Biomacromol. J., Vol. 5, No. 2, 129-139, December 2019.

Biomacromolecular Journal
www.bmmj.org

Lack of Antioxidant Effect of Selenium on Interaction of Methyl Tert-butyl Ether (MTBE) with Cytochrome c

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(Received 16 March 2020, Accepted 6 July 2020)

ABSTRACT

Antioxidants are of great importance because they can protect the body from oxidation agents. Reactive oxygen species (ROS) are constantly producing as a result of metabolic processes. However, environmental factors such as methyl tert-butyl ether (MTBE) can enhance oxidation stress. MTBE is a widely used fuel oxygenation liquid that has been shown to cause potential cancer. The use of antioxidants may help to reduce the oxidation condition caused by MTBE. Selenium is an essential element with antioxidant property. In this study, the interaction between Cyt C and MTBE has been investigated in the absence and presence of Se. Molecular level examinations are extremely important to investigate the structural change of proteins by MTBE. In this research, molecular behaviour of Cyt c as an important model of protein, in the presence and absence of MTBE and Se is detected by biophysical methods such as UV-Vis, fluorescence and FTIR spectroscopy, chemiluminescence and molecular docking. The results showed that MTBE is able to alter Cyt c tertiary structure by ROS production with no change in secondary structure and Se could not protect the protein from structural perturbation. Molecular docking was also confirmed by the prominent interaction of Cyt c with MTBE and Se at a different site. Then Se can cause the structural change of Cyt c.

Keywords: Selenium, Reactive oxygen species, Antioxidants, Cytochrome c, Methyl tert-butyl ether

INTRODUCTION

Production of reactive oxygen species (ROS) normally occurs in the body, and the immune system balances the condition, and no harms threaten the healthy body. However, the imbalance of ROS production and deletion causes the appearance of high ROS amount and disease comes out [1,2]. The surrounding environment is affected by the presence of tremendous factors including Methyl tert-butyl ether (MTBE) which is largely the result of ROS production as approved by researches [3-5]. MTBE is a colourless water soluble liquid that is mostly added to gasoline. Fuel oxygenation is the major cause of MTBE usage. This property results in better fuel combustion and less carbon monoxide generation. People who work at gas stations and police officers are the main group exposed to MTBE exposure danger because it can easily evaporate and enter the breath system. Human exposure to MTBE is also possible in different ways. MTBE leaking to groundwater may cause great pollution as a result of its high water miscibility [4-6]. Different experimental reports have shown the adverse effects of MTBE. It disrupts cell growth [7], influences the reproductive system [8-10] and damages DNA [11]. All these effects have been caused by ROS production in the presence of MTBE [12]. Although MTBE has not yet been determined to be human carcinogenic, clinical studies have shown its adverse effect on the human central nervous system (CNS) such as lightheadedness, headache and respiratory problems [13-16]. Contrary to various *in vivo* and cell line studies, there is a lack of molecular level information about MTBE and protein interactions. MTBE can easily enter the bloodstream and

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interact with different proteins and alter protein structure and function as previous research has been confirmed [3,4]. However, more experiments need to be done to understand the effect better. Here we candidate cytochrome c (Cyt c) as a protein model to detect MTBE induced impact on the protein. Cyt c is a small globular metalloprotein. It contains a covalently bound heme prosthetic group which results in a highly stable protein structure. Different significant functions are related to Cyt c. It is known as an electron carrier protein and triggers cellular respiration since it is located in the mitochondrial membrane space. Furthermore, Cyt c plays an important role in the planned death of the cell (apoptosis) by ROS production. Normal ROS production activates signalling pathways, but excessive production causes destructive consequences in the cell, including protein oxidation, mitochondrial malfunction and DNA damages [17-19]. Besides these noticeable roles, Cyt c has a conserved 3D conformation which makes it a suitable model protein for various studies, specially protein-ligand interactions.

As an essential element, Se plays an important role in antioxidant proteins like selenoproteins, glutathione peroxidase and thioredoxin reductase. Different selenoproteins are known in humans who further identify Se importance in the body. Based on studies, there is an association between low levels of Se with immune system defects, cancer, reproduction potency and mortality risk [20-22]. The antioxidative property of Se makes it an interesting case study especially in ROS generating problems like cancer and immunity diseases. However, there is a need to investigate further the molecular behaviour of antioxidants, such as Se to clarify their general effectiveness. Since MTBE is a ROS producer agent, we have attempted to investigate its effects on cytochrome C in the presence and absence of Se.

