Investigations on the characteristics of BSA-lipid oxidation product interactions: role of three dien-aldehyde with different chain lengths

wenjuan Li[1], Yan Wu[2], Conghu Li[2], Yiqing Xu[2], Meifeng Wang[2], and liangliang Zhu[1]

[1]Affiliation not available
[2]Anqing Normal University

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Abstract

Protein modification by aldehydes has been generally associated with aging and onset of various chronic and cardiovascular diseases. The characteristics of bovine serum albumin (BSA) incubated with three dien-aldehydes (trans, trans-2,4-heptadienal, trans, trans-2,4-nonadienal, trans, trans-2,4-decadienal) of different chain lengths at different concentrations were examined. The results were as follows: loss of the amino group and increased carbonyl value were indicative of BSA side chain damage by these three unsaturated aldehydes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis results showed that all the three aldehydes led to formation of BSA aggregation; the most significant effect was observed for heptadienal. Changes in intrinsic fluorescence and surface hydrophobic value of BSAs indicated that aldehydes modified protein structures of BSAs. Moreover, the three aldehydes used in this experiment caused BSAs to form yellowish-brown adducts and fluorescent lipofuscin. The heptadienal-BSA adducts exhibited increased ultraviolet–visible (UV-Vis) absorbance at 270-280 nm and 300-400 nm, similar to the two other aldehyde-BSA adducts. We also examined the correlation between the various oxidation parameters and the concentration of modifiers. Strong correlations were observed between formation of protein-bound carbonyls, the retention ratio of free amino content, maximum UV-Vis absorption value, and concentration of aldehydes. Finally, Principal component analysis (PCA) analysis was conducted on oxidation parameters and the comprehensive effect of these parameters on BSA modification. In general, greater BSA damage was observed when incubated with aldehydes with shorter chain length at higher concentration.

Headings:

1. BSA modified by long-chain unsaturated aldehydes without oxygen-containing side chains was studied.
2. The effect of aldehyde chain length on BSA was studied.
3. Various characteristics of aldehydes-BSA adducts were evaluated.

1 Introduction

Lipid is an important component of food and the carrier of fat-soluble substances, which can not only provide heat and essential fatty acids, but can also enhance the flavor of food (Coca et al., 2011). Lipid oxidation is very common in the food system, especially during food processing. Various aldehydes are generated as secondary products of lipid oxidation. Compared with free radicals, these aldehydes are relatively stable and

have a longer lifetime. Therefore, they can diffuse from the site of formation and migrate over long distances to react with various biological macromolecules (e.g, protein), thereby acting as "toxic second messengers" of lipid oxidation (Hidalgo et al., 2017; Wu et al., 2009).

The main hazard of these active aldehydes is that they can react with nucleophilic macromolecules such as proteins, resulting in protein aggregation, dysfunction, immunogenicity, and activation of specific receptors (Colzani et al., 2016). Protein modification by aldehydes is generally considered to be closely related to formation of lipofuscin, which is associated with aging, and play a significant role in the onset and progression of various chronic diseases, such as neurodegenerative and cardiovascular diseases(Colzani et al., 2013).

Among the aldehydes with high reactivity, the most extensively studied were acrolein (ACR), 4-hydroxy-2-alkenals, and malondialdehyde (MDA). Today, 4,5-epoxy-2-alkenals and 4-oxo-2-alkenal have become a hot research topic (Hidalgo et al., 2000).

Among the active aldehydes generated from unsaturated fatty acids, there is also a large number of other aldehydes in addition to those mentioned above. The oil in unsaturated fatty acid produced a variety of unsaturated aldehydes under hot working conditions. One such example is E,E-2,4-alkadienals with 6-10 carbon atoms (Guillén et al.,2012; Hidalgo et al.,2016). Oxidation of n-6 series of polyunsaturated fatty acids (such as linoleic acid) was found to produce a large number of E,E-2,4-decadienal (Poyato et al., 2014; Sousa et al., 2017); however, oxidation of linolenic acid and its esters produced E,E-2,4-heptadienal, especially under heat stress (Hidalgo et al., 2016; Poyato et al., 2014). Some unsaturated aldehydes with a large number of carbon atoms without oxygen-containing side chains have relatively high molecular weight, which results in lower reaction activity than the widely studied acrolein (ACR), which has a small molecular weight and is a 4-hydroxy-trans-2-nonenal (HNE) with a hydroxyl side chain. Therefore, these unsaturated aldehydes have received relatively low attention (Sousa et al., 2017). Protein modification by these aldehydes have rarely been studied, and chemical reactions between these aldehydes and protein nucleophiles are not completely clear.

Previous reports have shown that long-chain aldehydes with oxygen-containing side chains (e.g, HNE, ONE et al) and short chain side aldehydes with no oxygen side chain (e.g ACR, Butenal et al) can cause significant protein damage. This prompted us to investigate whether modification by long-chain unsaturated aldehydes without oxygen-containing side chains have any effects on proteins, including how their chain length and concentration affect the degree of protein modification, and what the characteristics of these aldehydes are after binding with proteins.

While *in vivo* oxidative damage is a long term and cumulative process, most of the *in vitro* research are short-term studies. Therefore, the concentration of *in vitro* modifiers are usually much higher as compared with that *in vivo* . Although there are some differences with the real system, this procedure is common in scientific literature when an *in vitro* protein modification is analyzed (Traverso et al.,2004) . In addition, the focus of basic research is to discover all possible changes (and mechanisms), which will be different from those that occur in nature. We have reviewed a large number of studies that examined the interaction between protein and aldehydes produced by lipid oxidation. We found that the concentration range of aldehydes added in these experiments is 0.01-100 mm. Taking into account all past *in vitro* experiments in literature, as well as own pre-experiment pilot studies, the concentration range of the three aldehydes we used was set to be 1-50 mM.

