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Article

Design and synthesis of novel peptides to protect ferulic acid against ultraviolet radiation based on domain site ⅡA of bovine serum albumin

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Abstract: Ferulic acid (FA) is known for its excellent antioxidant properties that can provide a lot of health benefits. One of its drawbacks is being unstable under UVA light that limits its potency. In this study, new peptides LW2 (QNKRFYFRKNQ) and CW2 (a cyclic form of LW2) have been designed based on bovine serum albumin site ⅡA conformation. UVA irradiation experiment was performed to investigate the protective ability of these peptides towards FA against UVA damage. The percentage of FA remaining under UV irradiation by the protection of CW2 and LW2 was 83%, 76% respectively. The results showed the importance of the cationic residues and hydrophobic residues included in the peptide sequences. Moreover, the cyclic rigid structure showed more protecting ability over that of the linear counterpart.

Keywords: Ferulic acid; UVA light; Cyclic peptide; Bovine serum albumin

1. Introduction

Ferulic acid (4-hydroxy-3-methoxycinnamic acid; FA) is a kind of phenolic compound, it exists in fruits, vegetables, and grains as free form or bonded to the cell wall [1]. FA has an excellent antioxidant capacity, showing many physiological functions such as anti-inflammatory, antioxidant, antimicrobial activity [2], anticancer [3], and antidiabetic effect [4]. Therefore, it has a wide range of applications as a food supplement, in many pharmaceutical formulations [5], and the cosmetics industry [6]. Nowadays, people are taking care of their health situations and they became aware of using antioxidants such as FA in their food regimen [5,7].

However, its applications are limited because of some unfavorable physicochemical properties such as autoxidation [8], poor water solubility, and light sensitivity. FA is extremely sensitive to ultraviolet (UV) rays, which is naturally existing as a part of sunlight. It has been reported that when FA was exposed to UVA, photo-oxidation happened and reactive oxygen species (ROS) were formed [9]. Those reactions may destroy the hydroxyl group attached to the benzene ring and the alkene group included in the FA structure [10]. Thus, all these drawbacks should be taken into consideration during the processes of formulation, storage, and transportation. To solve these problems, a suitable protective excipient for FA is usually considered an ideal strategy. Many materials have been investigated as an appropriate agent for such purposes as the hydroxypropyl-β-cyclodextrin [11], solid lipid nanoparticles [12], and gelatin [4].

But, using the above materials complexed with FA to form a stable complex, the over-consumer reagents and long-term stirring are always required. Proteins, which are major

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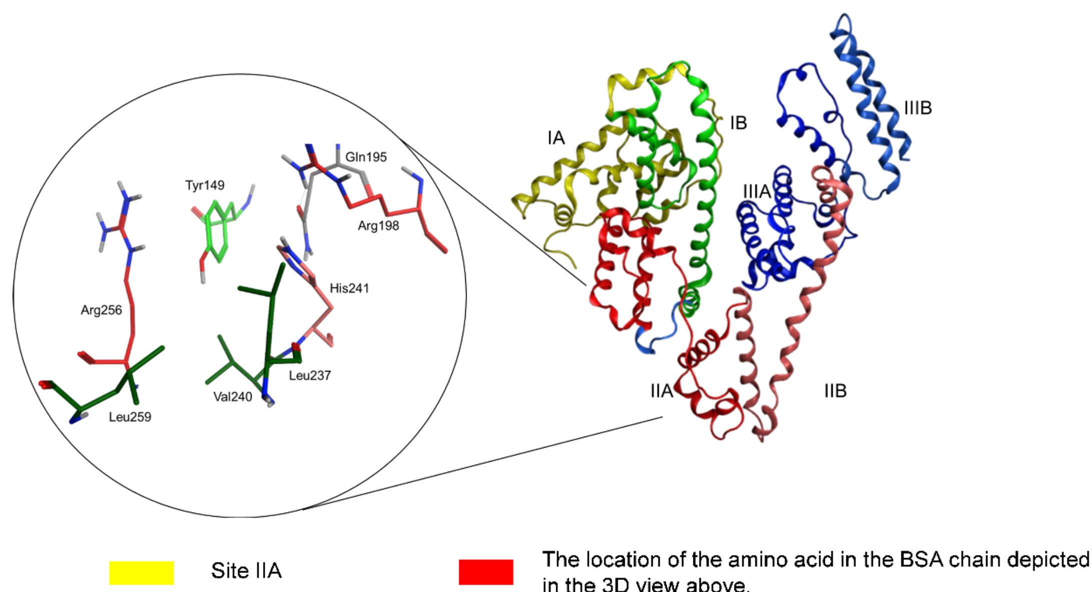
biomolecules, have a variety of functions, and one of these functions is a protective function. In a sense, proteins can be said to be mild materials with protective functions, because they are environmentally safe, and their structure naturally contains certain subdomains that allow target molecules to conveniently bound or covered in. Bovine serum albumin (BSA), which is often used as a protective protein, and its applications are diverse, ranging from protection of macroscopic to microscopic molecules. BSA is well regarded as a standard model protein *in vivo* carrier, and its active binding site has already been studied [13]. It has reported a high possibility of FA binds to BSA happened at subdomain site ⅡA of BSA due to the interaction forces including hydrogen bond, hydrophobic interactions, van der Waals force, and electrostatic force. As shown in Figure 1, the subdomain IIA structure of BSA including, Leu 237, Tyr 149, Arg 256, Glu 152, Ser 191, Tyr 156, Glu 291, Ala 290, Arg 198, Arg 217, and His 241, shaped a hydrophobic pocket. In a previous report, the main interaction force between BSA and FA was hydrogen bond donned by Arg 256 [14], and the hydrophobic amino acids strengthen these interactions by hydrophobic interaction forces [15]. The more interesting thing is that the damage of FA caused by UVA decreased after BSA has been added to the solution. In other words, BSA offered some protective support to FA against UVA in the solution. There is no doubt that BSA is an excellent carrier material, but BSA belongs to macromolecules and FA are small molecules, using BSA to protect FA is overkill.