MATERIALS AND METHODS

Materials

Cyt c from the bovine heart (95%) and sodium selenite pentahydrate 98% ($Na_2SeO_3.5H_2O$) were purchased from sigma. MTBE, phosphate buffer salts (K_2HPO_4 and HCl), H_2O_2 30% (standardized at 240 nm by UV-Vis spectroscopy) and Luminol (5-amino-2,3-dihydro-1,4-phthalazine), were obtained from Merck. All experimental solutions were prepared by the use of distilled water.

Method

UV-Vis spectrophotometry. UV-Vis spectra of all absorption measurements were gathered using Cary Series UV-Vis Spectrophotometer (Agilent Technology). Spectra were recorded from 235 to 295 nm and 350 to 450 nm by use of 1 cm path length quartz cell. Cyt c solutions were prepared in 50 mM potassium phosphate buffer and pH 7.4. The measurements were done in the presence and absence of MTBE and Se at 25 °C. Protein concentration in all experiments was 0.05 µM.

ATR-FTIR measurements. To increase FTIR (fourier transform infrared spectroscopy) measurements accuracy, ATR (attenuated total reflectance) is used. ATR is an accessory in FTIR to eliminate the need for pellet preparation for analysis. ATR-FTIR data was collected by Thermo Nicolet Fourier Transform Infrared Spectrophotometer (Thermo Fisher Scientific, USA). In this experiment, the desired amount of MTBE was added to Cyt c solution in the presence and absence of Se.

Fluorescence spectroscopy. Cyt c intrinsic fluorescence was measured by Cary Eclipse Spectrofluorometer (Varian, Australia). At first Cyt c samples were prepared by the concentration of 0.05 µM in 50 mM phosphate buffer (ph 7.4 and 25 °C). Then MTBE was titrated to protein solutions, and samples emission was recorded between 300-500 nm. Finally, the measurements were done in the presence of desired Cyt c- MTBE- Se concentration. Samples were excited at 280 nm.

Chemiluminescence Assay

In order to detect the level of reactive oxygen species (ROS), the chemiluminescence spectroscope (model Synergy H4 Hybrid Multi-Mode Plate Reader: BioTek Instrument, USA) was applied. The amount of ROS generated upon the interaction of Cyt c with MTBE was measured before and after Se addition. 50 mMluminol was solubilized in the phosphate buffer, and 5 µl of this stock was added to all solutions in the plate. The amount of samples emission was recorded at 430 nm. H_2O_2 standard curve was used to calculate the final level of reactive oxygen species. The emission data were repeated at least three times.

Molecular Docking

To investigate the binding sites of Cyt c in the presence of MTBE and Se (sodium selenite pentahydrate), molecular docking was performed, using AutoDock 4.2. The PDB file of Cyt c (pdb id code: 2B4Z) was extracted from RCSB protein data bank (http://rcsb.org/). SDF files of Se (PubChem CID: 134930) and MTBE (PubChem CID: 15413) were obtained from PubChem database (http://pubchem.ncbi.nlm.nih.gov/). Flexible- ligand docking studies were carried out by using AutoDock 4.2. In order to find potential binding sites Cyt c, a sufficiently large grid box size was chosen to include the whole protein molecule. The points of the grids were 40 Å × 35 Å × 40 Å (x, y and z) with a grid spacing of 1 Å through AutoDockVina. For the docking process, the 'number of the generation' was reduced to 200 conformers for both ligands. A structure with the lowest energy content and the best binding site was selected. All the molecular images and animations were produced using MGLTools and discovery studio software. Hydrogen bonds and hydrophobic interaction sites were measured with its binding partner using discovery studio and ligand scout software.

RESULT AND DISCUSSION

Based on recently published articles, Se displays numerous biological roles including ROS scavenging, being a cofactor of selenoproteins, contribution to thyroid hormone production and anti-inflammatory effects. As a natural antioxidant, Se has been suggested to ameliorate oxidative conditions in renal ischemia-reperfusion injuries in rats [23]. Experiments were also demonstrated that Se is able to alleviate hepatotoxicity induced by bisphenol A in rats. Se administration could modulate the liver disorder by increasing of some enzymes in the liver such as glutathione peroxidase and catalase. These enzymes are involved in oxidative processes. It means that Se is able to induce reactions involved in free radicals scavenging [24]. A review on Se-dependent antioxidant enzymes has explained thoroughly about selenoproteins and the importance of Se intake on dietary for their synthesis and function. Selenoproteins are involved in antioxidative status, responses of the body immune system and many other functions [25]. Se not only has been shown to have antioxidant behaviour *in vivo* experiments but also revealed a protective effect on cadmium-induced oxidative stress in plants [26]. Antioxidation property is attributed to a compound or element relied on repeatable experiments, but it is also possible to encounter contradictory behaviour as reported in some studies. In the case of sodium selenite, it is mentioned to induce cell toxicity and ROS production, which depends on experiment condition such as Se concentration [27,28,29]. Also, curcumin showed lack of antioxidation property in hemoglobin- MTBE solution [30]. Since there is a lack of in vitro molecular level studies on Se, our purpose was to detect the effect of Se in protein-MTBE solution (ROS condition).

