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Laccase-catalyzed cross-linking of BSA mediated by tyrosine

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Abstract

Tyrosine was explored as a cross-linking agent to form cross-linked bovine serum albumin (BSA) using laccase as a catalyst. Liquid chromatography-mass spectrometry (LC-MS) and fluorescence spectra indicated that tyrosine can be mainly oxidized to be dityrosine. Spectra analysis and molecular weight were used to characterize the BSA treated with tyrosine and laccase. Both SDS-PAGE and size exclusion chromatography confirmed the formation of cross-linked BSA, while most of the protein products existed as BSA–tyrosine conjugates. The MALDI-TOF analysis revealed that five tyrosine units were grafted on one BSA monomer, however one cross-linked BSA consists of two BSA monomers and 18 tyrosine. Furthermore, the content of the amino acid of BSA was identified using amino acid analysis, among those the percentage of lysine presented a visible decline from 12.36% to 11.43%, corresponding to 4-5 lysine residues. The pure and modified BSA were hydrolyzed by trypsin and the corresponding peptides were obtained. Different mass of five peptides from LC-MS spectra after hydrolysis indicated that tyrosine could react with Lys-136, Lys-204, Lys-224, Lys-322 and Lys-537 in BSA, promoting the formation of BSA–tyrosine conjugates and cross-linked BSA.

Keywords: Cross-linking; Bovine serum albumin; Tyrosine; Laccase

1. Introduction

Protein cross-linking is the process of binding two or more protein molecules together via covalent bonds which can lead to increasing stability of protein structure and resistance to chemicals [1-5]. The cross-linking of proteins into one large macromolecule has been studied in the field of food chemistry [6-9], textile [4, 10-13-9] and others. To achieve this, specific crosslinking reagents are used to form new covalent bonds. Both chemical reagents and enzymes can be used for protein cross-linking reactions. Transglutaminase (TGase, E.C. 2.3.2.13) is described to cross-linking of proteins based on lysine and glutamine residues: specifically, TG catalyzes the acyl transfer reaction between γ-carboxamide groups of glutamine and the ε-amino group of lysine residues in protein to form the ε-(γ-glutamyl)-lysine bond [5, 14-17]. Laccase enzymes are also described to catalyze the crosslinking of proteins with phenolic type moieties [6-8, 18-22].

Laccase (EC 1.10.3.2) can catalyze a wide range of substrates such as phenols, aromatic, aliphatic amines and their derivatives [23-25]. Catechol [26-29] was used to explore the mechanism of laccase catalyzed reaction. Cinzia *et al*. described a biosynthetic pathway to synthesis resorcinol/2,5-diaminobenzenesulfonic dyes using *Pleurotus ostreatus* POXA1b laccase [30]. Hossian *et al*. studied the effect of alkyl chain length on the polymerization of gallic acid [31]. On the other way, Yu *et al*. proved β-cyclodextrin can be grafted on to tyrosine and wool fabric via laccase/2,2,6,6-tetramethylpiperidine-1-oxyl [32]. Jiang *et al*. investigated laccase-catalyzed cross-linking of α-lactalbumin and ferulic acid [18]. Zhou *et al*. grafted chitooligosaccharide onto silk fibroin. Meanwhile D-Glucosamine and p-hydroxyphenylacetamide were used as the model compound of tyrosine residues in silk fibroin to disclose the grafting mechanism [33]. The primary OH groups of phenols are oxidized by laccase with or without mediate to obtain quinones that can react with ε-amino groups to form Schiff bases or Maillard reactions. In addition, laccase was proved to oxidize tyrosine [34, 35], cysteine and tryptophan. Vimbainashe *et al*. proved laccase-mediated crosslinking of amadumbe dough for potential gluten-free applications [36]. Wang and Zhu *et al*. proved tyrosine-containing peptide was covalently grafted onto the fibroin surfaces using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride [19] and genipin [37], respectively.

Bovine serum albumin (BSA) is an α-helix globular protein and its structure is quite different from α-lactalbumin, α-casein and silk fibroin [38]. BSA contained 40 tyrosine residues and 75% of those tyrosine residues are located inside the globular structure [38]. As BSA showed poor reactivity with laccase, grafting tyrosine-containing peptide, or phenolic materials on BSA is an effective method to promote the formation of cross-linked proteins. Some reports indicated that phenolic materials such as caffeic acid [8], ferulic acid [6, 7, 18, 20, 21], vanillin acid, gallate and *p*-coumaric acid [7], can be used in laccase-catalyzed cross-linking of proteins. Quercetin and

catechin showed the ability to react with *α*-casein and BSA, promoting the formation of protein conjugates or dimers [22]. The polymerization of phenolic materials was not controllable in the enzymatic process, leading to the different colors of the reaction solution and inevitable dyeing of cross-linked protein products [39, 40]. Otherwise, tyrosine was oxidized to dimers whose solution are transparent without additional oxygen. Till now no report adopted tyrosine as a cross-linking agent to accomplish the cross-linking of BSA.