Peptides are a general term for molecules consisting of short chains of amino acids connected by amide bond, and many peptides with various functions have been reported [16]. Among them, peptides with protective functions have also been reported [17], some protecting against protein aggregation and others protecting small molecules [18]. However, peptides in general have a linear structure and are easily degraded by proteases. On the other hand, cyclic peptides are expected to stabilize the steric structure and improve stability against enzymatic degradation. In this study, we designed a novel cyclic peptide CW2 based on the structure of BSA siteⅡA and evaluated the protective function of FA from UVA.



RDTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPFDEHVKLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLRETY
GDMADCCEKQEPERNECFLSHKDDSPDLPKLKPDPNTLCDEFKADEKKFWGKYLYEIARRHPYFYAPELLYYANKYNGVFQE
CCQAEDKGACLLPKIETMREKVLASSARQRLRCASIQKFGERALKAWSVARLSQKFPKAEFVEVTKLVTDLTKVHKECCHGD
LLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSFLYE
YSRRHPEYAVSVLLRLAKEYEATLEECCAKDDPHACYSTVFDKLKHLVDEPQNLIKQNCDQFEKLGEYGFQNALIVRYTRKVP
QVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTEDYLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKA
FDEKLFTFHADICTLPDTEKQIKKQTALVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEACFAVEGPKLVVSTQTALA

Figure 1. Domain structure of BSA site IIA, and full amino acids sequence of BSA. The heart-shaped BSA consists of three homologous α-helical domains (I, II, III) and each domain contains two subdomains (A and B).

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To mimic BSA site IIA structure, QNKRFYFRKNQ (CW2 or LW2) was engineered with four cationic residues (2 Arg and 2 Lys), four polar residues (2 Asn and 2 Gln), and three hydrophobic amino acids (2 Phe and Tyr). Two Arg were added to facilitate hydrogen bonding between peptides and FA. Simultaneously, two more Lys were applied to improve the electrostatic interaction. Furthermore, three hydrophobic amino acids, Phe-Tyr-Phe, were used to mimic BSA's hydrophobic field. Since CW2 and LW2 are both cationic peptides, we have created an anionic cyclic peptide CEG (EGWGEG) for contrast.

2. Materials and Methods

2.1 Materials

The calculated masses of CW2, protected LW2, deprotected LW2, and CEG were computationally determined as 1509.795, 3259.025, 1528.743, and 615.229, respectively, using ChemDraw 20.0 software. The preparation of linear peptide followed the standard Solid-Phase Peptide Synthesis method (SPPS) using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry and 2-chlorotrityl resin as the solid support [19]. All Fmoc-protected amino acids, resin, piperidine, O-benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole hydrate (HOBt·H_2O), N,N-diisopropylethylamine (DIEA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), and 2,2,2-trifluoroacetic acid (TFA) were purchased from Watanabe Chemical Industries, Ltd, Japan. Ferulic acid and other reagents were purchased from Wako Pure Chemical Industries, Ltd, Japan.

