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Antioxidative screening of fractions from the mucus of garden snail *Cornu aspersum*

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Antioxidative peptides have been utilized by various species to combat pathogenic microorganisms and stress. In the present study, the antioxidant potential of peptide fractions obtained from the mucus of the garden snail *Cornu aspersum* was evaluated. Bioactive compounds from the mucus of the garden snail were separated into four fractions with different molecular weight (MW): Fraction 1 (compounds with MW<5kDa), Fraction 2 (compounds with MW<10kDa), Fraction 3 (compounds with MW<20kDa) and Fraction 4 - in the region 10-30kDa. Three complementary test methods were employed for preliminary antioxidative screening, including measurement of the radical scavenging activity on the 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH), total antioxidant activity (ABTS method) and the inhibition of nitro blue tetrazolium (NBT) reduction by photochemically generated superoxide radicals ($^{\bullet}O_2^-$). The results demonstrated that the lower MW fraction of <5kDa exhibited better antioxidant potential compared to the others.

Key words: *Cornu aspersum*, Peptides, Mucus, Antioxidant activity, Mass spectrometry

INTRODUCTION

During the organism's metabolism, reactive oxygen species (ROS) or free radicals are naturally produced by oxidation reactions through breathing. These ROS include the superoxide anion radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$) and can cause peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately lead to cell death [1]. The cell defense system includes enzyme components that directly detoxify ROS. The enzymes superoxide dismutase (SOD), catalase (CAT), peroxidase and glutathione peroxidase are among the most important protection agents [2, 3]. Non-enzymatic antioxidants are reported as well, most common of which are glutathione, thioredoxin, ubiquinones, carotenoids and ascorbic acid [4-6]. Prevention or inhibition of oxidative effects are key functions of the system [7, 8]. The aim is to avoid the imbalance between oxidants and antioxidants in the cell, since this could lead to „oxidative stress" [9,10].

Antioxidants from natural origins have been found to possess the ability to effectively prevent the damage caused by ROS [11] and hence, there is a growing interest towards the discovery of novel natural antioxidant compounds, especially among naturally derived peptides.

Naturally derived peptides with antioxidant (AO) properties are well-known for their contributions to human health improvement through the prevention and treatment of non-communicable chronic degenerative diseases [12]. Also, AO peptides exert effective metal ion (Fe^{2+}/Cu^{2+}) chelating and lipid peroxidation inhibitory abilities in addition to having very few side effects. These features render them with potential properties as food processing additives [13]. It has been proposed that the majority of nutrient-based AOs act as chain-breaking AOs by stopping or slowing down the oxidative process once it has begun [14]. AO peptides can behave as sacrificial peptides by reacting with a radical before vital biomolecules are damaged or as donor AOs in which they are preferentially oxidized to stable products that cease the propagation of the radical chain reaction (i.e., uric acid, vitamins C, E) [14].

Terrestrial slugs and snails produce mucus which performs a variety of functions, including facilitating movement along the ground, communication and a non-specific, defensive response to physical or chemical irritation [15]. The excreted biological fluid is a rich source of bioactive natural compounds and is being commercialized in many skin care compositions

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and other formulations for the treatment of a number of skin ailments, for example wounds, burns, scars, keratosis, psoriasis, acne, wrinkles and age and skin damage [15, 16]. Moreover, snail mucus is well known for its anti-aging properties and widely investigated for antibacterial potency [17, 18]. In general, both of these properties are bound to the ability of the organism to cope with the generation of free radicals. However, to this date, there are no sufficient data on the antioxidant potency of the compounds in snail species mucus, a fact that opens a variety of perspective investigations in this aspect.

Herein, we present an initial antioxidant screening of different fractions derived from the mucus of the garden snail *Cornu aspersum*. The purified mucus was subdivided by molecular weight cut-off filters into fractions. Then the total antioxidant potential of the tested fractions was assessed by the DPPH and ABTS radical scavenging activity methods and the nitro blue tetrazolium (NBT) reduction assay. Based on the obtained results SO and CAT activities were evaluated as well.