In this study, bovine serum albumin (BSA) was used as a model protein and tyrosine was applied as a crosslinking agent. The aim of this study is to explore a new approach to obtain cross-linked BSA using tyrosine as a cross-linking agent by laccase.

2. Material and methods

2.1 Materials

Laccase (EC 1.10.3.2) from *Myceliophthora thermophila* was supplied by Novozymes, Denmark. Bovine serum albumin Fraction V (BSA) was provided by Sigma-Aldrich and used without further purification. Tyrosine, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and Trypsin (EC 3.4.21.4) were also purchased from Sigma-Aldrich. Reagents used in SDS-PAGE analysis were purchased from Bio-Rad Laboratories, Inc. (Hercules, USA). All other reagents were of analytical grade and provided by Sinopharm Chemical Reagent Co. Ltd (Shanghai, China).

2.2 Laccase activity

Laccase activity was measured by incubating 0.1 mL of diluted laccase and 0.1 mL of freshly prepared 5mM ABTS in 0.1 M acetate buffer (pH 5.0). The absorbance of ABTS was measured at 420 nm using a Synergy^{TM} H1 and recorded every 1 minute for 10 minutes. One unit of laccase (U) is defined as the amount of laccase required to oxidize 1 μmol of ABTS per minute [22].

2.3 Tyrosine catalyzed by laccase

Tyrosine (0.4 mg/mL) was diluted in 0.1 M acetate buffer (pH 5.0) with or without laccase (6 U/mL) in a shaker metal bath. The reaction temperature, time and speed were set at 40 ^{o}C, 6 h and 500 rpm, respectively. After the reaction, laccase was removed by ultrafiltration. The obtained liquid was divided into two parts, one part was kept at 4 ^{o}C for UV-Vis, fluorescence and LC-MS measurement. The other part was collected by freeze-drying and analyzed by FTIR.

2.4 Preparation of BSA-tyrosine complex using laccase

Mixture solution of 0.05% w/v BSA and tyrosine complex (ratio of BSA/tyrosine was 0.25) [41] was adopted for laccase treatment. 6 U/mL laccase was added to the mixture with no laccase added as controls. The mixed solution was equilibrated at 40 ^{o}C for 6 h with a speed of 500 rpm in a shaker metal bath. To separate unreacted tyrosine from the solution, samples were ultrafiltrate with 30 kDa. The filtrate (down layer) was collected for UV-Vis and fluorescence detection, while proteinic products (upper layer) were frozen dry to obtain the BSA-tyrosine complex.

Untreated BSA and part of BSA-tyrosine complex (1 mg/ml) were hydrolyzed by trypsin at 40 ^{o}C for 24 h and followed by a water bath at 100 ^{o}C for 5 min. Hydrolyzed samples were centrifugated at 8000 rpm for 10 min, and the obtained supernatants (contain peptides) were prepared for LC-MS analysis. The remaining BSA-tyrosine complex was kept for characterization.

2.5 Characterization of self-assembly tyrosine and tyrosine-BSA complex

2.5.1 FTIR spectroscopy

The spectra of self-assembly tyrosine and tyrosine-BSA complex were detected using a Thermo Nicolet iS10 spectrometer (Thermo Fisher Scientific Co., Waltham, MA, USA). Potassium bromide (KBr) powder was used as the matrix for background signal scanning. The samples mixed with KBr with a weight ratio of 1:100. The spectra were recorded at a wavelength from 4000 to 600 cm^{-1}, with a resolution of 8 cm^{-1} and 32 scans.

2.5.2 LC-MS

The filtrates of tyrosine treated with and without laccase (mentioned in 2.3) as well as the supernatants of hydrolyzed BSA samples (mentioned in 2.4) were clarified through 0.22 um Whatman microfilter and 1.5 ml aliquots were transferred into clean vials. All samples were confirmed by LC-MS analysis on an Acquity UPLC separation module coupled with Quattro Premier XE^{TM} Mass Spectrometer (Waters, USA). Separation of samples was carried out on an Acquity BEH C18 column (2.1 mm × 50 mm, 1.7 um) at the column temperature of 45 ℃, with a flow rate of 0.3 mL/min. The elution solvents consisted of 0.1% formic acid and methanol. Peaks were analyzed by software MassLynx V4.1. The reacted residues in BSA can be determined by comparing the detected molecular mass of peptide between BSA and BSA-tyrosine complex. The predicted molecular mass of peptide of pure BSA hydrolyzed by trypsin was provided by ExPASy resources [42].