2.2 Synthetic procedure

A standard SPPS was used to synthesize those peptides: LW2, CW2, and CEG in Scheme1. The synthetic process started from loading 1 mmol Fmoc-glutamine amino acid onto 1.6 mmol 2-chlorotrityl chloride resin, adding 0.7 mL DIEA in 30 mL dichloromethane (DCM) for reaction, then rotated for 1 hour (all the reaction presented at room temperature, same as below). Adding 1 mL methanol to cover the unreacted sites on the resin, the reaction was continued by rotating for 30 min. The following sidechain protected amino acids were coupled to the loaded glutamine residue using 2 mmol HBTU, 0.7 mL of DIEA, and 2 mmol of HOBt in 30 mL of N, N-dimethylformamide (DMF) as a solvent for the coupling process. The coupling process was carried out for two hours. The Fmoc protecting groups were removed by 20% (v/v) piperidine in DMF (30 mL) which was accomplished in 30 minutes. The cleavage of the sidechain protected linear undecapeptide from the resin was performed using a mixture solution including 2,2,2-trifluoroethanol (TFE) (18 mL)/acetic acid (6 mL)/dichloromethane (DCM) (6 mL) at a ratio of 3:1:1 (v/v/v). The removal of the sidechain protecting group was carried out for two hours. Purification was carried out by using a semi-preparative RP-HPLC Hitachi L-7100 apparatus fortified with a Chromolith® performance RP-18e (4.6 × 100 mm, Merck, Germany). The mobile phases were acetonitrile containing 0.1% TFA (solvent B), and H_2O containing 0.1% TFA (solvent A). The elution gradient was 0-100% of solvent B in 30 min. The flow rate was adjusted to 1.300 mL/min at room temperature. The peak intensity was determined both at a wavelength of 220 nm. The removal of the side chain protecting groups was achieved using a mixture of TFA/trisisopropylsilane (TIS) at a ratio of 99:1 (v/v). Lyophilization was carried out in an FD-1000 freeze dryer (Tokyo Rikakikai, Japan). Cyclization reaction was carried out following a low concentration (0.5 mM) of LW2 which efficiently avoids dimer formation. 2 equiv. of EDCI·HCl, and 4 equiv. DIEA was used for the cyclization process. The removal of the sidechain protecting groups of CW2 was carried out as mentioned before. Purification was achieved by a semi-preparative RP-HPLC followed by lyophilization to yield the targeted pure final cyclic product [16]. CEG synthesized follow the same process. The synthetic process was elucidated by HPLC as shown in Figure S1-S3. All of the synthesized peptides were confirmed by Fast atom bombardment (FAB^+), and Time of flight (TOF) mass spectrometry and the masses were found as

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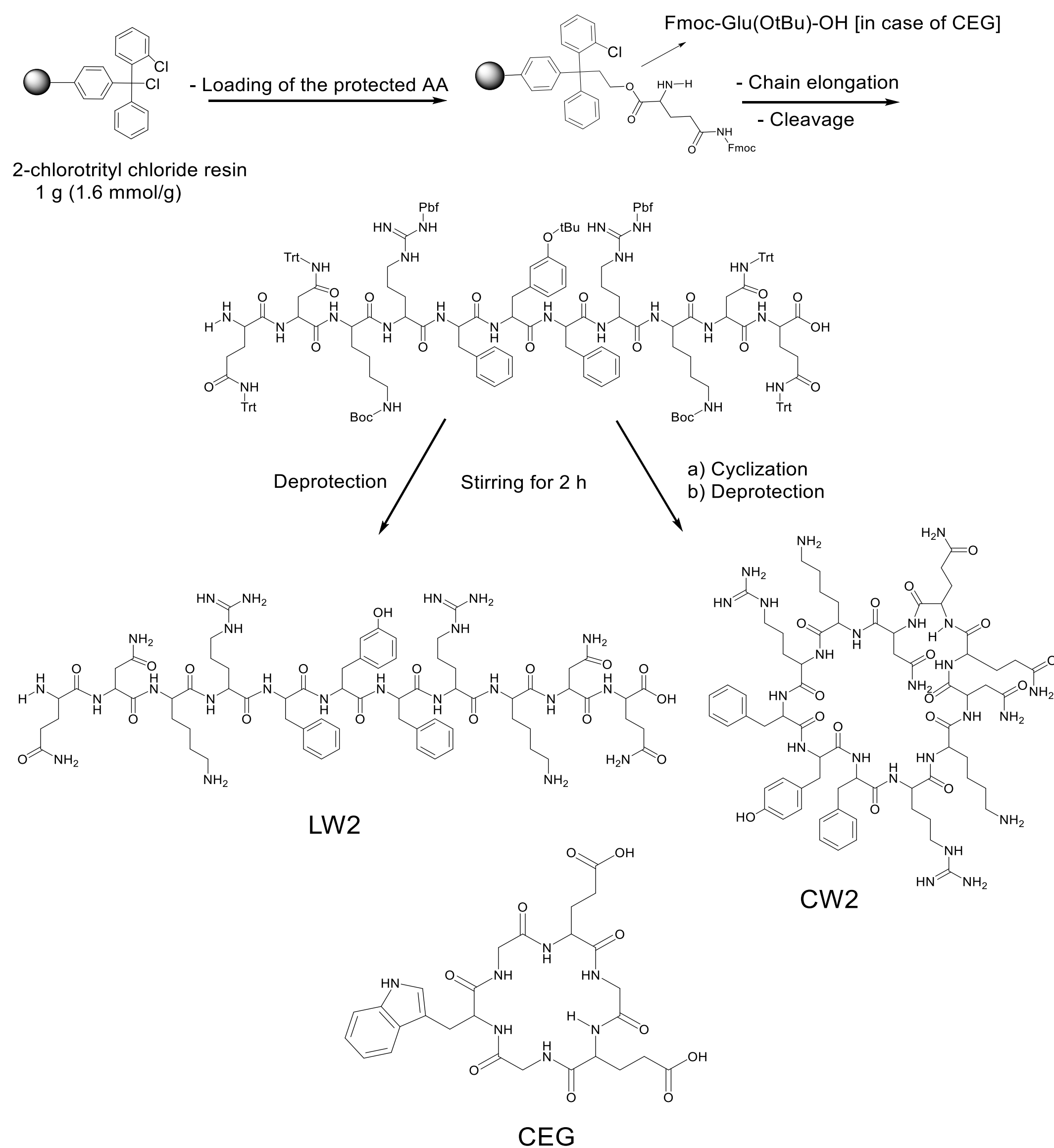
follow; LW2 (side-chain-protected), LW2, CW2, and CEG were 3259.600[M]$^+$, 1528.812 [M]$^+$, 1509.400 [M]$^+$, and 638.217 [M+Na]$^+$, respectively, as depicted in Figure S4.