The objective of this study was to characterize the changes produced in BSA incubated in different concentrations of heptadienal, nonadienal and decadienal. A variety of methods, such as fluorescence, UV visible absorption, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and colorimetric analyses were combined to characterize the aldehyde-protein adducts obtained. The results of this work extended findings from other studies, demonstrating that aldehydes from lipid oxidation can contribute significantly to protein damage. Our findings should be help define specific roles of long-chain unsaturated aldehydes without oxygen-containing side chains from lipid oxidation in the formation of degenerative proteins. This can provide a basis for a deeper understanding of the effect of these modified proteins on cells.

2 Materials and methods

2.1 Materials

BSA was purchased from Phygene Life Sciences Company (Fuzhou, China); ammonium persulfate and tetramethyl ethylenediamine (TEMED) were purchased from Amersham Biosciences (Uppsala, Sweden); trans-trans-2,4-heptadienal, trans-trans-2,4-nonadienal, and trans-trans-2,4-decadienal were purchased from Aladdin Industrial Corporation (Shanghai, China); protein ladder for SDS-PAGE was purchased from Beyotime Biotechnology Company Limited (Shanghai, China); all other chemicals were of analytical reagent grade.

2.2 Preparation of aldehyde-BSA adducts

Aldehyde-BSA adducts were prepared as follows: BSA was dissolved in a buffer containing 50 mM potassium phosphate (pH 7.4) to a final concentration of 10 mg/ml. Aldehyde-BSA adducts were prepared by incubating 10 mg/ml BSA with the modifiers trans,trans-2,4-heptadienal, trans,trans-2,4-nonadienal, or trans,trans-2,4-decadienal at various concentrations (1, 5, 10, 20 ,50 mM) for 24 h at 37. Pure BSA was incubated under same conditions as control. Following the reaction, unreacted aldehydes were removed via PD-10 column.

2.3 Determination of protein carbonyl

Protein carbonyls of native and modified BSAs were quantified according to the method described by Indurthi et al.(2012). and Suji et al.(2008) with slightly modifications. Proteins (200 µl at 2.5 mg/ml) were mixed with 1 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCL or 2 M HCl (blank control), and were incubated at room temperature (25) for 2 h (shake every 15 min). Equal volume of 20% TCA (to a final concentration of 10%) was added to precipitate the protein. The precipitate was collected by centrifugation at 8,000 g for 10 min. The precipitate was washed three times with 5 ml ethanol/ethyl acetate solution (1:1, v/v) to eliminate free DNPH. Protein precipitate was centrifuged again (8,000 g, 5 min) before being suspended in 3 ml of 6 M guanidinium chloride. Samples were incubated at 37 for 15-30 min for proteins to completely dissolve. Absorbance was measured at 370 nm. The results were expressed as nmol of carbonyl groups per milligram of soluble protein, with a molar extinction coefficient of 22,000 M^{-1} cm^{-1}.

2.4 Determination of free amino content

The free amino content of modified and native BSA were determined by the o-phthalic dialdehyde (OPA) method, as described by Adams et al.(2008). The OPA reagent was freshly prepared by dissolving 40 mg of OPA in 1 ml of methanol, followed by the addition of 25 ml of 0.1 M sodium borate, 2.5 ml of 10% SDS, and 0.1 ml 2-mercaptoethanol, finally adjusting to a total volume of 50 ml with distilled water. Protein (120 µl at 2.5 mg/ml) was mixed with 3 ml OPA reagent. After 2 min in the dark at room temperature, the absorbance at 340 nm was recorded against the OPA reagent. A calibration curve was obtained by using L-leucine as the standard.

2.5 SDS-PAGE

Protein crosslinking was analyzed by SDS-PAGE under reducing condition; 5% stacking gel and 10% resolving gel was used for protein separation. Samples were dissolved in the reducing buffer solution containing SDS, and were then boiled for 5 min before loading. Migration was carried out at 20 mA constant current. After separation, proteins were stained with 0.1% (w/v) Coomassie brilliant blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid, and were de-stained with 10% methanol (v/v) and 10% acetic acid (v/v).

2.6 Determination of surface hydrophobicity

The surface hydrophobicity of control and aldehyde-BSA adducts were determined using the 8-anilino-1-naphthalenesulfonic acid method, as described by Tang et al.(2012). and Lv et al.(2016) . Briefly, 10 μl 1-anilino-8-naphthalene-sulfonate (ANS) was added to 0.5 ml of each protein sample (diluted to 0.005-0.5 mg/ml), and was allowed to react for 3 min at room temperature. Fluorescence intensity was measured with an excitation wavelength of 390 nm and emission wavelength of 490 nm. Surface hydrophobicity was determined as the initial slope of the curve plot of fluorescent intensity versus protein concentration. The result was expressed as follows: % relative value of surface = ANS aldehyde – BSA adducts*100 / ANS control BSA.

2.7 Determination of intrinsic fluorescence

The intrinsic fluorescence of control and aldehyde-modified BSA (0.1 mg/ml) were recorded from 300 to 400 nm with an excitation wavelength of 290 nm (slit 5 nm), as described by Chen et al.(2013).

2.8 ultraviolet–visible (UV-Vis) spectroscopic analysis

UV/Vis Spectra were recorded on a Thermo scientific UV/visible spectrophotometer, using quartz cuvettes with a 10 mm pathlength. Protein concentration was 0.2 mg/ml, and absorbance measurements were done in the 240 to 600 nm range.