2.5.3 UV-Vis and fluorescence spectra analysis

Synergy^{TM} H1 was used to record UV-Vis and fluorescence spectra of self-assembly tyrosine. For UV-Vis absorption measures, data were collected from 240 to 600 nm in 2 nm steps. Due to the fluorescence characteristics of dityrosine, its formation can be monitored by the fluorescence spectrum. The emission wavelength was fixed at 360 nm [43], spectra were scanned in the range of 390 to 600 nm with 2 nm path length.

2.5.4 Determination of amino and phenolic hydroxyl content

Unreacted tyrosine and self-assembly tyrosine were separated from mix solutions by ultrafiltration (30 kDa), The obtained filtrates (down layer) were used to determine the amount of amino and phenolic hydroxyl following o-phthalaldehyde (OPA) [44] and Folin-Ciocalteu [45] method, respectively. The Folin-Ciocalteu method was described as followed. 0.2 ml of samples were mixed with 1 ml of 10% Folin-Ciocalteu solution for 7-8 min, then 0.8 ml of 7.5% sodium

carbonate was added. The samples were incubated for 1 h at room temperature. Absorbance was detected at 765 nm, and the amount of phenolic hydroxyl can be calculated from the calibration curve measured using gallate.

The OPA solution was prepared first. 1.9005 g of borax was completely dissolved in 37.5 ml of deionized water, then 1ml of 4% OPA (w/v in ethanol) and 100 ul of *β*-mercaptoethanol were added. The mixture solution was diluted to 50 ml by deionized water. 0.1 ml of samples were mixed with OPA solution for 1-2 min and then measured at the wavelength at 340 nm. Calibration curves were carried out using L-serine.

2.5.5 Circular dichroism (CD) spectroscopy

Circular dichroism (CD) spectra were used to evaluate the changes in the secondary structure of BSA products. Samples were dissolved in 50 mM acetate buffer (pH 5.0) to form a concentration of 0.05 mg/mL followed by recorded from 190-280 nm with 1 cm path length using Chiraxcan V100. SELCON3 method included in CDPro software was used to analyze the CD spectra [46].

2.5.6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight distribution of BSA before and after tyrosine grafting were analyzed by SDS-PAGE using 12.5% separating Tris-HCl gels on mini-protein tetra electrophoresis (Bio-Rad Laboratories, Inc., Hercules, USA). The range of molecular mass maker is 10-250 kDa. The 30 μl of protein products (0.1 mg/ml) was mixed with 9 μl of 4X Laemmli sample buffer and 1 μl of *β*- mercaptoethanol, and then heated in boiling water for 5 min. 10 ul of samples were loaded onto the wells and SDS-PAGE was operated at 120 voltage. After electrophoresis, Coomassie Bright Blue R250 was adopted to gel staining for 1 h followed by decolorization with water overnight.

2.5.7 MALDI-TOF mass analysis

MALDI-TOF was also used to detect the molecular mass of BSA products using a Bruker ultraflextreme MALDI-TOF Mass Spectrometer (Bruker AXS Co., Germany). The BSA products were dissolved in 1% trifluoroacetic acid (TFA) and sinapic acid (SA) was used as the matrix. The samples and matrix mixed with the volume ratio of 1:1 (1 ul each). The sample/matrix mixtures were deposited on a steel target matched with MALDI followed by air-dry at room temperature. The dried samples were conducted of positive-ion mass spectra.

2.5.8 Size exclusion chromatography

The ultrafiltration solution of pure BSA and BSA-tyrosine complex were further detected with an Ultrahydrogel Waters liner column (Waters, USA). The size exclusion chromatography (SEC) mobile phase was 50 mM phosphate buffer, containing 0.3 M NaCl at pH 7.0. 10 μl of samples (0.5 mg/ml) were injected with a flow rate of 0.5 mL/min. Spectra were collected at 214 nm with a UV-Vis detector linked to the SEC system (Waters, USA).

2.5.9 Amino acid analysis

Amino acid composition of BSA products was carried out using an Agilent 1100 series HPLC system (Agilent Technologies, USA). 100 mg of each sample was dissolved in 8 ml of 6 M HCL, and hydrolyzed for 22 h at 120 ^{o}C. After that, 4.8 ml of 8 M NaOH was added into the tube for neutralizing and diluted to 25 ml by deionized water. Obtained samples were filtered and then centrifugated at 15000 rpm for 10 min. 400 µl of supernatant was transferred to vials for measuring the amount of amino acid.

3. Results and discussion

3.1 Analysis of tyrosine oxidation products

The oxidation of tyrosine can be conducted in two ways as previously reported [34, 35, 43, 47]. Two tyrosine units linked via a C-C biphenyl bond named dityrosine, while the other coupled dimer of tyrosine linked via a diphenyl ether bond called isodityrosine [47]. Furthermore, the identification of dityrosine was done based on its characteristic fluorescence [43, 48-51]. And isodityrosine can be measured by the amount of free phenolic hydroxyl contents.