Scheme 1. SPPS synthetic protocol of the LW2, CW2, and CEG peptides.

2.3 Preparation of Protein -FA complex solution

The stock solution of FA was prepared in MOPS (3-(N-morpholino) propanesulfonic acid, 20 mM, pH 7.0, 0.1% TFA) buffer at a final concentration of 100 µg/mL. CW2, LW2, CEG (tested compounds), and BSA (positive control) were also prepared in MOPS buffer, each at a final concentration of 1 mg/mL. FA (25 µM) was mixed with CW2 (200 µM), LW2 (200 µM), CEG (487 µM), BSA (10 µM) solution, the molar ratio of FA: Peptide/BSA followed as: 1:8, 1:8, 1:20, 1:0.4. The blank control including 25 µM FA in MOPS, 200 µM CW2/LW2/CEG, and 10 µM BSA also be prepared.

2.4 UVA irradiation

The tested group (LW2-FA, CW2-FA, CEG-FA), BSA-FA, and only peptide/protein groups were irradiated with UVA by using a UVA lamp (365 nm, UVL-56 Handheld UV Lamp, 6 W; UVP, USA) at the same time. The distance between the lamp and the samples was fixed at 20 cm. All the samples were measured at 319 nm (UV-vis 1200, Shimadzu, Japan) using 10mm cuvettes with MOPS buffer as blank. The content of FA samples was

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checked at 0, 2, 4, 8, 12 hours, respectively. The reservation ratio (RR) of FA was determined according to the following formula:

$$RR=\{(Abi-Abj)/(Abo-Abj)\}\times 100\%$$

Where Abi is sample absorbance including FA at 319 nm as time, Abj is sample absorbance without FA at 319 nm as time, and Abo is the absorbance of the initial sample at the same wavelength. The UV-visible absorption spectra also scanned at the same time, in the range from 250 to 400 nm. All experiments were carried out in triplicate.

2.5 Simulation analysis

The molecule docking was simulated by using Molecular Operating Environment 2018.0 (MOE, Chemical Computing Group, Canada) software. Following the MOE operation 2016 version and simulated the interaction between FA and BSA/CW2, respectively. Briefly, the crystal structure of BSA acquired from Protein Data Bank (PDB Code: 3V03) and Chain A of the structure was selected for further simulation. The protein receptor and ligand atoms were prepared by using Quick Prep and minimize the energy at the force field of default values (Amber 10: EHT). The binding site was selected by the site finder using the alpha center. Default values (Triangle Matcher) were selected for the docking parameters [20].