2.9 Fluorescent pigments measurement

Fluorescent pigments were determined by methods from Meynier et al.(2004), with slight modifications described as follows. Formation of fluorescent pigments was first investigated by 3D scanning, with excitation and emission wavelengths of 300 – 410 nm and 350 – 550 nm, respectively, adjusted in 2 nm increments. The maximum excitation wavelength obtained from the 3D scanning image was used as a reference. The maximum excitation and maximum emission wavelength were then obtained by combining the wave scan method. Finally, the emission spectra of native BSA and aldehyde-BSA adducts were scanned from 350-550 nm with the excitation wavelength fixed at the maximum excitation wavelengths of various aldehyde-BSA adducts. The spectra were measured at the following conditions: PMT voltage: 700 V; scan speed: 600 nm/min; EM slit: 5 nm; response time 0.1 s.

2.10 Determination of colorimetric values

Colorimetric values of samples were obtained via a colorimeter (model NS810 spectrophotometrically, 3nh); a*(redness/greenness), b*(yellowness/blueness), and L*(lightness) were measured.

2.11 Statistical analysis

Each experiment was performed in triplicates. Data were expressed as means ± standard deviation (n = 3). Analysis of variance (ANOVA) was performed using the SPSS 16.0 software. Statistical significance was set at $p < 0.05$. Principal component analysis (PCA) was used to standardize and determine the relationship between the variables using the SPSS 16.0 software. This promoted comprehensive evaluation of the effects of different aldehydes on BSA modification.

3 Results and discussion

3.1 Effect of aldehydes on side chain modification of BSA

Formation of protein-bound carbonyls and retention ratio of free amino group confirmed the covalent binding of the three aldehydes to proteins, and determined the effect of carbon atom number and concentration on degree of modification.

3.1.1 The formation of protein bound carbonyls

The effects of the three kinds of aldehydes on BSA carbonyl contents are shown in Fig.1. An increase in carbonyl groups content was observed in the presence of all three aldehydes. This modification was significantly greater in aldehydes with shorter chains. Incubation of BSA with heptadienal resulted in the most dramatic increase in protein carbonyl content. The difference between the BSA carbonyl values caused by the three aldehydes at 1-10 mM were not significant, however, this was not the case at high concentrations (50 mM). The carbonyl content of BSA incubated with 10 mM heptadienal, nonadienal, and decadienal were approximately 4.83 ± 0.48, 4.08 ± 0.62, and 3.59 ± 0.32 times, respectively, that of the control. When treated with 50 mM heptadienal, nonadienal, and decadienal, the content of carbonyls reached 14.65 ± 0.93, 8.66 ± 0.43 and 6.32 ± 0.61 times, respectively, that of the control. The formation of protein bound carbonyls correlated well with aldehyde concentrations (R= 0.984, $p < 0.001$ for heptadienal-BSA; R = 0.912, $p = 0.011$ for nonadienal-BSA; R = 0.868, $p = 0.025$ for decadienal-BSA).

The addition of α,β-unsaturated aldehydes to nucleophilic amino acids on proteins occurs in two ways: Michael addition and Schiff base addition. The strong electron-withdrawing capability of carbonyl groups polarizes the double bond. This makes the - carbon more electrophilic and susceptible to Michael addition. The carbonyl group is maintained, and the conjugated double bond is lost after Michael adducts. When the - carbon atoms become saturated due to Michael addition, a Schiff's base reaction will occur, which will cause the introduced carbonyl to become undetectable(Yuan et al., 2007).

Wu et al.(2010). found that incubation with acolein induces a concentration-dependent increase of protein carbonyls. For example, the soybean protein carbonyl increased 10 times due to incubation with 10 mM acrolein. Similar result was obtained with BSA exposure to crotonaldehyde (an unsaturated aldehydes); the increase in protein carbonyl concentration was highly associated with loss of lysine and histidine residues (Ichihashi et al., 2001).

3.1.2 Retention ratio of free amino content

Fig.2 shows the concentration and chain length of modifier-dependent reduction of amino groups. The three unsaturated aldehydes at concentration of 1–50 mM had a significant effect on the amino retention ratio ($p < 0.05$). The results indicated that heptadienal has a stronger influence on the amino group when compared to the other two aldehydes. Free BSA amino group was rapidly decreased with increasing concentration of heptadienal, especially when it was at the 20-50 mM range. Incubation of BSA with 50 mM heptadienal resulted in an amino group retention ratio of only 33.99 ±3.61%.

Chopin et al.(2007). found that 2-hexenal and 2,4-hexadienal significantly reduces the number of amino groups. However, the number of double bonds exerts little effect on the retention ratio of free amino groups. In our study, loss of free amino group in BSA was relatively less after incubation with various concentrations of nonadienal and decadienal. After 24 h incubation with 50 mM nonadienal and decadienal, the rentention ratio of BSA amino group was 62.81 ± 7.96% and 77.29 ± 1.25%, respectively. A strong negative correlation was observed between the retention ratio of free amino content and the concentration of aldehyde for heptadienal (R= -0.958, $p = 0.003$), nonadienal (R = -0.844, $p = 0.035$), and decadienal (R = -0.765, $p = 0.076$).

3.2 Effect of aldehydes on BSA aggregation and cross-linking

Does covalent modification of proteins caused by these three aldehydes only exist on protein monomolecules or will it also lead to cross-linking between protein molecules? We used SDS-PAGE to determine whether protein modification by these three aldehydes will cause cross-linking, as well as the relationship between degree of aggregation and modifier concentration. This will also plays an auxiliary role in determining conformational change of proteins.

SDS-PAGE was performed in order to monitor BSA aggregation that occur due to the interaction with these three aldehydes (Fig.3). The electrophoretic pattern of the control BSA was characterized by one major band with a molecular mass of 66 kD. Aside from the major band, there were also a small amount of high molecular weight protein stemming from impurity. We found that all types of aldehydes decreased the BSA monomer band at 66 kD, and resulted in some form of aggregation; the degree of aggregation was closely associated with the type and concentration of the aldehydes.