MS analysis, the mass-to-charge (m/z) ratio at 182 and 165 attributed to tyrosine and tyrosine loss of NH_2 (Fig.1 (a)). Tyrosine oxidized by laccase showed two peaks at m/z=362 and m/z=378 were probably contributed dimer tyrosine and dimer tyrosine with Na^+ (Exact mass $[2Tyrosine+Na]^+$= 383). The fragment ions at m/z=340 and m/z=322 could be explained by the loss of COOH and NH_2-CH-COOH from parent ion of $[2Tyrosine+Na]^+$ [36], respectively. As we can see in Fig. 1(b), peaks at 2930~3000 cm^{-1}, 1545 cm^{-1} and 1419 cm^{-1} were attributed to C-H stretching, C=C stretching vibration of the benzene ring and C-H bending, respectively. The absorbances of phenolic hydroxyl in tyrosine (3208 cm^{-1}) decreased, indicating that self-assembly of tyrosine by laccase occurred in the enzymatic process. Moreover, ether bonds were not detected, indicating no isodityrosine formed.

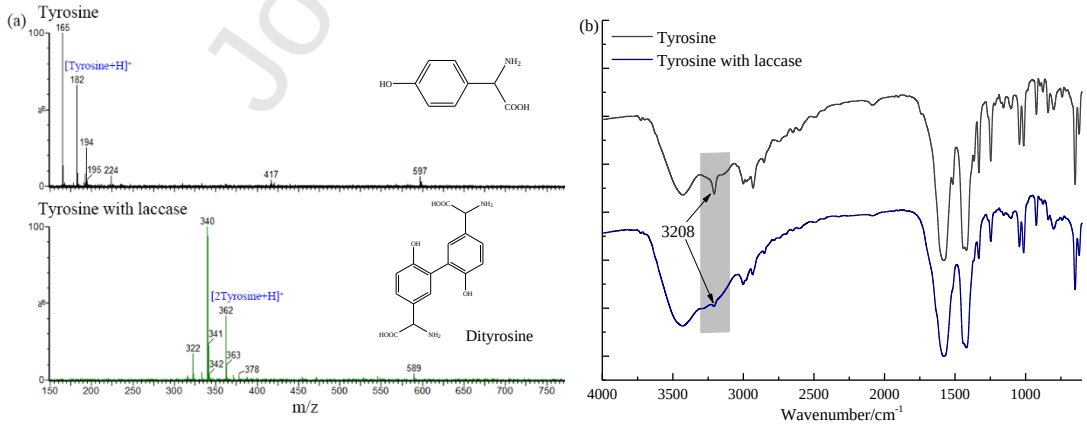

Fig. 1 LC-MS (a) and FTIR (b) spectra of tyrosine treated with or without laccase

Although, no addition peak presented in UV-Vis spectra (Fig. 2(a)), the fluorescence spectra (Fig. 2(b)) showed a significative peak around 450 nm attributed to dityrosine. While the free

phenolic hydroxyl contents showed in Table 1 decreased slightly. The results suggested that two tyrosine were formed into dityrosine other than being isodityrosine. The ability of laccase to oxidize tyrosine resulting in dimerization provided a new approach for cross-linking of protein.

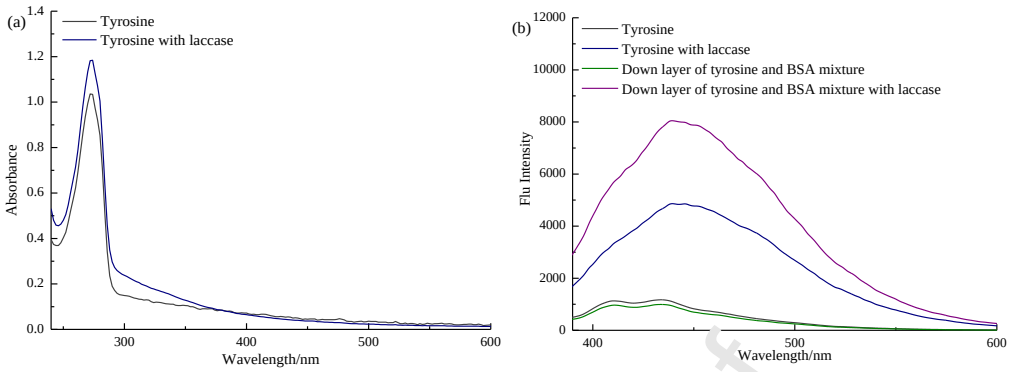

Fig. 2 UV-Vis of tyrosine (a) and fluorescence spectra of tyrosine and down layer of tyrosine with BSA mixture solution by ultrafiltration (b)

Table 1 Free phenolic hydroxyl contents of tyrosine solution treated with or without laccase

Sample	Free phenolic hydroxyl content (mg/L)
Tyrosine	260.72±1.41
Tyrosine with laccase	248.61±3.38

3.2 Spectroscopic analysis by FTIR, UV-Vis and fluorescence

To achieve the cross-linking of BSA, both self-assembly of tyrosine and tyrosine grafting on BSA need to be accomplished. As shown in Fig.2(b), dityrosine also could be seen when BSA was included in the tyrosine solution with laccase. In addition, the success of grafting tyrosine on BSA was proved by the decreasing of free amino content (Table 2).