3. Results

3.1. The reservation ratio of FA under UVA irradiation

The content of FA was decreased depending on the exposure time and the pattern of decrease slowed down steadily in Figure 2. At the blank, the descending portion happened within the first 2 hours, its RR was only left 55.4 % after 2-hour exposure. It was shown that almost half of the FA were destroyed in this period. Because the FA is unstable under neutral/alkaline conditions and that makes UVA depredates FA easily[21,22]. Keeping the UVA irradiation until 12 hours later, the solution became stable and its RR value holding at 29.6 %. At the BSA-FA group, the RR improved 2.8 times, and the value was increased from 29.6 % to 86.5 %, compared with blank control. BSA has shown highly effective protection and stability against the damage of FA from UVA. Comparing with the BSA-FA, the tested group CW2-FA was shown a similar protected ability but the RR value was a little lower than BSA-FA which was kept at 83 %. Another tested group LW2-FA also had a positive effective result that the RR was kept at 73 %. To point out the main factors influencing the interaction between peptides and FA, especially cyclic peptides. A cyclic hexapeptide (CEG) was selected. This cyclic peptide includes one tryptophan, two glutamic acids, and three glycine residues. In theory, this peptide should be negatively charged in physiological solution and that would make FA hardly attached to the peptide. In the CEG-FA group, after 12 hour UVA exposure, the RR remained at 63 %. In summary, the protective effect: BSA > CW2 > LW2 > CEG.

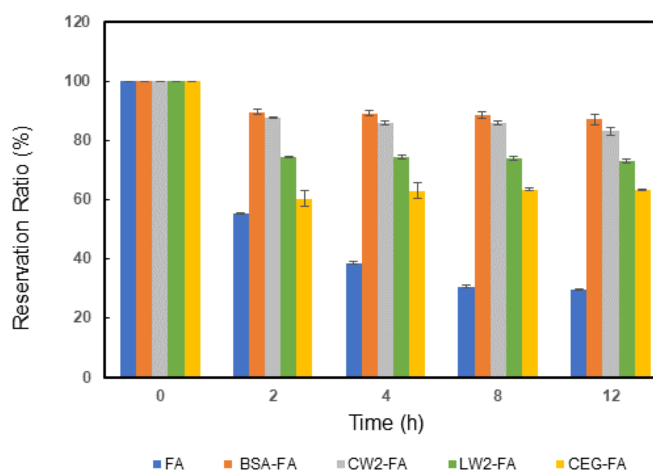

Figure 2. Time dependence of relative FA concentration under UVA radiation. The concentration of FA was 5 µg/mL at all samples. Except the blank control, the other group including 0.6 mg/mL BSA, 0.3 mg/mL CW2, 0.3mg/mL LW2 and 0.3 mg/mL CEG. Error bars represent mean ± SD (n=3).

3.2. UV scanning spectra

Figure 3a has shown the UV spectrum of blank control, BSA-FA, CW2-FA, CEG-FA, and LW2-FA before UVA irradiation. The UV spectrum of FA exhibits three λmax peaks at 230 nm, 287 nm, and 310 nm, respectively. In the BSA-FA group, the λmax 310 nm bathochromically shifted 2 nm and λmax 287 nm was covered since protein has a strong absorption peak at 280 nm [22]. And at the CEG-FA group, three λmax peaks were bathochromically shifted 2-3 nm. CW2-FA/LW2-FA was a similar UV spectrum with CEG-FA, but more degree bathochromically shifted certain peaks shifted down and up [6]. That indicated the different mechanisms of interaction between CW2/LW2-FA and BSA/CEG-FA.

Figure 3b has shown the blank (only FA) scanning spectrums at different irradiation times. The 287 nm peak was disappeared and a new peak was shown at 260 nm. The λmax 310 nm peak has certain hypsochromic shift and the degree of shift depending on time, and the absorption gradually decreased at the same time.

In BSA-FA, CW2-FA, and LW2-FA groups, FA was slightly damaged by UVA. After 12-hour exposure (Figure 3c, d, e), the spectrum was shown only a little degraded and no bathochromic or hypsochromic shift. This is because the damaged FA may exist in the solution as free form means no attachment to the protein/peptides. However, in the CEG-FA group (Figure 3f), the spectrum was a similar decreasing process and not so seriously, compared with blank control. CEG has some protective function but not so efficient.

