eFood
Vol. 2(4); August (2021), pp. 193–200
DOI: https://doi.org/10.53365/efood.k/143990; eISSN 2666-3066
https://efood.visagaapublishing.com/

Original Research

Active sites of peptide from Arg-Ser-Ser protect against oxidative stress in HepG2 cells

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ARTICLE INFO

Article History

Received 24 October 2021
Accepted 16 November 2021

Keywords

cellular antioxidant
peptide
quantum chemistry
active site

ABSTRACT

Peptide Arg-Ser-Ser (RSS) was derived from *Lactobacillus amylolyticus* co-incubated with edible *Dendrobium aphyllum*. Here, we further examined the antioxidative effects of RSS in HepG2 cells subjected to 2,2-azobis(2-methylpropanimidamidine) dihydrochloride-induced oxidative stress. RSS protected cells by eliminating the level of reactive oxygen species (ROS). The protein expression of antioxidant enzymes, Nrf2 and Keap1 determined by western blot, indicated that RSS might maintain cellular homeostasis by directly scavenging free radicals instead of by enzymatic system. Furthermore, quantum chemistry calculations and a characterization of electronic-related properties showed that the highest occupied molecular orbital energy distribution was on arginine residue. Pre-treatment with RSS with the active site methylated resulted in increased ROS levels, thereby verifying that N_2-H_3 is the active site for antioxidant activity. Our findings provide valuable insights into the antioxidant activity of RSS and a basis for developing antioxidant functional foods.

1. INTRODUCTION

Peptides can eliminate excess free radicals *in vivo* and inhibit lipid peroxidation, thus helping the body to resist various diseases [1]. Antioxidant peptides have few side effects and have favorable nutritional and processing properties [2]. In a previous study, we isolated Peptide Arg-Ser-Ser (RSS) from edible *Dendrobium aphyllum* after *Lactobacillus amylolyticus* solid-state optimized fermentation [3]. Dietary arginine-rich supplementation has been reported to attenuate oxidative stress in mice [4]. Therefore, it is meaningful to develop antioxidants from natural sources such as arginine-rich peptides and investigate their antioxidant mechanisms.

Reactive oxygen species (ROS), oxygen-containing free radicals can traverse cell membranes and can even lead to cell death [5]. The antioxidant defense system deployed by organisms to resist ROS in vivo can be concluded two ways (enzymic and non-enzymic systems) [6]. The enzymic system includes superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT), whereas the non-enzymic system mainly comprises endogenous antioxidants such as glutathione (GSH) and vitamins E and C [7]. A peptide derived from grape seed have anti-oxidant effect, which can directly scavenge free radicals [8]. Another component such as betaine [9] and Diosmetin [10] can also have ability of eliminating cellular free radicals. It has been reported that the mechanisms whereby free radicals are scavenged, are based on providing hydrogen atoms to block free radical reactions [10, 11].

Atomic-level structures and related information on system energetics can be determined using quantum chemistry and computational methodologies [11]. Theoretical calculations can be used as a new type tool to investigate the structure–activity of bio-active peptides [12]. Quantum chemical calculations such as density functional theory (DFT) have been widely employed. The application of quantum chemical parameters to the study of antioxidants generally includes highest occupied molecular orbital (HOMO) energy and lowest unoccupied molecular orbital energy, bond length and dihedral angle, and molecular shape parameters [13]. For example, Cheng et al. analyzed the peptides AQIPQQ, RVF, and NRYHE with their active sites with the support of parameters of HOMO energy and bond length [14].

In this study, we determine the mechanisms of antioxidative protective activities of RSS. Fluorescence-labeled peptides were used to observe the dynamic changes when peptides contacted HepG2 cells and to examine whether they could directly cross the membrane. The cytoprotective capacities of the peptides were evaluated based on malondialdehyde (MDA), ROS, antioxidant enzyme expression, and the Nrf2/Keap1 signaling pathway. The structure–activity relationships of the RSS were determined through quantum chemistry theoretical calculations and the active sites were predicted, which were verified through the methylation of predicted sites and an evaluation of the ROS levels.