Table 2 Free amino contents of upper layer of tyrosine and BSA mixture untreated and treated with laccase

Sample	Free amino content (mmol/L)
Tyrosine + BSA	3.10±0.11
Tyrosine + BSA with laccase	2.12±0.17

The protein product was analyzed by FTIR spectroscopy. As shown in Fig. 3(a), all the protein products showed the typical peaks near 1,650 cm^{-1} and 1,562 cm^{-1} attributed to amide I and amide II, respectively. The peak of amide I moved to a lower wavelength due to the reduction of α-helix content [52]. The cross-linking between tyrosine and BSA catalyzed by laccase was confirmed by a carbonyl bond presenting near 1700 cm^{-1} attributed to C=O stretching vibration bond [32]. Also, the absorption peak was observed near 804 cm^{-1} corresponding to the benzene ring in tyrosine.

Untreated BSA and BSA incubated with laccase performed similar spectra with the typical

peaks of α-helix (192 nm, 209 nm and 222 nm). BSA incubated tyrosine showed two peaks around 194 nm and 197 nm (Fig. 3(b)) and their molar ellipticity values were declined due to the decreasing of α-helix content. The results were consistent with FTIR analysis and other reports about BSA treated with metal ions [53,54] or chemical reagent [55-57]. The secondary structure contents of each sample were calculated and shown in Table 3. In contrast with untreated BSA, the α-helix and β-sheet of the BSA complexes decreased by 6.4% and 3.6%, respectively, while the structure of β-turn and random curl increased by 0.4% and 8.8%. Tyrosine grafting had a slight impact on the structure of BSA complexes, still showed a high content of α-helix (58.3%) after grafting. Compare to the data from PBD 3V03 (www.rcsb.org), BSA products performed the decrease in the amount of α-helix and β-sheet increase. It could be explained by water competed for the hydrogen with amino and carboxyl groups of protein in the freeze-drying process [54, 58].

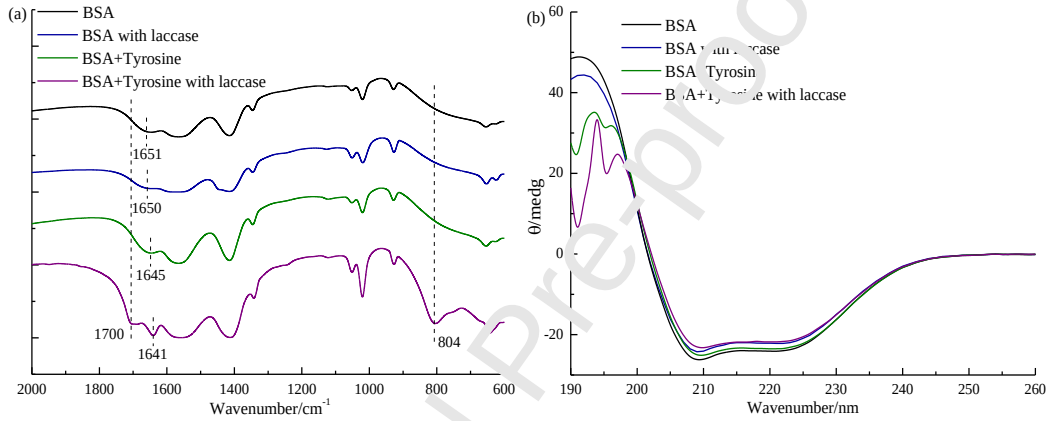

Fig.3 FTIR (a) and CD (b) spectra of BSA complexes

Table 3 The secondary structures of BSA complexes estimated by circular dichroism (CD) spectra

Sample	α-helix (%)	β-sheet (%)	β-turn (%)	Random coil (%)
BSA	64.7	25.9	4.8	5.4
BSA with laccase	63.7	25.4	4.3	7.7
BSA + Tyrosine	60.9	23.5	5.8	10.1
BSA + Tyrosine with laccase	58.3	22.3	5.2	14.2