A faint new band with a size of 95 kD appeared when BSA was treated with a low concentration aldehydes (1 mM). As the concentration of the three aldehydes increased to 5 mM, the intensities of the aggregate bands with molecular weights of 95 kD and >130 kD were increased, and the bands of the BSA monomer was decreased (Fig.3). Similar to our results, Liu et al (2007) also found that BSA treated with oxidation DHA for various length of times led to formation of a single narrow band with a molecular mass of approximately 93 kD, signifying the presence of a high-molecular-weight-protein. In addition, binding of acrolein to BSA also resulted in formation of two broad protein bands with molecular masses of approximately 80 kD and $>$ 130 kD; binding of malondialdehyde to BSA resulted in formation of two broad protein bands with the molecular masses of approximately 97 kD and 200 kD (Liu et al., 2007).

In heptadienal-BSA reactants, when the heptadienal concentration was 10 – 20 mM, bands of the 95 kD aggregates and BSA monomers became blurred and diffused, and bands of protein aggregates with molecular weight above 175 kD was intensified; when the concentration of heptadienal reached 50 mM, the bands of the aggregates and BSA monomers almost disappeared. This was accompanied by a band of $>$ 270 kD on the top of the resolving gel, as well as protein aggregates that were too large to enter the stacking gel (Fig.3(A)).

Similar results have been previously reported when the soybean protein was treated with 1-100 mmol/l MDA (Wu et al.,2009). Moreover, this phenomenon in SDS-PAGE was also observed when unsaturated aldehydes were allowed to interact with proteins. In the presence of high concentration of t-2-hexenal, a-lactalbumin disappeared almost completely, and a high molecular weight protein aggregate band appeared between the stacking gel and the main gel (Meynier et al., 2004).

When BSA was treated with 10-50 mM nonadienal, the 95 kD aggregate band and the BSA monomer band were also blurred and diffused; the bands at $>$ 175 kD were increased. However, when BSA was treated with 50 mM nonadienal, there was no obvious aggregates trapped in the sample adding port (Fig.3(B)). Similar aggregation pattern was also observed for proteins in the presence of decadienal, but to a lesser extent. In addition, the BSA monomer band and 95 kD aggregate band were relatively clear. No trapping of protein aggregation was observed in the sampling port at all tested decadienal concentrations (1-50 mM), (Fig.3(C)). Our study suggested that exposure to heptadienal induces significantly greater BSA crosslinking than exposure to either nonadienal or decadienal, especially when the concentration of modifiers are in the range of 20-50 mM.

3.3 Effect of aldehydes on the structure change of BSA

The change in protein surface value and intrinsic fluorescence reflects whether conformation of proteins have changed due to covalent modification and aggregation, and whether the number of carbon atoms and the concentration of aldehydes has an effect on protein conformation.

3.3.1 Change of protein surface properties

Hydrophobicity is an indicator of the number of hydrophobic groups on the surface of the protein that is in contact with the polar aqueous environment. It is known to be significantly related to the function of proteins (Tang et al.,2012; Liu et al., 2012). ANS are widely used for detecting protein surface properties, determining the refolding and unfolding processes, as well as characterizing aggregations, fibrillations and molten globule intermediates (Ahmed et al., 2017; Sattarahmady et al., 2007).

As shown in Fig.4, the extent of decline was closely correlated with the concentration and type of modifiers (R= -0.827, $p = 0.043$ for heptadienal-BSA; R = -0.730, $p = 0.099$ for nonadienal-BSA; R = -0.712, $p = 0.112$ for decadienal-BSA); in general, the higher the concentration of the modifiers, the lower of the surface hydrophobicity. Surface hydrophobicity of BSA was found to be greatly reduced by low concentration of aldehydes; modified with 1 mM heptadienal, nonadienal, and decadienal, the surface hydrophobic values were 80.14 ± 8.35%, 82.61 ± 4.16%%, and 79.22 ± 4.50% of control BSA, respectively. There were significant differences between the three groups of aldehyde-protein adducts and control BSA ($p < 0.05$), however, no significant differences were observed among the three groups of aldehyde-protein adducts ($p > 0.05$) when the concentration of modifiers was 1 mM. The surface hydrophobic value of BSA was steadily decreased steadily with increased concentration of the three modifiers. All three aldehydes caused sharp decrease in the surface hydrophobicity of BSA when their concentrations reached 50 mM. The surface hydrophobicity of heptadienal-BSA, nonadienal-BSA, decadienal-BSA were only 3.23 ± 0.55%, 20.04 ± 0.82%, and 33.20 ± 2.74% that of BSA control, respectively; the difference between each of those was significant ($p < 0.05$).

Based on the change of surface hydrophobicity, it can be inferred that the native conformation of BSA has also been altered by the three aldehydes. The decrease in hydrophobic values may be due to modification of surface hydrophobic groups or extensive protein aggregation, which resulted in the hydrophobic groups being buried into the aggregates. Wu et al. (2010). found that adding of 0.01-10 μmol/l acrolein (α, β-unsaturated aldehyde) to soybean protein also significantly decreased the surface hydrophobic value. According to the previous report, surface hydrophobicity was steadily decreased with increased MDA concentration from 0 to 100 mM , which has been considered to be the result of formation of hydrophilic groups (e.g protein carbonyls groups), protein aggregation via hydrophobic interactions, and structural modification of exposed hydrophobic residues of soy protein (Wu et al., 2009).