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Peer review under responsibility of the *International Association of Dietetic Nutrition and Safety*

2. MATERIALS AND METHODS

2.1. Materials

The peptides (RSS, FITC-acp-RSS, methylated-RSS for modifying active site N_2-H_3in RSS) assessed in this study were synthesized from Shanghai Science Peptide Biological Technology Co., Ltd (Shanghai, China). Human hepatoma cells (HepG2) were from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). The compounds AAPH and Trolox were both from Sigma (St. Louis, MO, USA). MDA (A003-1) SOD(A001-3), CAT(A007-1), glutathione peroxidase (GSH-Px) (A005), MDA test kit (PAB180007)were from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). ROS (PAB180052) and GSH/GSSG assay kits (S0053) were from Beyotime Institute of Biotechnology (Shanghai, China). Cell Count Kit (CCK-8,PAB180031) and Anti-SOD (PAB31525), anti-CAT (PAB30815), anti-Nrf2 (PAB30175), anti-Keap1 (PAB33016), anti-GAPDH (PAB36269) were purchased from Wuhan Hualian Biotechnology Co. Ltd (Wuhan, China). Anti-GSH-Px (Ab22604) was purchased from Abcam (Cambridge, UK).

2.2. Cell culture

HepG2 cells were incubated in DMEM high glucose medium at 37 °C in a 5% CO_2 humidified incubator. The culture medium was renewed at 2-day intervals, with the cells being subcultured at 80% to 90% confluency using a solution of 0.25% trypsin and 0.02% EDTA .

2.3. Evaluation of HepG2 cell viability

HepG2 cells were cultured at a density of 5×10^3 cells /mL. RSS and methylated RSS were dissolved into different concentrations (0.023 to 1.5 mg/mL), and co-incubating with the cells and then placed in a humidified incubator at 37 °C with 5% CO_2 for 24, 48, and 72 h, respectively. CCK-8 method was employed to evaluate the cell viability.

2.4. Dynamic observation of RSS in HepG2 cell

HepG2 cells were treated with fluorescence-labeled peptide (FITC-acp-RSS, 1.5 mg/mL) to observe whether RSS enter into HepG2 cells. Nucleus in HepG2 cells were also labeled in blue (DAPI), and the structure in HepG2 cells were labeled in red (actin red), then they were observed under a laser scanning confocal microscope (Leica SP8, Wetzlar, Germany).

2.5. Determination of ROS levels

AAPH-induced ROS in HepG2 cells was determined using dichlorofluorescin-diacetate (DCFH-DA) assays. Briefly, HepG2 cells (5×10^3 cells /mL) were cultured in 96-well plates and treated with 0.375-1.5 mg/mL of RSS, then the medium was discarded and washed three times with PBS, followed by 10 μM DCFH-DA in a 37 °C cell incubator for 20 min. After that, the cells were washed three times with PBS and treated with 200 μM AAPH or DMEM. The relative levels of ROS after treatment were determined using the ROS assay kit with DCFH-DA as the fluorescent probe [15].

2.6. Determination of MDA content and activities of GSH, and GSSG

HepG2 cells (5×10^3 cells/mL) were seeded in 24-well plates and treated with peptides (0, 0.375, 0.75, and 1.5 mg/mL) or Trolox(1.00 mg/mL) for 2 h. Thereafter, the cells were washed twice with PBS and then treated with 200 μM AAPH for 3 h. Following a further three washes with PBS, the cells were lysed with RIPA lysis buffer. MDA contents and the activities of GSH, and GSSG in the supernatants were determined according to assay kit instructions.