MATERIALS AND METHODS

Purification of the fractions from the mucus of garden snail Cornu aspersum

The snails *Cornu aspersum* were collected in Bulgaria and the mucus was purified. After that the mucus from the snails was subjected to ultrafiltration on Millipore filters (5, 10 and 30 kDa) to obtain fractions with different molecular mass: Fraction 1 (compounds with MW < 5kDa), Fraction 2 (compounds with MW <10kDa), Fraction 3 (compounds with MW < 20kDa) and Fraction 4 (compounds with MW between 10-30kDa).

DPPH assay

Each extract was evaluated for the ability for electrons (or hydrogen atoms) donation by using the bleaching level of purple-colored solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) – a stable radical used as a reagent. This spectrophotometric assay was done according to the method of Murthy *et al.*, [19] with small modifications. A volume of 0.5 ml from each of the three fractions was added to 1 ml of DPPH (100 μM) solution in ethanol. The absorbance was read against a blank at 517 nm, after 30 min of incubation period at 37 °C. Inhibition of free radical DPPH in percent was calculated according to the formula:

$$\text{Inhibition\%} = (A_{blank} - A_{sample} / A_{blank}) \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all the reagents except the test compound) and A_{sample} is the absorbance of the test compound.

ABTS radical scavenging activity

Total antioxidant activity was measured by ABTS radical cation ($ABTS^{\cdot+}$) assay as well, using the method of Re *et al.* [20] with some modifications. Shortly, 12 hours prior to use, $ABTS^{\bullet+}$ was generated in a mixture between 7 mM stock solution of ABTS and 2.45 mM potassium persulfate at room temperature. The stock $ABTS^{\bullet+}$ solution was diluted with methanol to achieve absorbance of 0.7 ± 0.01 at 734 nm. *Cornu aspersum* samples were added in 1 ml volume to 2 ml of the $ABTS^{\bullet+}$ solution. The final absorbance was measured after 1 min at 734 nm. Caffeic acid and ascorbic acid were used as reference compounds.

Superoxide anion scavenging activity

Determination of superoxide anion scavenging activity was done by inhibition of NBT reduction by photochemically generated superoxide ($^{\bullet}O_2^-$) [21]. The reaction mixture contained 56 μM NBT, 0.01 M methionine, 1.17 μM riboflavin, 20 μM NaCN and 0.05 M phosphate buffer with a pH of 7.8. Superoxide presence was evaluated by the increase in absorbance at 560 nm at 30°C after 6 min of incubation from the beginning of the illumination. Caffeic acid was used as a reference substance applied in a concentration of 5 μg/ml. The tested fractions and the caffeic acid were assayed by quantities of 0.02 ml in triplicate (n=3).

Enzyme activity determination

SOD activity in the tested fractions from *Cornu aspersum* was determined by NBT reduction [21]. One unit of SOD activity was assigned as the amount of SOD required for inhibition of the reduction of NBT by 50% (A560) and was expressed as units per mg protein (U/mg protein).

CAT activity was evaluated by the method of Beers *et al.* [22] in which the decomposition of hydrogen peroxide was analyzed spectrophotometrically at a wavelength of 240 nm. One unit of catalase activity was determined as the amount of enzyme that decomposes 1 mmol of hydrogen peroxide per min at an initial hydrogen peroxide concentration of 30 mmol/L, at pH = 7.0 and 25°C. The specific activity was represented in units per mg protein (U/mg protein).

Protein content was measured by the Lowry procedure [23], using crystalline bovine albumin as a standard.