Absorbance Measurements

The UV-Vis spectroscopy results at 280 and 410 nm are shown in Fig. 1 and Fig. 2, respectively. Figure 1 shows that the absorbance of Cyt c at 280 nm is increasing gradually by MTBE titration. Absorbance at 280 nm stands for protein aromatic residues meant Trp, Tyr and Phe. As reporter elements, these residues give us information about protein environment. The increase at 280 nm represents for Cyt c environment alterations.

Figure 2 indicates the decrease in soret band absorption occurs at 410 nm. This absorption is related to Cyt c heme prosthetic group. The data shows a sensible decrease at 410 nm. Soret band absorption spectra provide us with useful information about heme iron oxidation and ligand bindings to the protein [31]. Result of Fig. 2 is evidence of Cyt C tertiary structure changes in the presence of MTBE. It has been shown that the decrease in wavelength around 410 nm is due to the loss of interaction between the heme and the globin, which results in tertiary structure change [4].

Selenium effect on Cyt C conformation was examined by UV-Vis spectroscopy. It is shown that Se alone can influence Cyt C tertiary structure by exposing heme group to the solvent. This is supported by the result presented in Fig. 3. The effect of Se on the protein was also detected after MTBE addition. This effect was examined in the presence of 0.05 μM MTBE as a selective concentration. As mentioned before Se is known to be a protective element in oxidative processes [20]. However, no protection was seen in the presence of Se. Cyt C absorption was deduced at 410 nm in the presence of Se and MTBE. Thus heme- globin interaction was further reduced in the presence of Se after MTBE addition (Fig. 3). It means that Se not only

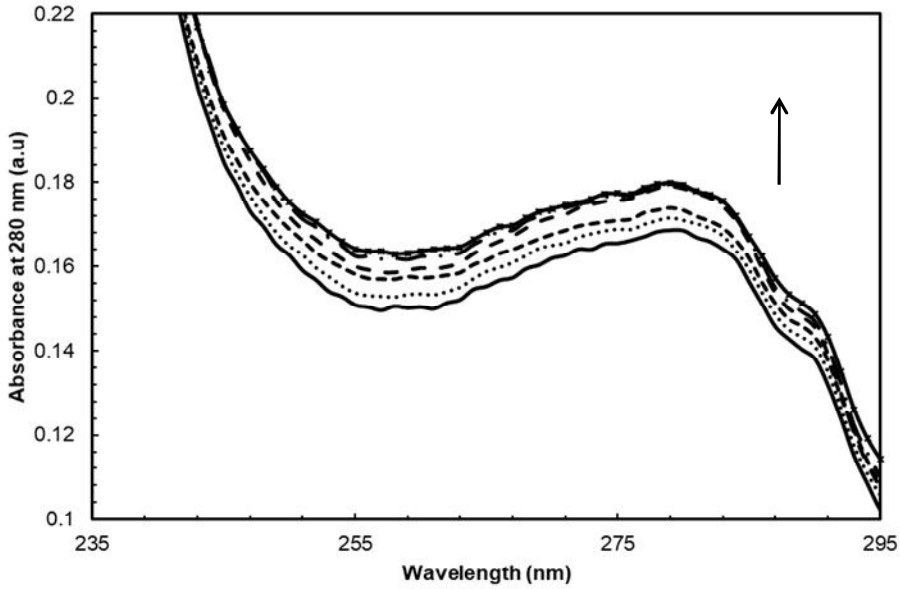

Fig. 1. UV-Vis spectra of Cyt C in the presence of different MTBE concentrations (0.05, 0.1, 0.15, 0.2 and 0.25 µM) at 25 °C. Samples were prepared in phosphate buffer 50 mM, pH 7.4. Spectra were gathered at 280 nm. (—) Cyt c; (…) Cyt c + 0.05 µM MTBE; (---) Cyt c + 0.1 µM MTBE; (- - -) Cyt c + 0.15 µM MTBE; (— · —) Cyt c + 0.2 µM MTBE and (—•—) Cyt c + 0.25 µM MTBE.