2.7. Western blotting

Sample were electrophoresed with a 10% sodium dodecyl sulfate polyacrylamide gel. The separated proteins were transferred onto a PVDF membrane (Millipore, USA) and blocked by incubating them in 5% skim milk for 2 h. Thereafter, the membranes were incubated with primary antibodies overnight at 4 °C. The following day, the membranes were incubated with secondary antibodies at 1:10000 dilutions for 1 h at room temperature. The treated-membranes were placed in an automatic chemiluminescence analyzer for detection.

2.8. Structure–activity analysis of peptides

Chemdraw Professional 16.0, GaussView 5.0, and Gaussian 09 were used in this study to calculate the structure of the RSS. Chemdraw software is used to simulate peptide geometry, preliminary geometry optimizations were performed using molecular mechanics MM+, a semi-empirical AM1 method, for further configuration optimization. Optimization results were obtained by using the Gaussian 09 input file. A small basis set was used to optimize the RSS conformation in the Gaussian 09 program. The geometries were extrapolated using B3LYP/6-31 and the optimized geometries were obtained for the RSS. Parameters (HOMO distribution, bond length, Mulliken charge distribution) were obtained, facilitate analysis of active sites.

2.9. Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA). We used Gaussian 09 and GaussView 5.0 to analyze the electronic-related properties of RSS.

3. RESULTS AND DISCUSSION

3.1. Cell viability

We determined the toxic effects on HepG2 cells using the CCK-8 assay. RSS (purity more than 90%) showed almost no cytotoxicity

at the different concentrations, with cell viability being higher than 80% [16]. Cell viabilities after treatment with RSS and methylated RSS for 24, 48, and 72 h showed a concentration-dependent decrease with increasing RSS concentrations (Fig. 1A). Based on the results, the RSS and methylated RSS concentrations of 0.375, 0.75, and 1.5 mg/mL were used for subsequent analyses (Fig. 1B).

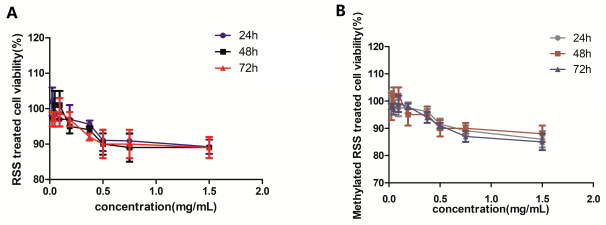

Figure 1 Cell viability afterpeptide treatment. HepG2 cells were treated with indicated concentrations of(A) Arg-Ser-Ser (RSS) and (B) methylated RSS.

3.2. Dynamic observation of RSS in HepG2 cells

RSS was fluorescently tagged to verify that it could enter HepG2 cells for labeling green in RSS, and the cell structure and nucleus were labeled Actin red (red) and DAPI (blue), respectively. It can be seen that the green spots were observed that it distributed inside the red frame-like cell structure (Fig. 2).

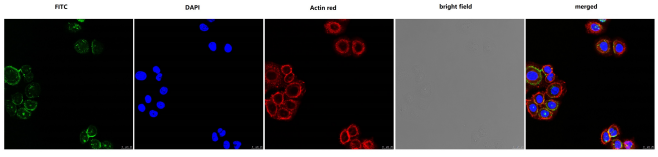

Figure 2 Fluorescenceimages of HepG2 cells treatedwith fluorescence-labeled Arg-Ser-Ser (RSS).

Fluorescence microscopy revealed that FITC-acp-RSS could enter the HepG2 cells. Membrane-permeable peptides such as arginine-rich peptides are well documented [17]. However, RSS contains the guanidinium side-chain groups on the arginine. The ability of arginine-rich peptides to passively penetrate across cellular membranes is due to the like-charge pairing of the guanidinium side-chain groups [18]. Based on this, the cell membrance penetrating activity of RSS might be related to its guanidinium side-chain groups on arginine.

3.3. ROS elimination of the RSS

ROS accumulation can have adverse effects on cells [19]. We adapted the DCFH-DA assay to determine ROS levels, with a lower fluorescence intensity indicative of fewer ROS. The results showed that there was a significant increase ($P < 0.05$) in ROS levels in the AAPH group (Fig. 3), compared with that in the control group, positive control group (Trolox), and groups pretreated with different concentrations of the peptides.