Determination of peptides by mass spectrometry analysis

MS was performed on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanoelectron spray ion source. MS analysis was run in data-dependent acquisition using a top five method (i. e., the five most intense ions with a positive charge between 2 and 4 analyzed during survey scan were selected for fragmentation during each scan cycle). Survey scans were performed in the Orbitrap at a resolution of 60,000 with a scan range of 450–650 m/z (450–750 m/z for absolute quantification). Peptides were fragmented using collision-induced dissociation (normalized collision energy, 35%; activation time, 30 ms; isolation width, 1.3 m/z) with resulting fragment ions (MS/MS scans) analyzed in the linear ion trap. Dynamic exclusion was enabled for all runs (maximal number of masses excluded at each time point [exclusion list size] 500; duration of exclusion for each mass: 40s).The mass spectra were analysed using Xcalibur Qual Browser (Thermo Scientific).

RESULTS AND DISCUSSION

Purification and mass spectrometric peptide sequencing

Four fractions were isolated after ultrafiltration of collected mucus from the snails on Millipore filters: Fraction 1 (with MW <5kDa), Fraction 2 (with MW <10kDa), Fraction 3 (with MW <20kDa) and Fraction 4 (with MW between 10-30kDa). Mass spectrometric peptide analysis was performed by LTQ Orbitrap XL mass spectrometer and several peptides were identified as shown in figure 1A. Figure 1B depicts the mass fragmentation spectrum of LLMGPEV following b- and y-ions in MS/MS spectrum of peptide with m/z 379.71 $[M+H]^{2+}$.

Most of the peptides appeared as z=2 and the amino acid sequences of some of them were confirmed by their MS/MS spectra. Following b- and y-ions in the MS/MS spectrum shown in figure 1C, the primary structure LPDSWEPGGGG of the peptide with m/z 1071.56 $[M+H]^{+}$ was determined. Peptide sequencing performed by MS/MS spectra showed that Pro (P), Gly (G), Tyr (Y) and Trp (W) – rich peptides are present in the mucus (Table 1).

Antioxidant screenings

The screenings of the antioxidant properties of the mucus from *C. aspersum* started with evaluation of the total antioxidant activity. At first, the free radical scavenging activity was measured by the DPPH method that is usually used for different natural extracts. The effects of antioxidants on DPPH radical scavenging are thought to be due to their hydrogen-donating ability. The decrease in absorbance of DPPH radical caused by antioxidants was compared to the control, because of the reaction between antioxidant molecules and radical progressed, results in the scavenging of the radicals by hydrogen donation [24]. Based on the data obtained, Fractions 2 and 3 of MW <10 kDa and 10-30 kDa, respectively, demonstrated scavenging activity (Fig. 2).

The tested fractions were able to reduce the stable free radical DPPH to the yellow-colored 1,1-diphenyl-2-picrylhydrazyl. Fraction 2 with MW <10 kDa exhibited better free radical DPPH reduction to the yellow-colored 1,1-diphenyl-2-picrylhydrazyl. Moreover, this fraction exhibited better free radical scavenging activity than the others with higher MW.

Similar results have been reported for peptides derived from meat muscles [25] and protein hydrolysates of blue mussel, salmon, grass carp skin, etc. [26]. Therefore, the present results suggest that fractions with MW < 10kDa and 10-30kDa are DPPH• inhibitors and primary antioxidants that react with free radicals.

The total antioxidant activity of the tested mucus fractions was calculated by another complementary method - the decolorization of $ABTS^{+}$ in the reaction mixture. The results were expressed as percentage inhibition of absorbance.