3.3 Characteristic of cross-linked BSA

The molecular weight distributions of pure BSA and modified BSA catalyzed by laccase were performed by SDS-PAGE. The band of laccase was hardly detected (line 3 in Fig.4(a)) due to its low amount (6 U/mL). When only laccase or tyrosine was incubated, it impacted on the secondary structure of BSA, but tyrosine residues in BSA cannot oxidize by laccase to form stable chemical bonds directly (line 4 and line 5 in Fig.4(a)) [22]. When adding both tyrosine and laccase in BSA solution, the band migrated to higher molecular weight (line 6 in Fig.4(a)), indicating tyrosine and BSA were linked by stable covalent bonds in the enzymatic process. The SDS-PAGE

results were not convincing enough to prove the success of cross-linked BSA formation, so the SEC was conducted for further research. As shown in Fig. 4(b), the major peak at 32 min and a small peak at 26 min of untreated BSA were assigned with BSA monomer and dimer BSA. The time of major in two curves was almost unchanged due to the small difference between their molecular weight. Furthermore, it also can be seen a wider peak at 24 min assigned to the cross-linked BSA linked by tyrosine dimers.

MALDI-TOF analysis was used to measure the molecular mass of protein products in quantity. Two significant peaks can be seen in Fig.4(c), the molecular mass near 67 kDa ($[M+H]^+$) and 134 kDa ($[M+2H]^{2+}$) represent BSA monomer and dimer BSA, respectively. When both tyrosine and laccase existed, the molecular mass shifted to 67.73 kDa ($[M+H]^+$) and 137.02 kDa ($[M+2H]^{2+}$) which were higher than that of untreated BSA. It revealed that most BSA monomer was reacted with tyrosine to form covalent bonds, but only part of BSA monomer was cross-linked to form BSA-[Tyrosine-Tyrosine]$_n$-BSA conjugate. The differences molecular weight between unmodified and modified BSA were 1.0 kDa and 3.0 kDa, corresponding to five tyrosine and 18 tyrosine were grafted on BSA monomer and cross-linked BSA, respectively.

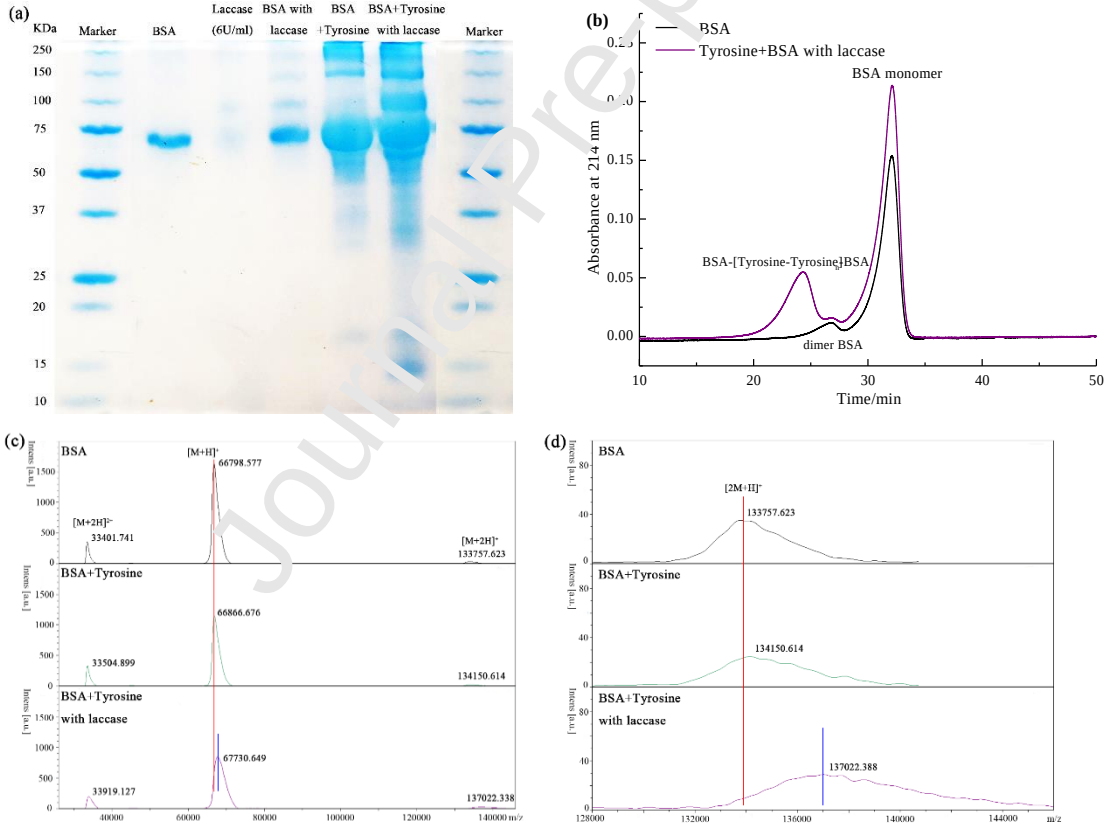

Fig.4 Molecular weight analysis of BSA complexes (a) SDS-PAGE, (b) SEC spectra, (c) MALDI-TOF: 27000~147000 m/z, (d) MALDI-TOF: 128000~147000 m/z

To sum up, the BSA products mainly existed in two forms, we called them BSA-tyrosine conjugate and cross-linked BSA. In this case, the enzymatic process could be described in two ways (Fig.5). The first way, tyrosine reacted with the primary amino groups in BSA monomer to

form BSA-tyrosine conjugate. On the other way, dityrosine was formed and followed by grafting on the BSA monomer to assemble cross-linked BSA. Thus, tyrosine can be used as a cross-linking agent.

