroxidation inhibitor may be related to its iron binding capacity.

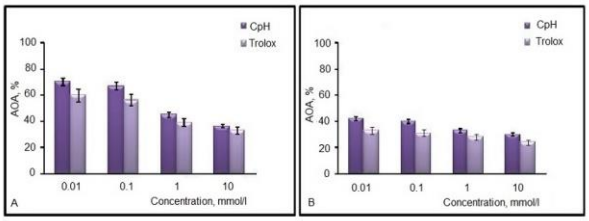

Fig. 2. Lipid peroxidation inhibitor ability of CpH in systems of egg liposomes (2A) and rat liver supernatant (2B). The ratio of the absorption at 560 nm for samples containing CpH in different concentrations and the same absorption for the controls (without CpH or Trolox) in percentage expresses the antioxidant activity (AOA).

The DPPH radical was one of the few stable radical sources and was widely used to test the free radical-scavenging ability of various samples. A freshly prepared DPPH solution exhibited a deep purple color with a maximum absorption at 517 nm. This purple color disappears when an antioxidant is present in the medium. Our results showed that CpH can quench DPPH free radicals and convert them to a colorless product, resulting in a decrease of absorbance at 517 nm.The scavenging activity of CpH was expressed as I (%). Based on the results in Table 1, there is a significant decrease ($p < 0.01$) in the concentration of DPPH radicals due to the scavenging ability of CpH.

Superoxide is an oxygen-centered radical which can generate more dangerous species and promote oxidative reactions due to its ability to reduce transition metals, release protein-bound metals and form perhydroxyl radicals which initiate lipid oxidation [20]. The generation of superoxide in the model system xanthine-xanthine oxidase and the changes occurring upon CpH were investigated spectrophotometrically by the NBT test. The decrease of absorbance in presence of CpH indicated the consumption of superoxide anion in the reaction mixture. Data were calculated in percentage as spectrophotometric scavenger index (SpSI) - the ratio of the absorption at 560 nm for the sample with CpH, and the same absorption for the controls (without CpH). From the results given in Table 1 it can be seen that CpH slightly inhibits the generation of superoxide anions in the system applied.

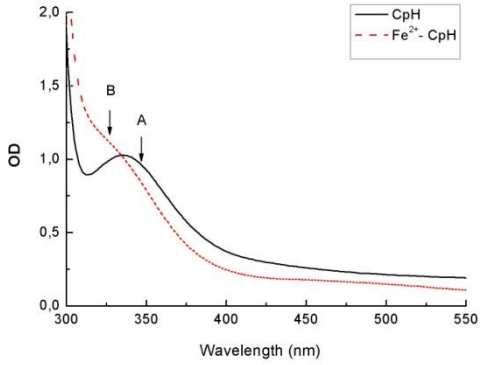

Fig. 3. Absorption spectra of CpH (A) and Fe^{2+}-CpH complex (B) in PBS buffer, pH 7.4.

Hydroxyl radical (OH•) is the most reactive among oxygen species. The statistically treated results for the scavenging activity of OH• radicals of CpH are shown in Table 1. The results are expressed in percentage of inhibition of hydroxyl radical-induced deoxyribose degradation (DI). They reveal that the OH• radical scavenging activity of the tested compound increases in a concentration-dependent manner.

DISCUSSION

In this work we investigated the antioxidative profile of the copper-containing protein CpH in some model systems. In general, antioxidant compounds can interact with free radicals, to scavenge them, removing catalytic metal ions, and inhibit or retard the process of lipid peroxidation [22]. Antioxidant power of natural products can manifest itself through prevention the degradation of biomembranes by oxidants in different ways, including the possibility to express capacity of defense from the action of the free radicals [23-26].

Table 1. Antioxidant capacity of different concentrations of CpH (0.01 – 10 nmol.l^{-1}). The ability of CpH to scavenge DPPH radicals is presented in percentage (I, %). Data for the superoxide scavenging activity are presented as spectrophotometric scavenger index (SpSI). Percentage inhibition of deoxyribose degradation was calculated as DI, %.

CpH, [mmol.l^{-1}]	I, [%]	SpSI, [%]	DI, [%]
0	100	100	100
0.01	68.2 ± 10.1**	96.6 ± 6.6	94.36 ± 8.12*
0.1	52.8 ± 9.1*	94.7 ± 5.4	93.02 ± 7.33
1.0	45.3 ± 8.7*	91.5 ± 7.1*	62.66 ± 6.37**
10	31.5 ± 7.2*	88.3 ± 7.5**	53.18 ± 4.24**

**$p < 0.001$ versus control; *$p < 0.05$ versus control

Although peroxidation in model membranes may be very different from peroxidation in cell membranes, the results obtained in model membranes may be used to advance understanding of peroxidation in biological membranes [26].