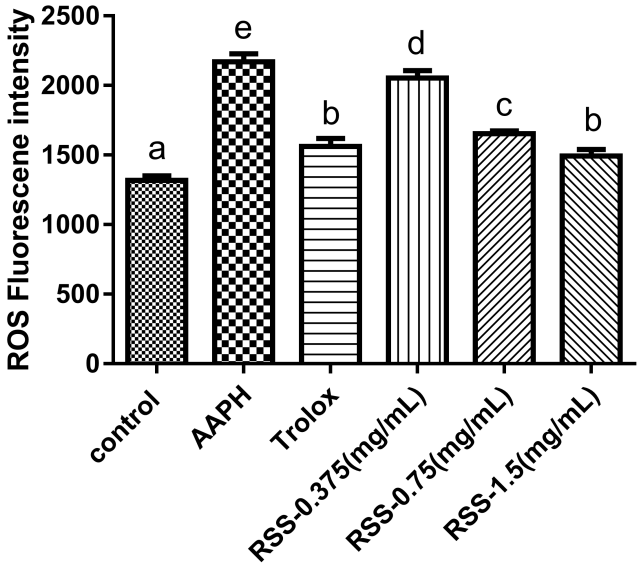

Figure 3 Reactive oxygen species (ROS) fluorescence intensities of RSS. Thedata marked by different letters are significantly different ($P < 0.05$). Data are presented as the mean ± standard deviation.

The ROS fluorescence intensity of the RSS-treated group at 1.5 mg/mL had no significant difference with Trolox, indicated that RSS was effective in protecting cells from oxidative damage as an antioxidant. Results have been reported by Wang et al. [20], wherein antioxidant peptides were found to protect HepG2 cells from oxidative damage by scavenging ROS.

3.4. Effect of the RSS on MDA, GSH and GSSG

MDA is a marker of cell membrane damage [16]. We found that with an increase in the concentration of the peptides used to treat cells, there was a concomitant decrease in the MDA content in the cells. Pre-treatment with 1.5-mg/mL of RSS resulted in low MDA content compared with that in the AAPH alone group. The 1.5-mg/mL RSS treatment group showed the lowest MDA content among the RSS groups and was similar to that in the Trolox control group. Together, these results indicate that all peptides show a protective effect against AAPH-induced cell membrane damage (Fig. 4A). Similar findings were obtained by Wu et al., where peptide treatment significantly reduced the increase in MDA contents. [16].

Depletion of GSH reflects a cellular non-enzymatic antioxidant defense mechanism, after which generating GSSG [16]. In our study, results of cells treated with AAPH and the control group showed that oxidative stress changes the GSH and GSSG levels. A comparison of RSS at 1.5 mg/mL and control groups showed no significant difference, whereas the AAPH group was significantly decreased. It indicated that 1.5mg/mL of RSS treatment group was in a normal status regarding the level of GSSG(Fig. 4B). As shown in Figure 4C, the level of GSH in the 1.5 mg/mL of RSS was lower than that of in

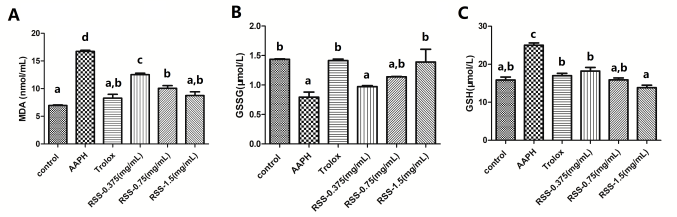

Figure 4 Concentration of (A) MDA, (B) GSSG , and (C) GSH inHepG2 cells after the indicated different treatments. Values shown are the mean± standard deviation, n = 4. Different letters indicate statisticallysignificant differences (P < 0.05) among the different treatments.