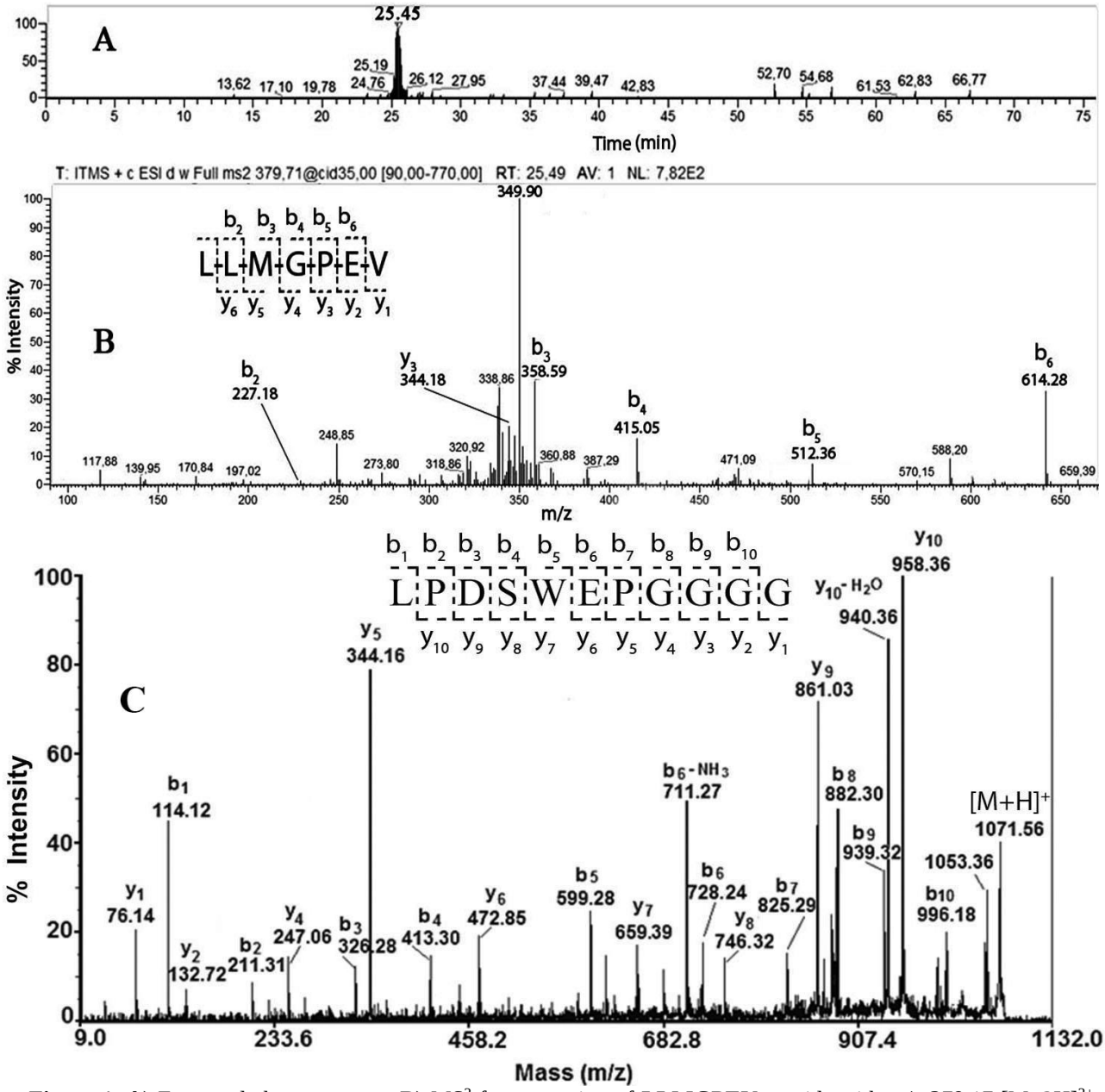

Figure 1. A) Extracted chromatogram, **B)** MS^2 fragmentation of **LLMGPEV** peptide with m/z 379.17 $[M+2H]^{2+}$, **C)** MALDI-MS spectrum (positive ion mode) of the peptide at m/z 1071.56$[M+H]^+$. Standard peptide solution was used to calibrate the mass scale of the AutoflexTM III, High Performance MALDI-TOF & TOF/TOF Systems (Bruker Daltonics).