Lipids such as free and ester forms of polyunsaturated fatty acids are vulnerable targets of free radicals. Liposomal system and rat liver homogenate are usually used as model systems to evaluate the antioxidant activities of compounds on lipid peroxidation. It is known that transition metal ions are involved in both initiation and propagation of lipid peroxidation. In this system, we found that CpH effectively inhibited ferrous ion-induced lipid peroxidation (Figures 2A, B). Ferrous chelation may render important antioxidative effects by retarding metal-catalysed oxidation [20].

The DPPH radical scavenging test is a sensitive antioxidant assay and depends of substrate polarity. CpH shows good ability to reduce the stable radical DPPH (Table 1). Undoubtedly, the DPPH test provides very important evidence of the antioxidant profile of the tested substances, but often this radical has little relevance to be present in biological systems [25, 26].

Hydroxyl radicals can be formed from superoxide anion and hydrogen peroxide in the presence of metal ions, such as iron or copper. Hydroxyl radicals could damage important biomolecules such as proteins, DNA, polyunsaturated fatty acids, nucleic acids and almost all biological membranes. Therefore, removal of hydroxyl radicals is one of the most effective defenses of a living body against various diseases. Our studies showed that CpH is a good scavenger of hydroxyl radicals (Table 1).

The fact that CpH possesses some antioxidant activities could be connected with the biological protective role of this protein. Reactive oxygen components are often generated under aerobic conditions. Hypoxia is known as a condition for generation of superoxide radicals [20]. Aquatic organisms routinely experience fluctuations in oxygen levels. In response to hypoxia exposure of *Cancer magister*, the concentration of respiratory pigment Hc significantly increased in hypoxic crabs. On the other hand, total oxy-Hc was significantly higher in crabs that had been exposed to hypoxic conditions [27]. Due to the high affinity to oxygen, several Hcs subunits play an important role as a regulator for oxidative homeostasis [11]. The beneficial effects of antioxidant marine bioactive peptides are well known in scavenging ROS and free radicals or in preventing oxidative damage. It is not surprising that proteins, such as CpH, can inhibit the lipid oxidation by biologically designed mechanisms (e.g. antioxidant enzymes and iron-binding proteins) or by nonspecific mechanisms [1, 2].

Proteins have excellent potential as antioxidant additives in foods because they can inhibit lipid oxidation through multiple pathways including inactivation of ROS, scavenging free radicals, chelation of prooxidative transition metals [24]. A protein's overall antioxidant activity can probably occur by disruption of its tertiary structure to increase the solvent accessibility of amino acid residues so that they can scavenge free radicals and chelate prooxidative metals [28]. Considering the destructive effect of lipid peroxidation processes in several disease conditions, the ability of CpH to inhibit peroxidation could perhaps partly constitute the basis for the pharmacological reasons for its implementing.

In conclusion, the present study elucidates the antioxidant activity of CpH. This work verifies that CpH is a natural antioxidant against lipid peroxidation in rat liver supernatant, as well as in the liposomal system. Its antioxidant activities are primarily attributed not only to its free radical scavenging actions, but also to iron ion chelation ability. It will be interesting to further investigate the effect of CpH on various radical-mediated injury models *in vivo*. CpH can be incorporated into lipid-containing foods as an antioxidant to minimize free radical-mediated lipid peroxidation. It can also be used as an alternative to conventional drugs for treating human diseases associated with free radical-mediated tissue damage. However, such usage must be adequately justified by animal and clinical studies, creating a need for further research.

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ИН ВИТРО ИЗСЛЕДВАНЕ НА АНТИОКСИДАНТНИТЕ СВОЙСТВАНА ХЕМОЦИАНИН ОТ РАК *CANCER PAGURUS*

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Постъпила на 17 май, 2016 г.; приета на 2 юни, 2016 г.

(Резюме)

Хемоцианините са мед-съдържащи протеини в хемолимфата на много членестоноги и мекотели, чиято биологична функция е основно свързана с транспортирането на кислород до тъканите. Като компонент на морската храна, която традиционно се свързва със здравословна диета, те са интересен обект за изследване на тяхната биологична активност и фармакологичен потенциал.

В настоящото изследване, хемоцианин от морски рак *Cancer pagurus* (CpH) е изолиран и пречистен, и са изследвани неговите антиоксидантни свойства ин витро. Използвани са три радикала - супероксиден, хидроксилен и 2,2-diphenyl-1-picrylhydrazyl (DPPH). Тестван е защитния ефект на CpH срещу окислително увреждане при две моделни липидни мембрани – чернодробен супернатант от плъх и липозомна суспензия, в условията на желязо-индуцирана липидна пероксидация. CpH показват добра радикал улавяща активност по отношение на DPPH и хидроксилни радикали и по-ниска способност за улавяне на супероксидни радикали, при физиологична стойност на pH. CpH показа много добра способност да инхибира Fe^{2+}-индуцирана липидна пероксидация в приложените системи, и хелатираща активност към железни йони.

Това изследване показва, че CpH има способността да хелатира метални йони, както и да осигури защита срещу оксидативен стрес и по този начин да намали риска от разрушаване на биомолекули, причинено от свободните радикали.