AAPH group, which was no significant difference compared with control group. It means that the GSH was not consumed under 1.5 mg/mL of RSS treatment. Accordingly, it can be concluded that at a concentration of 1.5 mg/mL, the cell membrance protection of RSS did not exert by GSH depletion.

3.5. Effect of the peptides on antioxidant enzymes, Nrf2, and Keap1

When cells undergo free radical attack, the Keap1 molecule is disrupted, leading to the dissociation of Nrf2 from Keap1.They initiated regulatory mechanisms involving phase II xenobiotic detoxification enzymes (e.g. SOD, CAT, and GSH-Px included) to degrade free radicals [21].

Cells treated with AAPH showed increased activities of SOD, CAT, and GSH-Px, indicating that the cells were subjected to oxidative stress, because they showed enhanced antioxidant enzyme activities to maintain cell homeostasis. In the RSS-treated groups, the activities of SOD, CAT, and GSH-Px were found to decrease (P < 0.05; Fig. 5).

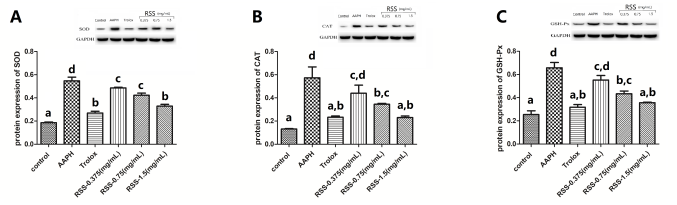

Figure 5 Effect of peptides onthe antioxidant enzymes in HepG2 cells. (A) SOD, (B) CAT, and (C) GSH-Px protein expression levels relative to the level of GAPDH after the different indicatedtreatments of HepG2 cells. Data are expressed as the mean ± standard deviation.Different letters indicate significant differences (P < 0.05).

These results again demonstrated that at 1.5 mg/mL, RSS exhibits similar antioxidant capacities to Trolox. One report showed that treatment with antioxidants results in a cytoprotective effect under oxidative stress in HepG2 cells. Decreasing SOD level was consistent with our results [22]. However, the cellular antioxidant system depends on the quenching of $O^{2\bullet-}$ by SOD and further protection by CAT and GSH-Px to convert H_2O_2 to H_2O, indicating that CAT and GSH-Px levels are related to SOD levels [23]. Therefore, it can be inferred that pretreatment with RSS resulted in effective control of HepG2 cells antioxidant defense systems as indicated by the lower SOD, CAT, and GSH-Px levels, which were maintained at normal levels.

Furthermore, treating cells with the RSS affected the protein expression levels of Nrf2 and Keap1 (Fig. 6). In the AAPH alone group, the level of Nrf2 was highest, whereas the level of Keap1 was lowest. The electrically sensitive cysteine structure of Keap1 is easily affected due to free radical-induced damage [24]. Keap1 protein levels were not reduced in AAPH-treated HepG2 cells that were pre-treated with RSS and Trolox, indicating that RSS could partially protect Keap1 from free radical-induced damage.

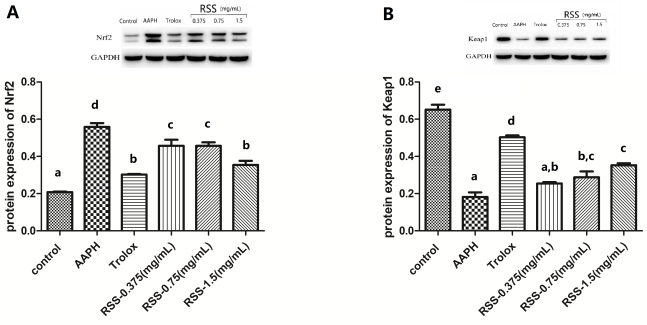

Figure 6 Proteinexpression by western blotting analysis. Protein expression of (A) Nrf2, (B)Keap1 in HepG2 cells after the different indicated treatments. Expression wasdetermined relative to that of GAPDH. Data are expressed as the mean ± standarddeviation. Different letters indicate significant differences (P <0.05).