Table 1. Some sequenced peptides from the mucus of *Cornu aspersum.*

Sequence	Modifications	Activation Type	Charge	m/z	$[M+H]^+$	ΔM [ppm]	RT [min]
LLMGPEV		CID	2	379.71	758.41	1.35	25.49
LmYQPP	M2(Oxidation)	CID	2	382.69	764.36	-0.28	5.22
GNGPTGLHmA	M9(Oxidation)	CID	2	485.72	970.44	-2.76	65.55
QSGKSPGFGL		CID	2	489.25	977.50	-2.63	45.50
LPDSWEPGGGG		CID	2	536.20	1071.56	-1.86	56.30
VGQGCDEmLQG	M8(Oxidation)	CID	3	384.83	1152.46	-1.74	79.64
YNGFRPGDCY		CID	3	397.83	1191.49	-1.94	69.68

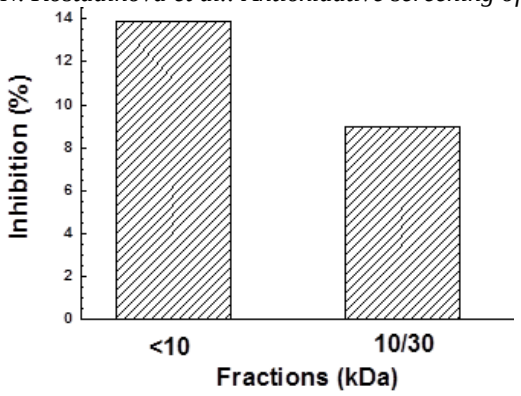

Fig. 2. DPPH scavenging power of Fraction 2 and Fraction 3 isolated from the extract from *C. aspersum*

The data in Fig. 3 clearly show the higher scavenging effect of the fraction with molecular mass below 20 kDa in comparison with the total extract. The values of the <20 kDa fraction were similar to those of the reference compounds vitamin C and caffeic acid. In addition, we evaluated the <5 kDa and 10-30 kDa fractions, which also expressed antioxidant activity by the $ABTS^{+}$ method. The results from Table 2 demonstrate two different dose-dependent trends. While the scavenging activity of the fraction of <5kDa slightly decreased with the increase in the protein concentration from 5 to 40 µg, the 10-30 kDa fraction demonstrated an opposite tendency. Concentration above 15 µg caused more than 20% increase in the antioxidant effect.

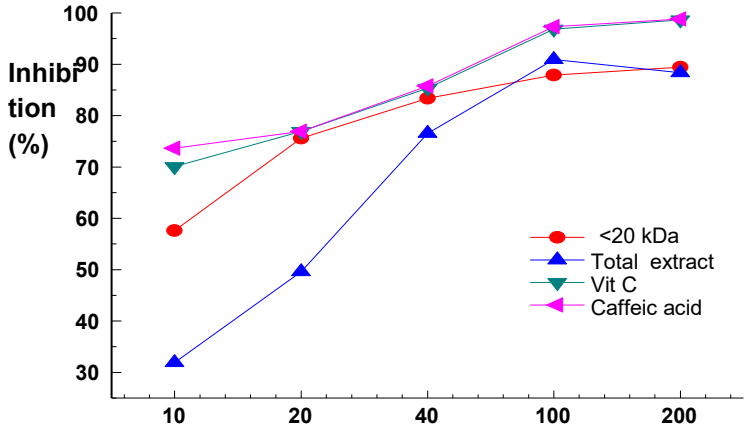

Fig. 3. Potential of the tested samples from *C. aspersum* for scavenging of ABTS radical.

Table. 2. Scavenging effect of *C. aspersum* according to the applied protein quantity.

Protein (µg)	Scavenging effect (%)	
	<5 kDa	10-30 kDa
5	82.42	70.00
10	75	72.92
15	67.55	83.96
20	67.27	84.,11
40	70.15	84.27

Moreover, the low-molecular fraction of <5 kDa was observed to have a more potent scavenging effect (82.42%) which was more strongly expressed at low concentrations than the others. Antioxidative peptides of <1 kDa from zein or *Struthio camelus* egg hydrolysate also displayed high ABTS activity [27, 28].