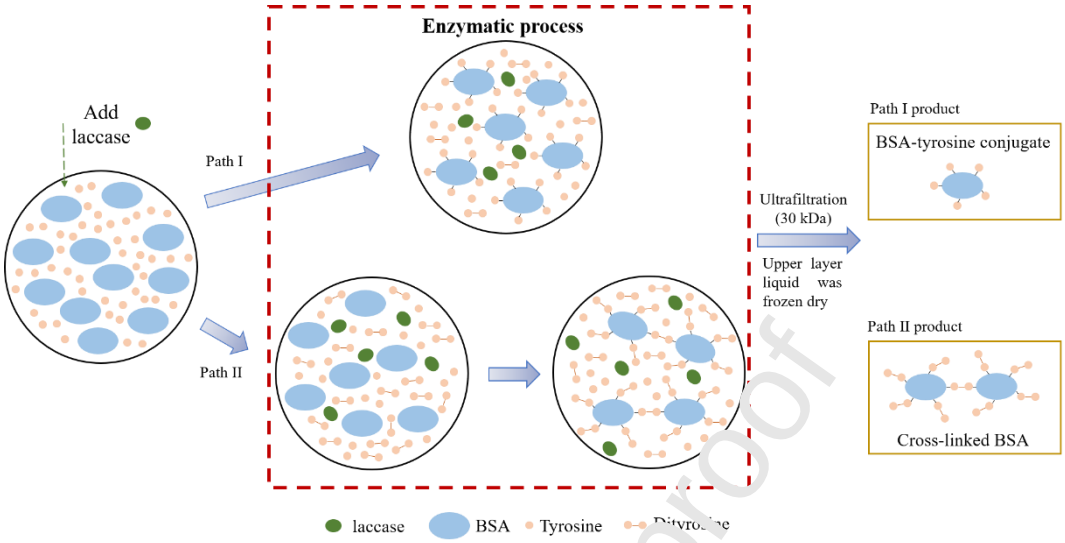

Fig. 5 Proposed reaction process and structure of BSA-tyrosine conjugate and cross-linked BSA

3.4 Identification of reacted amino acid residues in BSA

The amino acid contents of unmodified and modified BSA were measured to determine the certain amino acid residues in BSA reacted with tyrosine. Both lysine and arginine can provide primary amino groups. Comparing with untreated BSA, lysine content of cross-linked BSA presented a more visible decrease from 12.36% to 11.43% (4-5 lysine residues) than arginine (Table 4). In our study, tyrosine was mainly reacted with lysine in BSA. The relative solvent accessibility (RSA) of amino acid residues can be used to determine if the amino acid on the surface of the protein reacted or not. RSA of lysine in BSA (Table S1) was obtained by PYMOL software using the amino acid sequence based on crystal structure of bovine serum albumin (PDB ID: 3V03, Chain A). In addition, most of the lysine residues in BSA are on the surface (RSA> 7% [59]) which means they can react with tyrosine catalyzed by laccase.

Table 4 Amino acid contents of pure BSA and BSA treated with tyrosine and laccase

Sample	Arg (%)	Lys (%)
BSA	6.01±0.19	12.36±0.26
Tyrosine + BSA with laccase	6.11±0.62	11.43±0.45

Furthermore, LC-MS of the peptides obtained by trypsin hydrolyze can provide the specific reactive lysine of BSA. Five peptides showing different mass means that five tyrosine was grafted on BSA monomer by laccase which was consistent with results from MALDI-TOF analysis. On the contrary, none of the obtained peptides was related to dityrosine grafted on BSA or dimer BSA,

as the yield of BSA-[Tyrosine-Tyrosine]$_n$-BSA conjugate (cross-linked BSA) was low. As can be seen in Fig. 6, peptide T1 showed m/z=391 attributed to sequence FPK, referring to the report [42], and m/z=554 can be explained by [FRK + tyrosine], relating to Schiff base formation between tyrosine and Lys-224 in BSA. Other peptides (Fig. S1) with the mass of 508, 536, 649 and 752 were attributed to peptide sequence HKPK (T2), FWGK (T3), CASIQK (T4) and NYQEAK (T5) [42], respectively. Meanwhile, the mass of 728, 723, 847 and 952 can be speculated by [HKPK + tyrosine(K^+)], [FWGK + tyrosine (Na^+)], [CASIQK + tyrosine (Na^+)] and [NYQEAK + tyrosine (Na^+)], corresponding to Lys-537, Lys-136, Lys-204, Lys-224 and Lys-322 (Fig.7 (a)) in BSA were reacted with tyrosine by laccase formed Schiff base or Maillard reactions.