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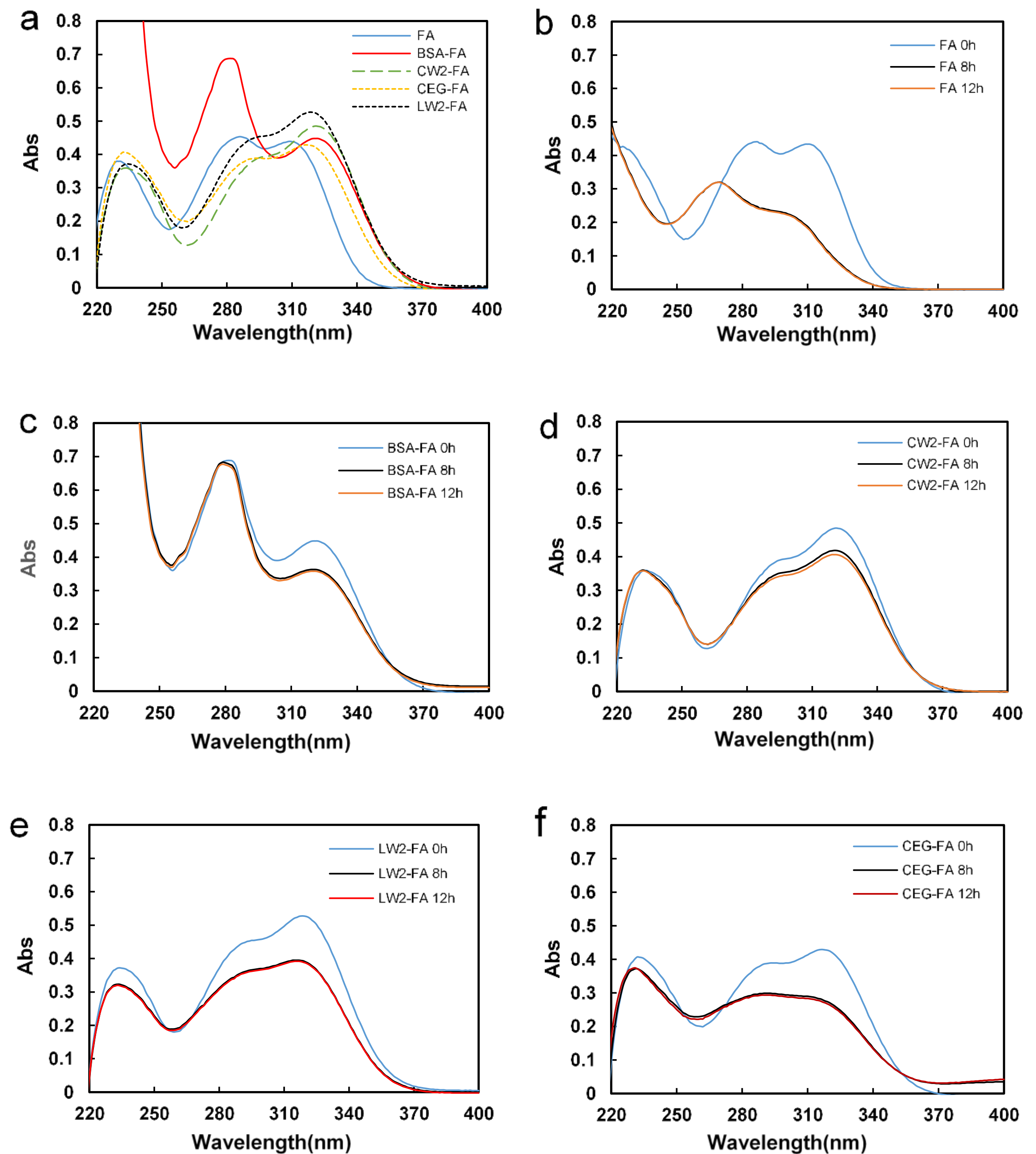

Figure 3. Time dependence of UV absorption of each sample in the wavelength range of 220 nm to 400nm **(a)** before UVA irradiation, **(b)** FA, **(c)** BSA-FA, **(d)** CW2-FA, **(e)** LW2-FA, and **(f)** CEG-FA.

3.3. Concentration dependence of the protection of FA

The RR of FA depending on dose after exposure 12 hours under UVA was shown in Figure 3. CW2-FA and BSA-FA both had no protective function for FA against UVA damage at 0.075mg/mL concentration of protein or peptide. BSA and CW2 started shown the protective function of FA at 0.45 mg/mL and 0.15 mg/mL, respectively. The dose-effect of the CW2-FA group was shown earlier than BSA-FA. As concentration increases, their protective ability was increased. The increasing carve of each other a little different, CW2

protective increasing gradually depending on dose and BSA sharply increasing at a certain concentration. These results show that CW2 effectively protected FA concerning the amount added.