Further, the antioxidant effects of the *C. aspersum* snail extracts were evaluated by suppression of the superoxide anion radicals generated in a photochemical system in the presence of the test samples (NBT assay) as is shown in Fig. 4. The best $^{\bullet}O_2^-$ scavenging activity was shown by the fraction with MW below 5 kDa. These preparations inhibited the development of the color produced during the reaction of $^{\bullet}O_2^-$ with NBT by 57, 42, 40 and 39%, respectively. These radicals are known to be highly reactive, mainly because of their role in the formation of more powerful and dangerous hydroxyl radicals and singlet oxygen, both of which are involved in the appearance of oxidative stress events [29].

The reducing power of a natural compound may serve as a significant indicator of its antioxidant activity level. A study on *Giant African Snail (Achachatina maginata)* antioxidant activities reports that its hemolymph possesses antioxidant activity and significantly inhibits the acute liver toxicity induced by CCL4 in rats [30]. The authors suggest that the hepatoprotective activity of the

hemolymph may be due to its free radical scavenging and antioxidant activity, resulting from the presence of flavonoids and phenolic compounds in the hemolymph that enhance the regeneration ability of liver.

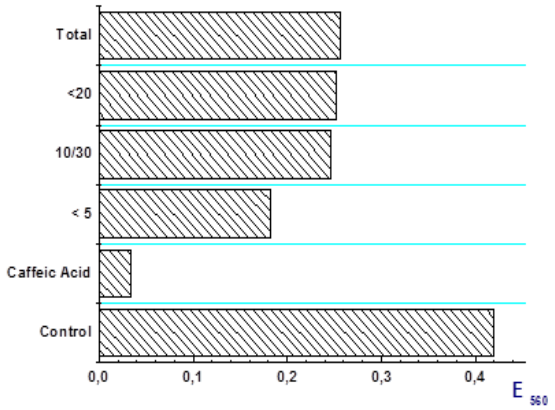

Fig. 4. Inhibitory effect of the fractions from *C. aspersum* on the reduction of NBT by photo-chemically generated superoxide anion radicals

The next step in the present investigation was the measurement of the potential SOD and CAT activities of the tested fractions. At first, SOD and CAT activity values were reported for the total mucus sample. These activities were low but they were detected in the total sample where high MW proteins exist (Fig. 5).

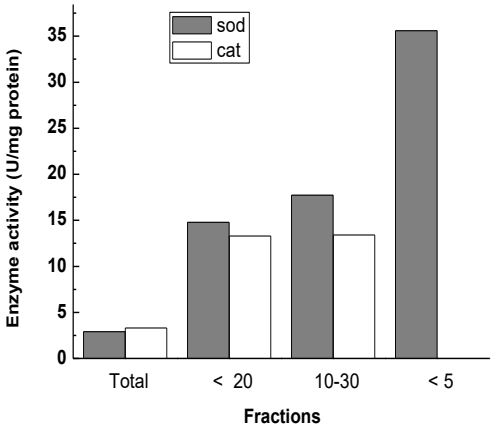

Fig. 5. SOD and CAT activities of the tested samples from ***C. aspersum***.

The fractions with MW <20 kDa and between 10-30 kDa demonstrated values similar to each other for both enzymes. The fraction of <5 kDa showed the highest SOD activity (of 35.6 U/mg protein), although there was no CAT activity detected. It is possible that the high SOD recorded in the latter fraction represents the so called "SOD-like activity" that could be displayed by some peptides with low molecular mass capable of superoxide scavenging. The real enzyme cannot be found in low-molecular fractions.

Over the last decade, various reports have been made for the possible use of snail slime in wound treatment. Brieva *et al.* [31] found that slime from *Cryptomphalus aspersa* (also known as *Helix aspersa* or the common garden snail) contains antioxidant superoxide dismutase and glutathione-s-transferase activity. As a whole, a few studies concentrate on the antioxidant enzymes in that type of natural products, which requires additional and complex investigation in that direction.