Some scholars believed that oxidative modification could also lead to protein unfolding and exposure of hydrophobic groups. Following that, the exposed hydrophobic groups can interact with each other, which leads to protein aggregation (Tang et al., 2012). The observed surface hydrophobicity value in this paper may be the result of an equilibrium reached from the exposure and aggregation of hydrophobic groups (aggregation of proteins shown by electrophoresis).

3.3.2 Change of intrinsic fluorescence

The maximum emission wavelength of tryptophan is highly dependent on the polarity of the surrounding microenvironment, which makes it a very suitable endogenous fluorescence probe to assess changes in protein conformation via change in its maximum emission wavelength (Girish et al., 2016; Ahmed et al., 2017).

The maximum emission of control BSA was approximately 340 nm. As shown in Fig.5, a sharp decrease in fluorescent intensity and a significant blue shift in its maximum emission wavelength were observed when BSA was incubated with these three kinds of aldehydes.

At a concentration of 1 mM, all the three aldehydes caused an abrupt decline in intrinsic BSA fluorescence, but did not lead to blue shift in maximum emission. The intrinsic fluorescence intensity of heptadienal-BSA, nonadienal-BSA, and decadienal-BSA was reduced to 48.25 ± 2.70%, 46.50 ± 0.41%, and 48.80 ± 1.49% that of the control sample, respectively. Heptadienal-BSA, nonadienal-BSA, and decadienal-BSA decreased to approximately 7.72 ± 0.49%, 8.77 ± 0.46%, and 19.88 ± 1.38% that of the control, respectively. Moreover, the maximum emission wavelength showed a significant blue shift to 325-326 nm in the presence of 5 mM aldehydes. At concentrations of 10-50 mM modifier, there was a continued reduction in intrinsic fluorescence

intensity, but no further blue shift was observed. The intrinsic fluorescence intensity of heptadienal-BSA, nonadienal-BSA, and decadienal-BSA was decreased to 2.65 ± 0.43%, 5.99 ± 0.42%, and 9.9 ± 0.65% that of the control, respectively, when the modifier was at the highest concentration (50 mM). At this concentration, these three dien-aldehydes almost completely extinguished the intrinsic fluorescence of BSA.

To summarize, all three dien-aldehydes (at 1-50 mM) demonstrated significant effects on the intrinsic fluorescence of BSA. When the concentrations of the dien-aldehydes were low (1 mM), there was no significant change in the BSA intrinsic fluorescence ($p > 0.05$). The intrinsic fluorescence intensity of BSA decreased in the following order: heptadienal > nonadienal > decadienal, when concentrations of modifiers were in the ranges of 5-50 mM; there were also significant differences among BSAs treated with different modifiers ($p < 0.05$).

The maximum intrinsic fluorescence emission wavelength denotes the relative position of tryptophan residues within proteins (Estévez et al., 2008).The blue shift of the maximum emission wavelength indicated that with greater concentration of the modifiers, the conformation of BSA was gradually destroyed, and the previously exposed tryptophan residues of the native protein were buried in the interior. The BSA protein thus became more hydrophobic and less polar, resulting in aggregation (Wu et al.,2009). However, the decrease in intrinsic fluorescence intensity is usually explained by protein folding, aggregation, or/and degradation of tryptophan (the aggregation of proteins shown by electrophoresis) (Lv et al.,2016).

3.4 UV/VIS, fluorescent spectroscopic and color properties of modified BSA

The spectral characteristic of aldehyde-BSA adducts were determined by ultraviolet visible spectrophotometer and fluorescence; the color characteristics of the modified protein were given by color parameters. The effects of length and concentration of aldehydes on these properties were also determined.

3.4.1 Changes in UV-Vis absorption characteristics

Fig.6 shows that the UV-Vis absorbance profiles for heptadienal-, nonadienal-, and decadienal-modified protein were identical; the absorbances was increased to 270-280 nm, as well as to 300-400 nm (Fig.6). This suggested formation of structurally similar chromophores of these aldehyde-BSA adducts (Vetter et al.,2011). Similar absorbance spectra shapes have been reported to be typical of melanoidins (Adams et al;2009).

Previous studies have reported that BSA reacts with methylglyoxal lead to formation of chromophores with absorbances at 300-400 nm and below 290 nm. The authors speculated that absorption at 320-335 nm occurs due to the newly generated argyrimidine structure, and absorption at 325-335 nm was due to the pentosidine structure (Vetter et al., 2011). The binding of whey protein to 2-hexenal or hexanal also resulted in the increase of the absorption at 280 nm and 300-360 nm (Meynier et al.,2004).

Glyceraldehyde incubated with acetyl-lysine (118 mM) produced new compounds with maximum absorption at 275.7, 232.2, 268.7, and 349.3 nm; the maximum absorption wavelength of 297 nm was accompanied by 260 nm shoulder peaks. Among the new compounds, the maximum absorption of trihydroxy-triosidine was at 275.7 nm; maximum absorption of Lys-hydroxy -triosidine was at 232, 269 and 349 nm; maximum absorption of triosidine-carbaldehyde was at 297 nm. The new compound Arg-hydroxy-triosidine, with a maximum absorbance at 330 nm, was produced when acetyl-lysine (55 mM) and acetyl-arginine (50 mM) were incubated with glyceraldehyde (Tessier et al.,2002).

As shown in Fig.6, light absorption at 270-280 nm by control BSA was mainly due to amino acid residues with optical properties, such as tryptophan, tyrosine, phenylalanine. When the concentration of these three modifiers were low (1 mM), the absorbance of the aldehyde-BSA adducts at 270-280 nm was slightly decreased, which may be caused by modification of chromogenic amino acid residues. Lv et al. (2016) showed that the maximum UV absorption of untreated shrimp protomyosin was at approximately 275 nm, which was largely due to Tyr and Trp residues. After shrimp protomyosin was treated with low concentrations of HNE (0.01-1 mM), the absorption wavelength of Tyr and Trp slightly shifted and decreased. This was mainly due

to modification of Tyr and Trp residues on the protein surface, which caused a change in the polarity of the microenvironment.