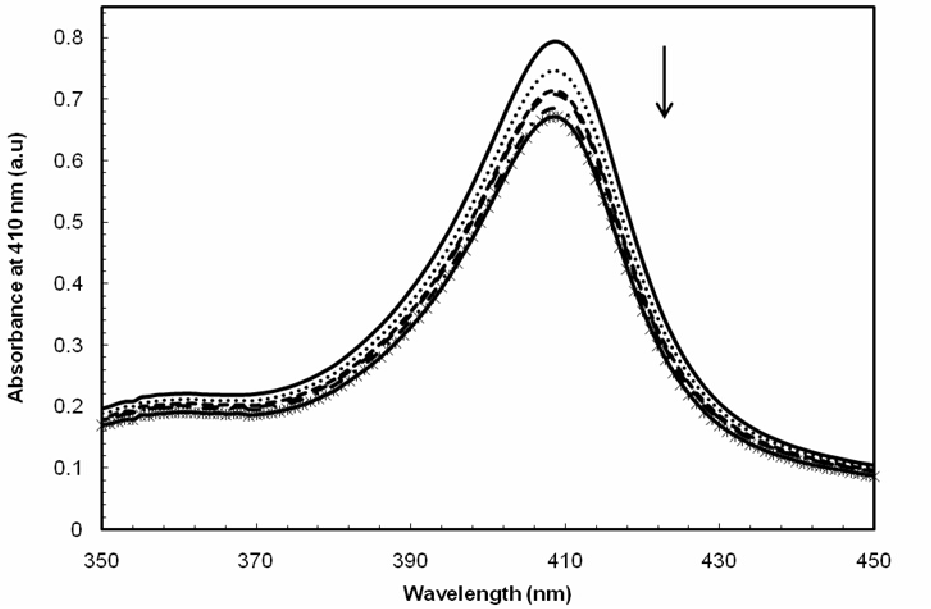

Fig. 2. Soret band spectra of Cyt C in the presence of different MTBE concentrations (0.05, 0.1, 0.15, 0.2 and 0.25 µM). Samples were prepared in phosphate buffer 50 mM, pH 7.4 and 25°C. Spectra were collected at 410 nm. . (—) Cyt c; (…) Cyt c + 0.05 µM MTBE; (---) Cyt c + 0.1 µM MTBE; (- - -) Cyt c + 0.15 µM MTBE; (— · —) Cyt c + 0.2 µM MTBE and (—•—) Cyt c + 0.25 µM MTBE.

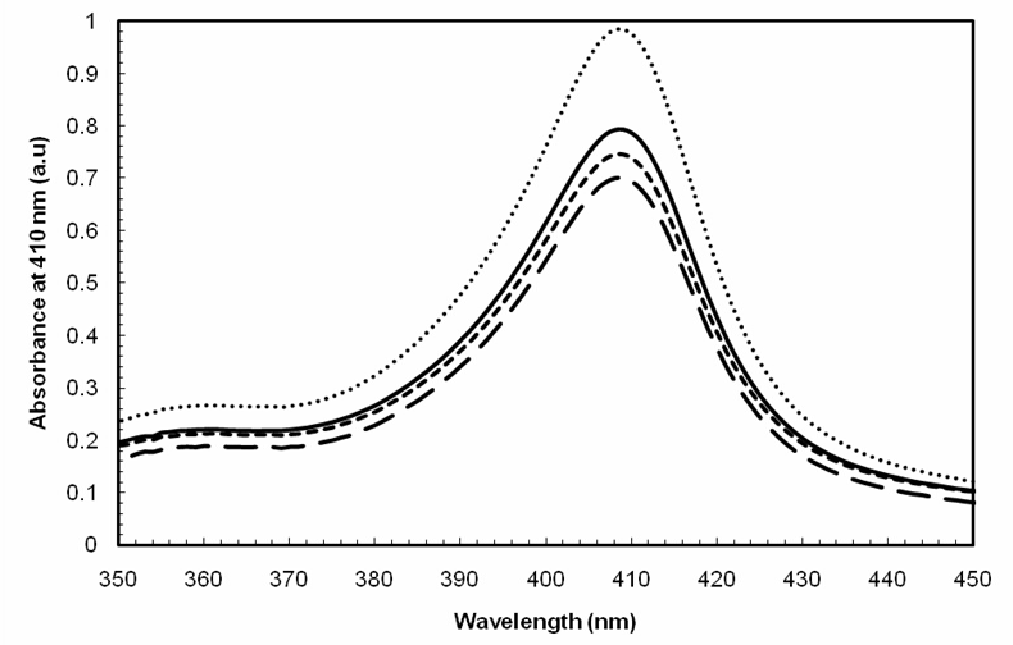

Fig. 3. Soret band absorption (410 nm) of Cyt C in the presence of 0.05 µM MTBE and 0.4 µM Se. Experiment was done at 25 °C using 50 mM phosphate buffer pH 7.4. (—) Cyt c; (…) Cyt c + 0.4 µM Se; (---) Cyt c + 0.05 µM MTBE; (——) Cyt c + 0.4 µM Se + 0.05 µM MTBE.