Based on these observations, the free radicals were scavenged by RSS pre-treatment, leading to maintenance of the Nrf2/Keap1 complex and less Nrf2 proteins, consequently resulting in low protein expression of SOD, CAT, and GSH-Px. Similarly, some antioxidant acted as a directly free radical scavenger to protect the cell which maintain the anti-oxidative enzyme in a normal status [9]. Therefore, the anti-oxidant effect of RSS may be not dominated by SOD, CAT, and GSH-Px, but RSS had free radical scavenging ability to maintain the HepG2 cells at a stable stage.

3.6. Electronic-related properties and active site prediction

The energy of HOMO (E_{HOMO}) is the highest occupied orbital energy [25]. However, the higher the E_{HOMO} value, the stronger the ability of a molecule to give electrons to an electron-poor species [26]. It has been reported that the E_{HOMO} can characterize the potential for electron donation; moreover, an electron removed from a frontier molecular orbital HOMO of the parent molecules gives rise to radical cation species, and thus, the E_{HOMO} can also be used to characterize antioxidant potential [14]. The E_{HOMO} of RSS was −5.86296 eV, which is considered good antioxidant capacity; this result is consistent with that obtained by Alaşalvar et al. [27].

Qualitative data for identifying a molecule's active site for radical scavenging activity can be derived from the HOMO composition,

due to the H abstraction reaction involving electron transfer [12]. C, N, and O, electronegative atoms, can attract electrons. The molecular fragments C-H, N-H, and O-H release hydrogen atoms, forming a proton, and a new hydrogen bond will attract another electronegative atom. Therefore, the hydrogen bond donor was C-H, N-H, and O-H, whereas the acceptor was an electron-rich species (an atom or anion) [28]. The formation of resonance-stable radicals and intra-molecular H-bonding from hydrogen bond donors facilitates a higher level of antioxidant activity. A previous study indicated that hydrogen bonds comprised an antioxidant parameter that could be assessed [29].

The HOMO was distributed on the guanidinium group of arginine (**Figure 7B**). As shown in **Table 1**, the highest HOMO contribution rate in RSS was on 2N. The most probable H-atom donation areas in the active site were at N_2-H_3. Furthermore, H is readily removed on account of the conjugated system between the guanidine double bond and the isolated electron pair of nitrogen [30]. Arginine and its derivatives can regulate peroxidation processes in membranes, owing to the cationic properties of the guanidinium group, which has the ability to undergo protonation [31]. In brief, the antioxidant properties of RSS are related to their structural characteristics for scavenging free radicals, because of the ability of the guanidinium group to act as an H donor. Therefore, it can be speculated N_2-H_3 was the active site in RSS.

Table 1 Arg-Ser-Ser (RSS) highest occupied molecular orbital (HOMO) contribution rate

Atom Number	Atom	Rate	Atom Number	Atom	Rate
1	C	9.860549%	25	C	0.000466%
2	N	47.077551%	26	H	0.000028%
3	H	3.472728%	27	H	0.000231%
4	N	14.299987%	28	O	0.000094%
5	N	16.955031%	29	H	0.000011%
6	C	1.837789%	30	C	0.000130%
7	H	0.537511%	31	O	0.000074%
8	H	1.293938%	32	N	0.000366%
9	C	0.411392%	33	C	0.000070%
10	H	0.108710%	34	H	0.000027%
11	H	0.035174%	35	C	0.000009%
12	C	0.087692%	36	H	0.000007%
13	H	0.017169%	37	H	0.000003%
14	H	0.032999%	38	O	0.000001%
15	C	0.024121%	39	H	0.000000%
16	H	0.005697%	40	C	0.000006%
17	N	0.020094%	41	O	0.000005%
18	H	0.002014%	42	O	0.000003%
19	H	0.001664%	43	H	0.000000%
20	C	0.014675%	44	H	1.065493%
21	O	0.025860%	45	H	1.373216%
22	N	0.005554%	46	H	1.428993%
23	C	0.001228%	47	H	0.000844%
24	H	0.000271%	48	H	0.000527%