Fig.6 shows that with greater concentration of the three aldehydes (5-50 mm), absorption of aldehyde-BSA adducts at 270-280 nm was rapidly increased. These results were similar to those of other studies. Khatoon et al.(2012). found that HSA modified by HNE led to increased absorbance at 280 nm, and was also accompanied by a slight increase in absorbance at 300-390 nm. It was speculated that the increase in absorbance at 280 nm may be due to structural expansion of HSA caused by the addition of HNE with lysine, histidine, and cysteine.

The absorption of aldehyde-BSA adducts were dependent on modifier concentration, and the increasing range was negatively correlated to length of aldehydes. On one hand, carbonylation may have distorted the native conformation of the BSA, thereby exposing the aromatic amino acid residues and causing an increase in absorbance at 270-280 nm. On the other hand, some new compounds may have been formed, which absorb at 270-280 nm (Ahmed et al.,2017).

As shown in Fig.6(A), the absorbance of heptadienal-BSA around 270 nm increased greatly with greater concentration of the modifiers, which increased from 0.33 ± 0.01 to 1.91 ± 0.06 and 4.79 ± 0.18 times that of the control. The maximum absorbance wavelength shifted from approximately 270 nm at low modifier concentration to approximately 276 nm at high modifier concentration (50 mM).

The absorbance of nonadienal-BSA and decadienal-BSA near 270 nm were increased from 0.33 ± 0.01 to 1.14 ± 0.08 and 0.69 ± 0.01, which was 2.45 ± 0.24 and 1.20 ± 0.03 times higher than that of the control. The maximum absorption wavelength of nonadienal-BSA and decadienal-BSA were shifted to approximately 275 nm and 273 nm, respectively, when the concentration of the modifiers reached 50 mM (Fig.6(B-C)). A significant correlation was found between maximum absorption and the concentration of aldehydes ($R = 0.991$, $p < 0.001$ for heptadienal-BSA; $R = 0.984$, $p < 0.001$ for nonadienal-BSA; $R= 0.854$,$p = 0.030$ for decadienal-BSA).

Moreover, as presented in Fig.6, these three aldehyde-BSA adducts have no characteristic absorption peaks in the range of 300-400 nm, and their optical density increased with decrease in wavelength. This is similar to the typical "tail pattern" of melanin, which has a tailing absorption rage that extends to the visible spectrum. The main reason for this phenomenon may be due to the many kinds of chromophore structures, which resulted in a system that has no characteristic absorption peak in this region (Hrynets et al.,2013).

3.4.2 Formation of fluorescent pigments

Accumulation of fluorescent pigments (also called lipofuscin) is usually closely related to aging and various chronic degenerative diseases. In this study, the fluorescent lipofuscin of aldehyde-BSA adducts and control were detected by fluorescence spectrophotometer. It has been reported that the maximum excitation and emission wavelengths of fluorescent lipofuscin fall between 340-375 nm and 420-490 nm, respectively (Trombly et al.,1975). In the presence of aldehydes, new fluorescent lipofuscin with the excitation and emission wavelengths at 340-400 nm and 400-500 nm, respectively, were observed (Meynier et al.,2004). We showed that incubation of unsaturated aldehyde and oxidized linoleic acids with BSA produced new fluorescent compounds, which showed maximum excitation and emission wavelengths at 350-380 nm and 420-450 nm, respectively, similar to previously reported values (Yamaki et al., 1992).

Based on the previous reports, we first used 3D scanning to determine the approximate maximum excitation and emission of lipofuscin. However, data obtained from 3D scanning is affected by the wavelength accuracy and Rayleigh scattering. Therefore, on the basis of 3D scanning, we further used two-dimensional of excitation and emission wavelength scanning to determine the maximum wavelength of fluorescent lipofuscin from aldehyde-BSA adducts (Considering the results of the pre-experiment, the aldehyde-BSA adducts lipofuscin fluorescence intensity was relatively higher when modifier concentrations was at 10 mM).

The fluorescent lipofuscin emission spectra for each kind of aldehyde-BSA adducts were obtained with

the excitation wavelength set at their own maximum excitation wavelength. As shown in Fig.7(A-C), the maximum excitation and emission wavelengths of BSA modified by heptadienal, nonadienal, and decadienal at 10 mM were 352.3/436.4 nm, 353.3/434.4 nm, and 343.4/430.5 nm, respectively. In addition, Fig.7(A) also showed that the spectra of heptadienal-BSA exhibited a shoulder peak at about 465-470 nm; BSA-nonadienal has a defined shoulder peak at 465-470 nm (Fig.7(B)); BSA-decadienal demonstrated two shoulder peaks at 405-410 nm and 465-470 nm ((Fig.7(C)). Moreover, the lipofuscin excitation and emission spectra were broad, which suggested the presence of several fluorophores (Hidalgo et al.1993) .

Fig.8 is the emission spectrum of fluorescent lipofuscin from aldehyde-BSA adducts. We selected the excitation wavelength of 352.3 nm for detection of heptadienal-BSA and control, as shown in Fig.8(A). Control BSA exhibited very low fluorescence emission with a broad range. On the other hand, fluorescence emission intensity of lipofuscin rose steadily after BSA was modified by heptadienal at concentrations from 0 to 10 mM. From the initial value of 2410.70 ± 103.45 (control), fluorescence intensity rose to 18716.23 ± 612.66 (concentration of heptadienal at 10 mM). However, lipofuscin fluorescence intensity was decreased to 4187.53 ± 452.88 when BSA was incubated with heptadienal at 50 mM. In addition, a red shift in the maximum emission wavelength of lipofuscin was observed when the concentration of heptadienal reached to 20-50 mM. The maximum emission wavelength of lipofuscin shifted to 468.9 nm when the concentration of heptadienal reach 20 mM, and further shifted to 477.8 nm when the concentration of heptadienal reached to 50 mM.