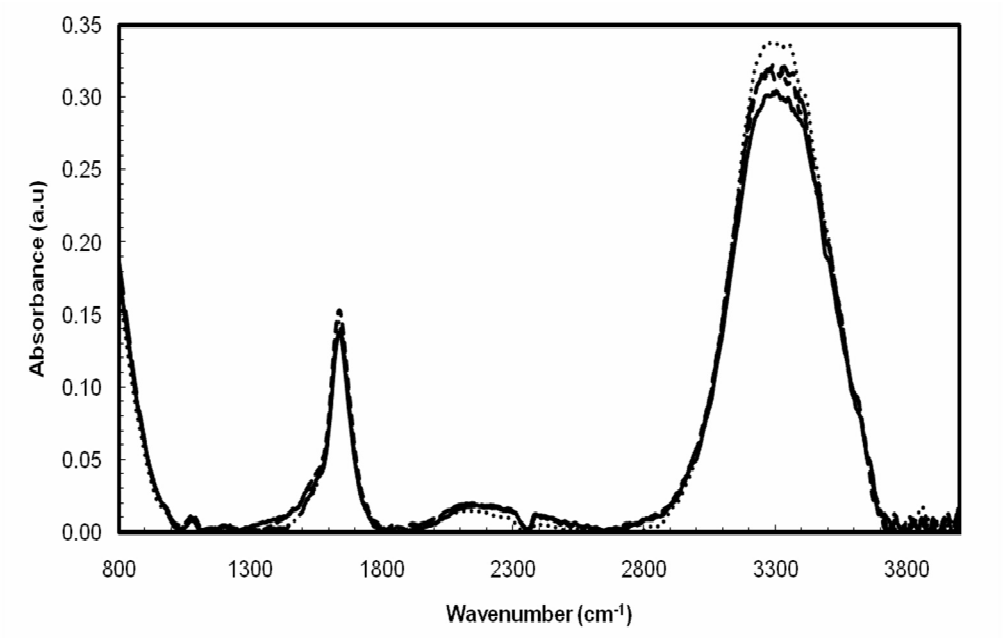

Fig. 4. ATR-FTIR spectra of Cyt c in the absence and presence of MTBE and Se. Samples spectra were measured at 25 °C in 50 mM phosphate buffer pH 7.4. (—) Cyt c; (---) Cyt c + 0.05 µM MTBE; (…) Cyt c + 0.4 µM Se; (——) Cyt c + 0.4 µM Se + 0.05 µM MTBE.

alters Cyt C conformation but also amplify MTBE destructive effect on the protein.

Secondary Structure Examination

To study the effect of MTBE on Cyt C secondary structure in the presence and absence of Se, ATR-FTIR technique was applied. FTIR is a precious instrument for the study of macromolecule structures. The ability to detect amide I and II bands makes it suitable for protein secondary structure detection. There is absorption near 1650 cm^{-1}, known as amide I band, emerges mainly from stretching of the C=O bond. Amide II is a band characterized mostly by absorption around 1550 cm^{-1}. It arises from N-H bending vibrations affected by neighboring groups (CO, CC and NC groups). Since both C═O and N-H bonds are involved in hydrogen bond formation, their vibration modes (amide I and amide II) could make useful information about the secondary structure of the proteins. Amide I band is generally used for protein secondary structure examinations, ranged from 1600-1699 cm^{-1} [32,33]. Figure 4 Represents the ATR-FTIR spectra of Cyt C before and after MTBE and Se addition. The data shows no significant changes in Cyt c secondary structure because there is not any wavelength shift in presence of MTBE and Se.

Fluorescence Measurements

For better conformation study, fluorescence measurements were done. Figure 5 shows the fluorescence intensity change of Cyt c after MTBE titration. Results show the decreasing of fluorescence intensity in the presence of MTBE. Among aromatic residues, Trp is the major fluorophore of intrinsic fluorescence in the protein structure (at 280 nm). The fluorescence intensity and maximum emission wavelength of Trp are extremely influenced by micro-environment polarity and consequent interactions. When protein hydrophobic patches are exposed to the solution, Trp fluorescence intensity (around 340 nm) may decrease, and emission wavelength shifts to the red region [34,35]. Therefore, reduction of Cyt c fluorescence intensity demonstrated in Fig. 5 is due to Trp exposure to polar solvent. It means that the protein tertiary structure is destructed by MTBE and its hydrophobic pockets are exposed to the polar solvent.