3.7. Active site verification

To verify the active site, N_2-H_3, involved in RSS cytoprotective effects, the methylation of RSS was employed to observe whether it lost a free radical scavenging ability, after its N_2-H_3 was substituted (**Figure 7C**). As shown in **Figure 7D**, the ROS level in the AAPH group increased significantly ($P < 0.05$) compared with that in the control group, indicating enhanced oxidative stress. Furthermore, the ROS fluorescence value increased with time upon treatment with methylated RSS, indicating an increase in the free radical content, similar to that observed after AAPH treatment alone. Thus, the quenching ability of free radicals by RSS (methylated) decreased, indicating that this active site was N_2-H_3 in RSS.

4. CONCLUSION

In this study, we demonstrated that peptide RSS characterized from edible Dendrobium aphyllum could protect HepG2 cells from oxidative stress. There are some evidence to prove that the RSS antioxidant mechanism is through the scavenging of free radicals directly in HepG2 cells. As we mentioned in the introduction, antioxidant mechanisms are divided into enzyme-dependent and enzyme-independent systems. Exogenous antioxidants can quench and reduce the accumulation of ROS in HepG2 cells [32]. Based on our findings, we propose that RSS can scavenge free radicals directly rather than dominated by SOD, CAT, GSH-Px, a enzyme-dependent systems (Fig. 7E). We also analyzed the free radical scavenging active sites of the peptide and showed that the active site of RSS was on N_2-H_3. Notably, the ROS content was increased after treatment with methylated RSS, wherein the active site was methylated, indicating that the N_2-H_3 active site is crucial for the antioxidant properties of RSS. Thus, our study provides information about the structure–activity relationship between the peptide and its antioxidant ability, and our findings lay the basis for further investigations on antioxidant peptides. We further anticipate that elucidating the structures of RSS peptides can make a meaningful contribution to the field of functional food and food antioxidants.

5. FUNDING

This study was funded by Guangdong Provincial Key Laboratory of Lingnan Specialty Food Science and Technology (Grant No. 2021B1212040013), the Fundamental and Applied Basic Research Fund for Young Scholars of Guangdong Province (Grant no. 2019A1515011283), Guangzhou Science and Technology Commissioner Project (Project No.GZKTP201937).

6. ABBREVIATIONS

Arg-Ser-Ser(RSS); 2,2-azobis(2-methylpropanimidamidine) dihydrochloride (AAPH); Reactive oxygen species (ROS); superoxide dismutase (SOD); glutathione peroxidase (GSH-Px); catalase (CAT); glutathione (GSH); density functional theory (DFT); highest occupied molecular orbital (HOMO) ; malondialdehyde (MDA); human hepatoma cells (HepG2) ; dichlorofluorescin-diacetate (DCFH-DA); phosphate-buffered saline (PBS).