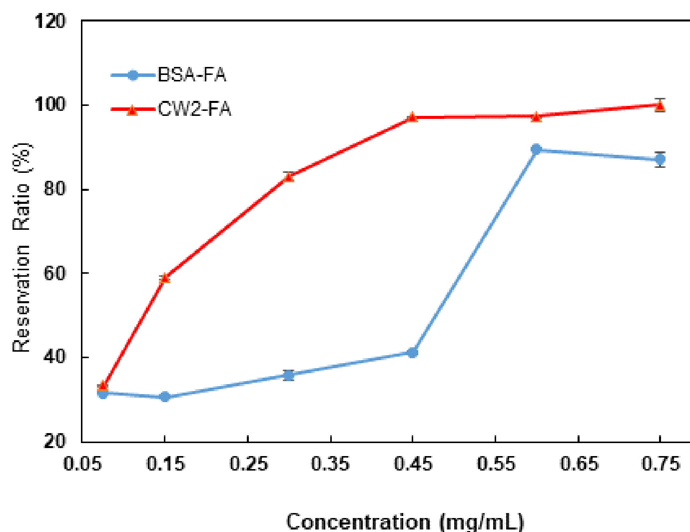

Figure 4. The reservation ratio of FA depending on dose after exposure 12 hours under UVA. The concentration of FA was 5 μg/mL in two groups. The blue line indicated the reservation ratio of BSA-FA groups after 12 hours UVA irradiation at a serials concentration of protein (0.075 mg/mL; 0.15 mg/mL; 0.3 mg/mL; 0.45 mg/mL; 0.6 mg/mL; 0.75 mg/mL). The red line indicated the reservation ratio of CW2-FA groups, follow the same treatment and concentration of CW2. Points represent means ± SDs (n=3).

4. Discussion

The subdomain IIA structure of BSA includes several hydrophobic amino acids (Leu 237, Tyr 149, Tyr 156, and Ala 290) and positively charged amino acid (Arg 256). As previously reported, Arg 256 role as a main contributor in the hydrogen bonding between BSA and FA [22], and the hydrophobic amino acids strengthen these interactions by hydrophobic interaction forces as shown in Figure 5a. CW2 was designed to behave similarly to BSA binding activity which exists within the subdomain IIA [20]. To mimic such functional structure, new peptides CW2 and its linear counterpart were designed to have 2 Arg, 2 Lys (cationic amino acids), and hydrophobic site containing (Phe-Tyr-Phe) to enhance the connection between peptide and FA in Figure 5b. The structural activity relationship was confirmed by the UVA exposure experiment. As the experimental results showed the protective effect of the newly designed peptides towards FA against UVA. The cyclic peptide showed a more efficient protective function than the linear peptide. Therefore, the cyclic structure of CW2 can work as a better protective shield towards FA because of its rigid structure [23]. And at the same time, the positively charged residues also very important to obtained protective function. That was proved by using CEG, which didn't exhibit a good protecting effect towards FA against UVA radiation.