Se effect on Cyt c conformation in the presence of MTBE was also examined by spectrofluorometer. As shown in Fig. 6 fluorescence intensity of Cyt c is more decreased in the presence of MTBE and Se compared to Cyt c and MTBE solution. This data is in the line of previous results (Figs. 3 and 4) which show no protective effect of Se on Cyt c in the presence of MTBE. On the other hand, molecular docking results showed that both MTBE and Se are in interaction with Trp and also Tyr residues. The results are tabulated in Table 1. MTBE interacts with Trp 67 and Tyr 48. Se interacts with Trp 59 and Tyr 67. Since Trp is the main fluorophore of the proteins intrinsic fluorescence, the interaction of MTBE and Se can quench its fluorescence. Furthermore, MTBE and Se interact with different Trp residues. This may be the reason that Cyt c fluorescence intensity is a little bit more decreased in the presence of MTBE and Se, because more Trp residues are in interaction with MTBE and Se and consequently more quenching happens.

Chemiluminescence Experiments

Chemiluminescence experiments were done to detect the amount of ROS produced in the presence of MTBE and Se. As shown in Fig. 7, ROS was more produced after MTBE addition to the protein solution. ROS production was also more in protein-Se sample. The amount of ROS was the most when both Se and MTBE were added to the protein sample. Previous studies have confirmed the destructive effect of MTBE on proteins by ROS production [3,4]. Hence the effect of MTBE on Cyt c secondary and tertiary structure is due to ROS generation. Furthermore, the immune system preserves the body against ROS, but in the case of oxidative stress (more ROS generation and deficiency of immune system) different side effects can occur. As a result, Se could generate ROS, and the amount was more when MTBE was added to Se protein solution. This data is also in agreement with previous results and confirms the lack of Se protective effect on Cyt c in oxidative condition.

MTBE and Se produce more ROS in protein solution, and this can damage proteins and other macromolecules. Protein oxidation can result from ROS accumulation in the body and cause the occurrence of conformational diseases such as Alzheimer's, Parkinson and Diabetes [36-38].

Molecular Docking Examinations

In order to investigate the binding site of Cyt c to MTBE

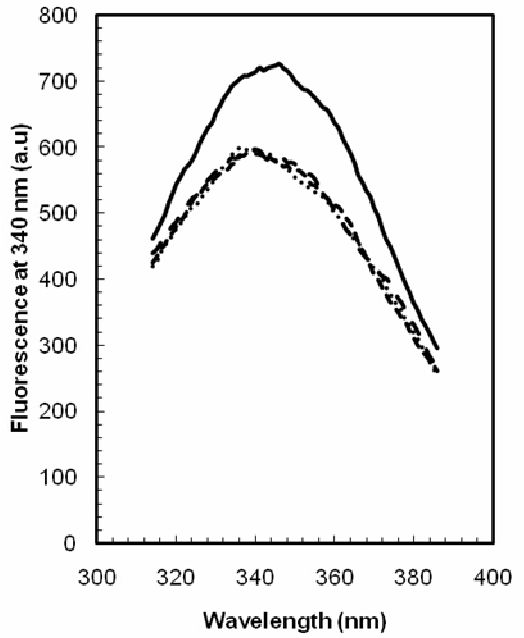

Fig. 5. Intrinsic fluorescence intensity of Cyt c in the presence of MTBE different concentrations prepared in Phosphate buffer 50 mM, pH 7.4 at 25 ºC. Excitation wavelength was set at 280 nm. (—) Cyt c; (…) Cyt c + 0.05 µM MTBE; (---) Cyt c + 0.1 µM MTBE; (- - -) Cyt c + 0.15 µM MTBE.

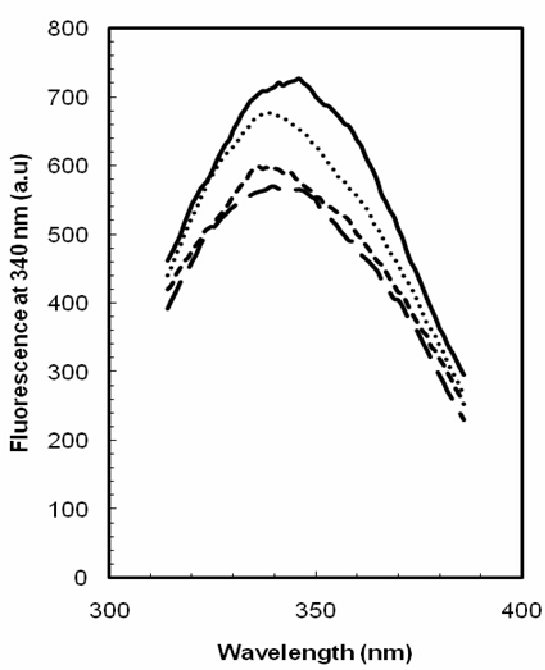

Fig. 6. Intrinsic fluorescence measurements of Cyt c in the presence of 0.4 µM Se and 0.05 µM MTBE dissolved in phosphate buffer 50 mM, pH 7.4 at 25ºC. Excitation wavelength was 280 nm. (—) Cyt c; (---) Cyt c + 0.05 µM MTBE; (…) Cyt c + 0.4 µM Se; (——) Cyt c + 0.4 µM Se + 0.05 µM MTBE.