Similarly, lipofuscin fluorescence emission intensity increased with greater nonadienal concentration (0-10 mM), from 2360.20 ± 64.79 (the control) to 19989.53 ±603.07 (concentration of nonadienal at 10 mM) (Fig.8(B)). As the concentration of the modifier continued to increase, lipofuscin fluorescence emission intensity decreased to 12295.30 ± 738.64 when the concentration of nonadienal reached 50 mM; the maximum emission wavelength exhibited a weak red shift to 439.3 nm.

As previously reported, in malondialdehyde/glycine reaction systems, there was a red shift in the maximum emission wavelength of fluorescence lipofuscin with increased aldehyde concentration (Yin et al.,1994). Gardner et al.(1979) found that increase in conjugated groups led to the red shift in maximum fluorescence emission wavelength of lipofuscin. However, Vetter et al. (2011) pointed out that the change in the maximum emission wavelength may be due to the existence of multiple fluorophores with overlapping absorbance and emission spectra. Similar to our results, the reaction of BSA with oxidized oil also produced fluorescent lipofuscin, initially with a maximum emission wavelength at 410 nm, but exhibited an increasingly prominent shoulder at 425 nm with increase in degree of oxidation (Rampon et al.,2001).

The lipofuscin fluorescence emission intensity increased with greater concentration of decadienal (0-10 mM) (Fig.8(C)), from the initial 1830.13 ± 122.76 (control) to 22668.23 ± 403.97 (concentration of decadienal at 10 mM). With the increase in the concentration of decadienal (20-50 mM), the lipofuscin fluorescence emission intensity decreased to 17505.17 ±761.58 (50 mM); the maximum emission wavelength did not show a significant red shift.

Overall, a weak correlation was observed between fluorescent pigments and concentration of aldehydes (R= -0.284, $p = 0.586$ for heptadienal-BSA; R = 0.190, $p = 0.719$ for nonadienal-BSA; R = 0.435, $p = 0.389$ for decadienal-BSA).

Only a few of aldehyde-protein adduct products have fluorescence properties, which are also affected by various factors such as the type and concentration of aldehydes involved in the reaction, the type of protein or amino acid, the nature of the medium, the reaction temperature, and the intermediate products (Chelh et al.,2007).

Fluorescent lipofuscin usually have certain structural characteristics, such as an electron-donating group in conjugation with an imine, which is the source of fluorescence (Gardner et al.,1979). Pyridinium and pyrrole were confirmed to be the main sources of fluorescence during lipid oxidation and protein reactions by NMR and LC-MS/MS. Notably, these molecular structures contain the imino-ene conjugation required for fluorescence, while the other non-major fluorescent structures are not well-studied (Schaich et al.,2008) .

Model systems are usually used to explain the structure and formation mechanism of fluorescent lipofuscin. The most widely studied system is the formation of AGEs (Advanced glycation end products, also one of the sources of lipofuscin) by reducing sugar (which also has aldehyde groups that can participate in electrophilic addition) with protein or amino acid.

The typical fluorescent structures of AGEs are pyrraline, pyrroles, pentosidines, and crosslines (Méndez et al.,2007;Yin et al.,1996). Reaction between MDA and protein (or amino acid) results in addition products formed by lipid oxidation products and proteins (or amino acids / biomacromolecules containing free amino acid), which have been extensively studied. The group of products from MDA/protein reactions with strong fluorescence properties was proposed to be 1,4-dihydropyridine-3,5-dicarbaldehydes, which has a conjugated electron donor structure (Itakura et al.,1996) .

In addition, the reaction of 4-hydroxy-2-nonenal (HNE, with hydroxyl group on the side chain of molecule), 4-oxo-trans-2-nonenal (ONE), and epoxaldehyde with proteins could also lead to production of fluorescent lipofuscin products, which usually contain pyridine or pyrrole structures (Li et al.,2015) . However, there are few reports on the fluorescence properties of lipofuscins formed by proteins and unsaturated aldehydes without oxygen-containing groups in the side chain. Meynier et al.(2004) . pointed out that the maximum excitation and emission wavelengths of fluorescent compounds produced by 2-octeral and lysine residues of BSA was 350 nm and 440 nm respectively, and that the fluorescent compounds were pyridinium adducts. However, fluorescent compounds produced by dien-aldehyde and protein (or amino acid) have been less studied. Decadienal was the main research object in previous reports. Leake et al.(1985). found that reaction between 2,4-decadienal and lysine or alpha-N-acetyl lysine resulted in strong fluorescence with excitation at 355 nm and emission at 420 nm, while 2,4-decadienal alone gave no fluorescence. Zhu et al.(2010). found that the reaction of 2,4-decadienal with L-lysine on β-LG could not only form a Schiff base structure, but could also form structures such as a pyridinium adduct, which may be the source of fluorescence.

3.4.3 Changes in color characteristics

As shown in Fig.9(A-C), our results demonstrated that the various concentrations of modifiers increased the value of a *and b *, and decreased the value of L* in BSA ($p < 0.05$). When the modifiers concentration was low (1-10 mM), the value of a *and b * of aldehyde-BSA adducts was rapidly increased, while the value of L * was sharply decreased. The greater b * value indicated that higher modifier concentration leads to increased yellowness of aldehyde-BSA adducts; The increase in a * value was indicative of an increase in the red degree of the samples, while the decrease in L * indicated that the aldehyde-BSA adducts complex became darker when the concentration of the modifiers was increased.