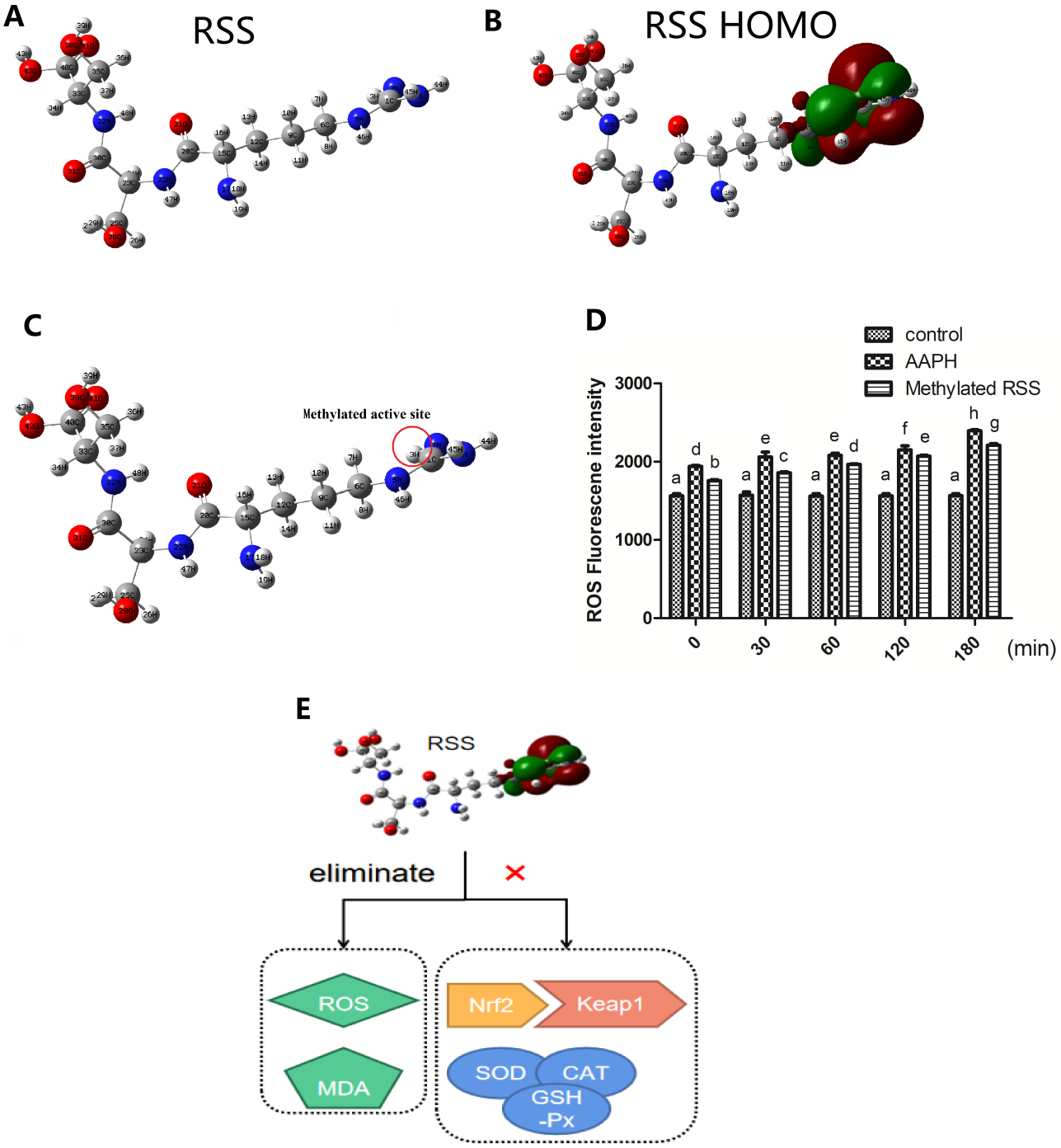

Figure 7 Electronic-related properties of Arg-Ser-Ser (RSS). (A) Peptide structure of RSS. (B) Highest occupied molecular orbital (HOMO)distribution of RSS. Confirmation of Arg-Ser-Ser (RSS)active sites. (C) Methylated active site of RSS. (D) Reactive oxygen species (ROS) scavenging capacity of methylated RSS after different incubation time points under conditions of2,2-azobis(2 methylpropanimidamidine) dihydrochloride (AAPH)-induced oxidative stress in HepG2 cells. The data marked by different letters are significantly different ($P < 0.05$). Data are presented as the mean ± standard deviation, n = 4. (E)The possible cytoprotective mechanism of Arg-Ser-Ser(RSS).

7. CONFLICT OF INTEREST

There are no conflicts to declare.

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