Table 1. The List of Residues Involved in Hydrogen Bonds and Hydrophobic Interactions of Protein-MTBE-Se

Complex	Interaction sites
Protein-MTBe	His 18, Pro 30, Leu 32, Leu 35, Gly 41, Tyr 48, Asn 52, Trp 59, Tyr 67
Protein-MTBE-Se	His 18, Leu 32, Leu 35, Asn 52, Trp 59, Leu 64, Tyr 67

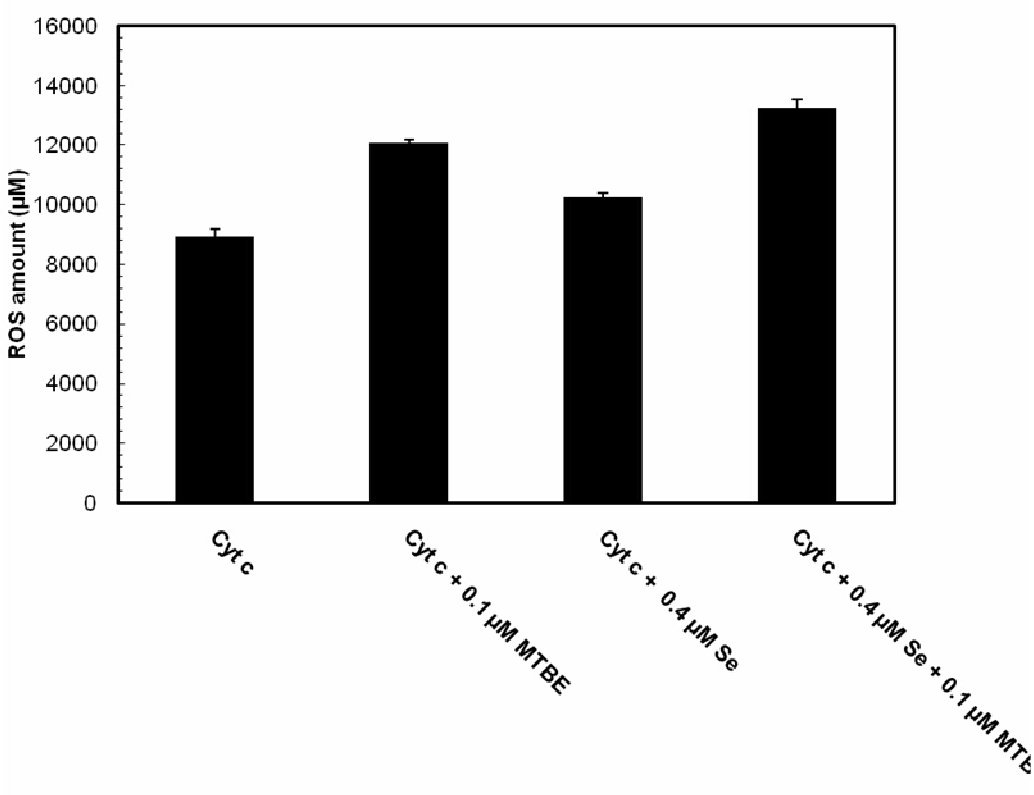

Fig. 7. ROS generation of Cyt c solution in the presence and absence of 0.4 µM Se and 0.05 µM MTBE. Samples were prepared in phosphate buffer 50 mM, pH 7.4 and measurements were done at 25 ºC.

in the presence and absence of Se, molecular docking was performed. The low energy binding site with the best fits MTBE and Se are shown in Figs. 8 and 9, respectively. As illustrated in Figs. 8 and 9, the following residues are in interaction with MTBE: His 18, Pro 30, Leu 32, Leu 35, Gly 41, Tyr 48, Asn 52, Trp 59 and Tyr 67. The residues that are involved in Cyt c- Se interaction are His 18, Leu 32, Leu 35, Asn 52, Trp 59, Leu 64 and Tyr 67. When comparing these two bindings, it is clear that the three residues Pro 30, Gly 41 and Tyr 48 are the main ones in Cyt c- MTBE binding which are not involved in Cyt c- Se binding instead the new Leu 64 binding site is