When the modifiers concentration was 20-50 mM, the color of aldehyde-BSA adducts changed slowly. The results showed that the yellowness and redness of the protein increased sharply at low aldehyde concentrations. However, the increasing range of color was weakened at high modifier concentrations, and the a * and b * values of heptadienal-BSA was decreased. When the concentration of the modifiers were 10 mM, the a * values of heptadienal-BSA, nonadienal-BSA, and decadienal- BSA increased from -0.03 ± 0.03 in the control to 8.39 ± 0.81, 9.17 ± 0.17, and 7.92 ± 0.33, respectively (Fig.8(A)); their b * values increased from 4.24 ± 0.04 in the control to 17.95 ± 0.45, 21.58 ± 0.28, and 20.87 ± 0.16, respectively (Fig.8(B)).

The value of L * decreased drastically with the concentration of the modifiers was increased (1-50 mM). The L* value of heptadienal-BSA decreased sharply with increase in modifiers concentration (20 mM), from 95.13 ± 0.26 to 71.98 ± 1.15, which was 24.33 ± 1.21% lower than that of the control. However, when modifier concentrations were in the range of 20-50 mM, the L * value of heptadienal-BSA was reduced as the concentration of modifiers was increased. L * decreased to 68.63 ± 1.50 when heptadienal concentration was 50 mM, which was 27.86 ± 1.58% lower than that of the control (Fig.9(C)).

The L * of nonadienal-BSA and decadienal-BSA with 10 mM modifier were 80.48 ± 1.42 and 82.64 ± 0.46, respectively, which were 15.40 ± 1.49% and 13.13 ± 0.48% lower than those of the control, respectively. When the concentration of the modifiers was increased to 50 mM, the L * of nonadienal-BSA and decadienal-BSA

were 77.96 ± 1.20 and 81.44 ± 0.64, respectively, which were 18.05 ± 1.26% and 14.39 ± 0.67% lower than that of the control, respectively (Fig.9(C)).

The nonenzymatic browning of biomacromolecules is mainly due to the reaction of carbohydrates or lipid oxidation products (such as aldehydes) with various nitrogen-containing substances, such as amino acids, peptides or proteins (Hidalgo et al.,2000). It has been previously reported that these two kinds of browning reactions are generally accompanied by an increase in a *, b *, and a decrease in L * , similar to our results (Bosch et al., 2007) . Moreover, some scholars proposed that there were no significant chemical differences between biomacromolecule browning caused by lipid oxidation products such as aldehydes) and sugars (Hidalgo et al.2000; Hidalgo et al.,1999). The main melanoidins generated from these two browning reactions are furans, pyrroles, pyrazines, and pyridines (Wang et al.,2011). Some researchers pointed out that the Schiff-base structure formed by amino groups and aldehydes produced from lipid oxidation are source of colored substances (Gardner et al.,1979) .

4 Principal Component Analysis (PCA)

PCA was conducted to explore the relationships between the variables, and to evaluate the effects of these variables on aldehyde-modified BSA. We obtained two principal components, which accounted for 97.496% of the total variance; PC1 explained 77.343%, while PC2 explained 20.153% of the total variance. As can be seen from the factor loading plot (Fig.10(A)), during formation of protein bound carbonyls, a* and b* were positively correlated with PC1, the retention ratio of free amino content, the protein surface value, and the intrinsic fluorescence; L* was negatively correlated with PC1, while lipofuscin fluorescence was positively correlated with PC2.

Fig.10(B) shows the total score plots of aldehyde-BSA samples, which represent the comprehensive adverse effects of these aldehydes on proteins. As shown by Fig.10(B), the overall adverse effect on BSA are aldehyde-mediated enlargements. When the concentration of the modifiers increased to 20-50 mm, heptadienal had the greatest effect on BSA, followed by nonadienal, and finally decadienal, which was consistent with results from previous analysis.

5 Conclusion

Incubation of BSA with three different long-chain dien-aldehyde (trans, trans-2,4-heptadienal, trans,trans-2,4-nonadienal and trans, trans-2,4-decadienal) all led to protein side chain modification and formation of fluorescent lipofuscin, as well as changes of protein structure and protein aggregation. The three aldehyde-protein adducts showed different degrees of a yellowish-brown color, and their UV-Vis absorption curves were similar. In general, the degree of protein modification by the three dialdehydes showed significant concentration dependence, and was also affected by modifier chain length. In addition, the reaction rules of the three aldehydes with BSA, as well as the product characteristics, were found to be very similar. In general, the three aldehydes without oxygen-containing side chains selected in the experiment were comparable in BSA modification at low concentrations. However, at high concentrations, we found that the shorter the chain length, the more significant the modification effect; this was also consistent with the PCA results. In the concentration range selected in the experiment, a correlation study was performed to establish links between oxidative modification parameters and concentration of aldehydes. We found relatively clear correlations between formation of protein bound carbonyls, the retention ratio of free amino content, the maximum UV-Vis absorption value, and the concentration of aldehydes. However, the correlation between LFLP and the concentration of aldehydes was not linear. PCA of the relationship between the various oxidation parameters demonstrated that fluorescence lipofuscin was highly correlated with PC2, while other oxidation parameters were highly correlated with PC1.

Results from this study are helpful for us to better understand the properties of proteins modified by long-chain unsaturated aldehydes without oxygen-containing side chains, and can enrich our knowledge about

aldehyde-protein adducts. We will consider further mechanism exploration in future research.

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Conflict of interest

The authors declare that there is no confict of interest.

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