It has been reported that the snail mucus contains allantoin (0.3-0.5%), collagen (0.1-0.3%), glycolic acid (0.05-0.1%), lactic acid (0.05-0.1%), anti-protease (1.3-1.8%), vitamins, trace minerals and a high content of glycine, hydroxyproline, proline and glutamic acid [32]. Recently, several peptides [17] and glycoproteins [33] have been isolated from the mucus of *Helix aspersa* and *H. pomatia* snail species and high concentrations of Asp, Glu, Gly, Leu, Pro and Lys were also reported [17].

As seen from the results, the low-molecular weight fractions exhibited better antioxidant potential compared to the other tested fractions which is in accordance with other observations where low-MW peptide fractions have displayed in general better antioxidant activity compared to fractions with higher MW. As reported, around 70% of the identified antioxidant peptides had MW ranging from 400 to 650 Da [13]. In another study [34], the DPPH radical scavenging activity of a 3–5 kDa fraction was exerted mainly by the sub-fraction dominated by peptides with masses below 600 Da. Also, the activity of the <3 kDa fraction was attributed mainly to the radical scavenging activity of the sub-fractions with lower MW. The highest reducing power was found in a sub-fraction containing peptides rich in Arg, Tyr and Phe. The authors state that both free amino acids and low-MW peptides thus seemed to contribute to the antioxidative potential. It has been recognized that peptides are more reactive than proteins, due to their lower MW and bioactive peptides mostly contain less than 20 amino acid residues [35]. In an attempt to identify and isolate antioxidant peptides Najafian *et al.* [36] reported that the highest antioxidant potential was displayed by peptides with 8–12 amino acid residues. Analyzing the amino acid composition of 42 antioxidant peptides, Zou *et al.* [13] have calculated the percentage distribution of the individual amino acids in these peptides. Gly (G), Pro (P) both having approximately 12% each distribution account for the majority of the amino acids, followed by Leu (L) and Ala (A) with 9.8% and 6.3%, respectively. Moreover, the antioxidant potential of the mucus

can also be attributed to the presence of allantoin, which has been shown to possess antioxidant properties [37].

CONCLUSION

The present investigation reports initial data on the antioxidant potential of *C. aspersum* mucus. The results from the antioxidant screenings of *C. aspersum* mucus and its fractions show that this naturally derived product, specifically the low-molecular weight fractions, possess the properties to counteract the formation of reactive free radicals. From the mucus, several peptides having predominantly Pro (P), Gly (G), Tyr (Y) and Trp (W) were sequenced by mass spectrometry.

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СКРИНИНГ НА АНТИОКСИДАНТНАТА АКТИВНОСТ НА ФРАКЦИИ, ИЗОЛИРАНИ ОТ СЛУЗ НА ГРАДИНСКИ ОХЛЮВ *CORNU ASPERSUM*

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(Резюме)

Природните пептиди, получени от различни животински видове проявяват и антиоксидантно действие, като част от механизма за борба с патогенни микроорганизми и оксидативния стрес. Настоящото изследване проучва антиоксидантния потенциал на пептидни фракции, получени от слуз на градински охлюв *Cornu aspersum*. Биологично-активните вещества от слузта са разделени на четири фракции с различно молекулно тегло (MW): фракция 1 (съединения с MW<5kDa), фракция 2 (съединения с MW<10kDa), фракция 3 (съединения с MW<20kDa) и фракция 4 (MW между 10 30kDa). За определяне на антиоксидантната активност на получените фракции са използвани три допълващи се метода: обезвреждане на 1,1-дифенил-2-пикрилхидразил (DPPH) радикал; обща антиоксидантна активност по ABTS метод и инхибиране редукцията на nitro blue tetrazolium (NBT) чрез фотохимично генериране на супероксидни радикали ($^{\bullet}O_2^-$). Установено е, че фракцията с MW<5 kDa проявява по-добър антиоксидантен потенциал в сравнение с останалите фракции.