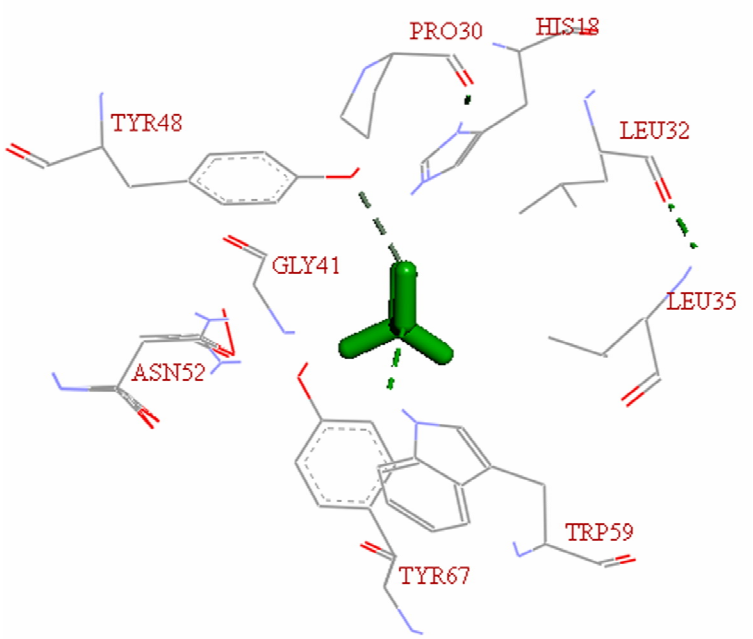

Fig. 8. Molecular docking image depicted Cyt c binding site for MTBE, using AutoDock 4.2.

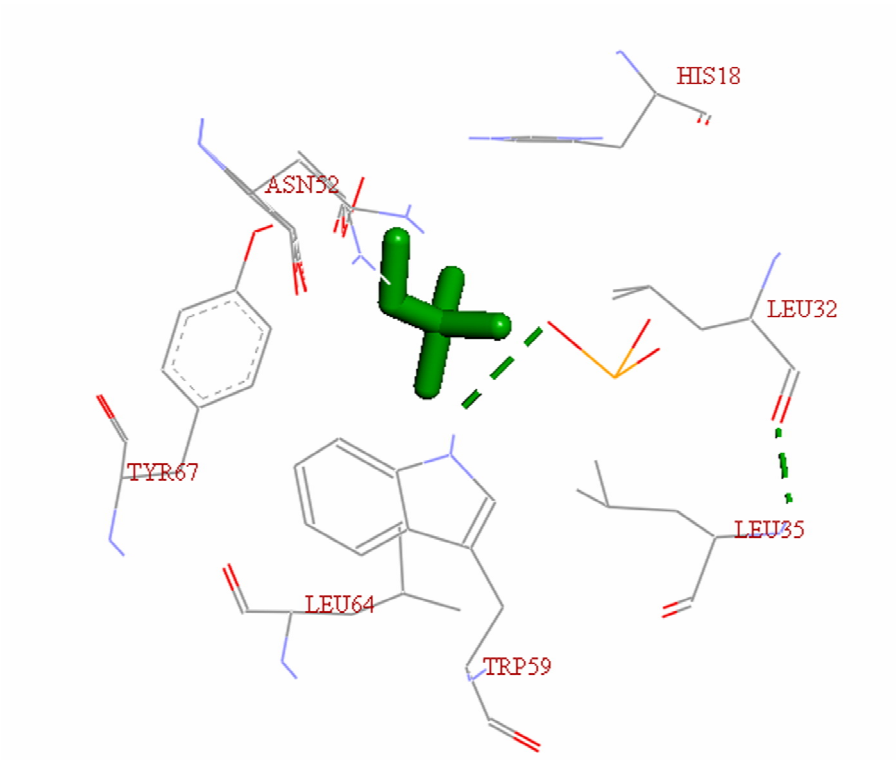

Fig. 9. Molecular docking image depicted Cyt c binding site for Se, using AutoDock 4.2.

available. These data indicate that Se is unable to lose Cyt c interaction with MTBE because Se binds to a different residue. So the Se and MTBE bind to the Cyt c and cause structural change. The examination was also showed that the interaction between MTBE, Se and Cyt c are hydrophobic and hydrogen bonds.

CONCLUSIONS

As a fuel additive, MTBE is extensively distributed in the environment, and several studies have confirmed the deleterious effect of MTBE on proteins. MTBE may influence proteins' secondary and tertiary structures. Cyt c conformation is altered in the presence of MTBE, which results from ROS generation. As the relation between protein structure and function is obvious then it can be concluded that Cyt c function may be disturbed by MTBE. Se as an antioxidant was applied to moderate MTBE side effects on Cyt c, but its antioxidation property was not efficient in this case. It is a good idea to make use of other antioxidants to see if they can help to decrease MTBE adverse effects.

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