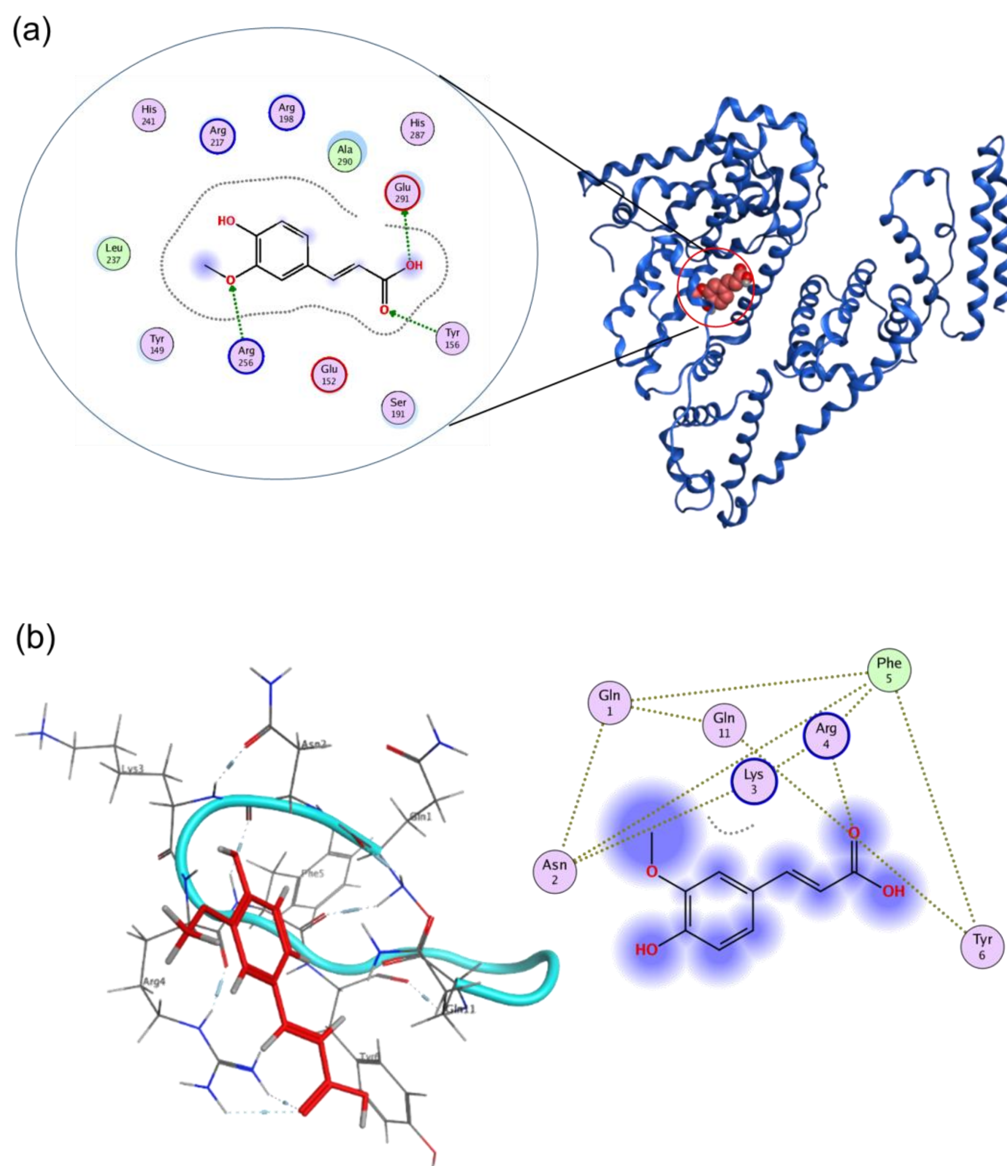

Figure 5. Molecular binding analysis by using Molecular Operating Environment, **(a)** 3D image of FA docked onto the BSA (PDB: 3V03), and 2D representation of the binding site and interacting residues between FA and BSA, **(b)** 3D image for FA docked onto the CW2 and 2D representation of the binding site and interacting residues between FA and CW2.

5. Conclusions

In this study, a new cyclic peptide CW2 was designed and synthesized to imitate BSA activity in FA protection against UVA. CW2 and LW2 (the linear counterpart) both showed the ability to protect FA against UVA damage. The RR was kept at 83% and 76%, respectively, for a reaction time of 12 hours. CW2 showed a higher capability to keep FA stable more than that of the linear form. The negatively charged cyclic peptide CEG has the lowest protective ability (RR was kept at 63%) compared with the others. Therefore, positively charged, and hydrophobic residues are required to initiate the interactions between FA and peptides. We suggest that the use of peptides to protect phenolic compounds against UVA damage requires the presence of a protective cyclic conformation, cationic, and hydrophobic residues. These functional peptides, which can be chemically synthesized to produce uniform molecules, will become useful as protective agents to replace proteins, but cyclic peptides are currently expensive for practical use. Over the years, peptide-ligation technologies have been developed that provide useful tools to facilitate successful cyclic peptide synthesis, such as enzyme-mediated cyclization, which is a cost-effective approach for large-scale synthesis [24]. In the future, it is hoped that such

peptide synthesis methods can be improved so that cyclic peptides can be synthesized inexpensively. As a prospect, it will be necessary to conduct the cytotoxicity analysis, antibacterial test, and molecular stability tests for the practical application to develop FA into food additives and cosmetics for skincare purposes due to their excellent antioxidant properties.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: HPLC chart of the protected sidechain [LW2] and [CW2], Figure S2: HPLC trace analysis of the deprotected-side-chain [LW2] and [CW2], Figure S3: HPLC trace analysis of the pure cyclic deprotected CEG, Figure S4: Mass spectra of peptides, Table S1: The score of molecule docking between BSA/CW2 and FA, Table S2: Yield and purity for synthesized compounds.

Author Contributions: Conceptualization, Y.W. and H.N.F.; Data curation, Y.W., and H.N.F.; Formal analysis, Y.W.; Investigation, Y.W.; Methodology, H.N.F.; Project administration, S. I.; Software, Y.W.; Supervision, T. K. and S. I.; Validation, Y.W., and H.N.F.; Visualization, Y.W.; Writing – original draft, Y.W.; Writing – review & editing, H.N.F., T. K., H. L., and S. I.; All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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