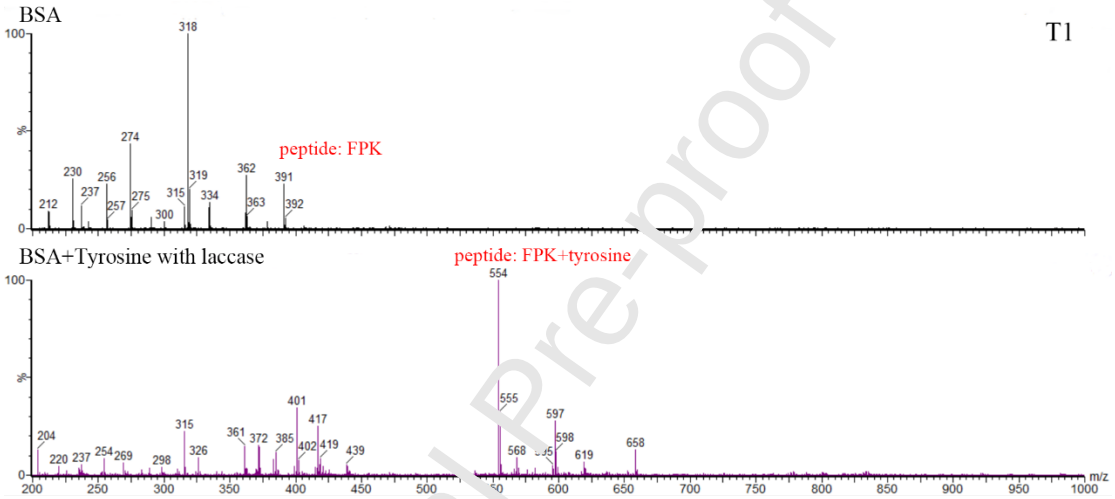

Fig. 6 LC-MS of peptide T1 obtained by trypsin hydrolysis of pure BSA and BSA treated with tyrosine and laccase

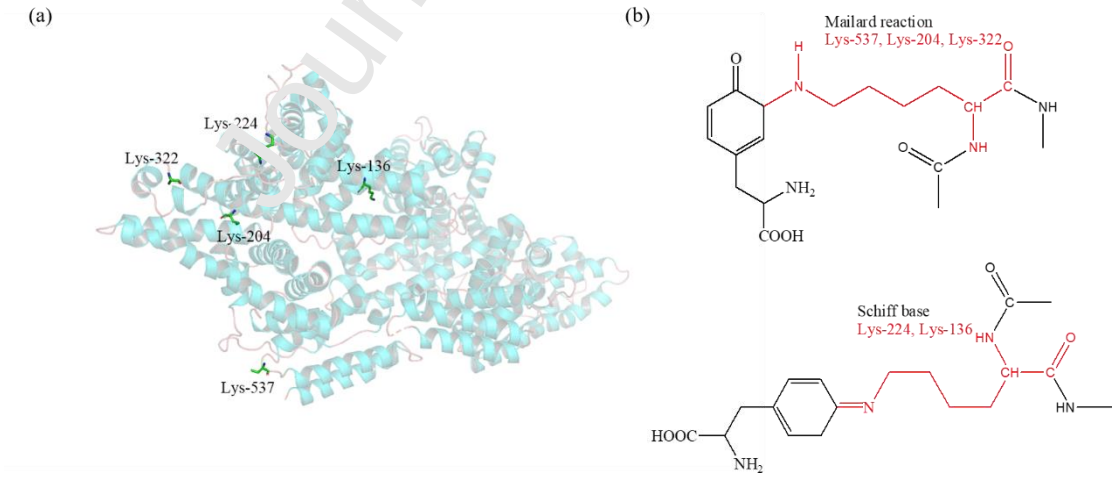

Fig. 7 Reactive lysine residues in BSA showed as sticks (a) and possible chemical reactions between tyrosine and lysine (b)

4. Conclusion

In this study, cross-linked BSA was formed successfully using tyrosine as a cross-linking agent. The spectra characterizations indicated that laccase could oxidize tyrosine to dityrosine

efficiently, it also catalyze Schiff base or Maillard reactions between tyrosine/dityrosine with primary amino groups in BSA. SDS-PAGE, SEC and MALDI-TOF analysis indicated the changes in molecular weight of BSA, showing the proposed structure of BSA-tyrosine conjugate and cross-linked BSA. Five tyrosine was grafted on BSA monomer, and cross-linked BSA was consist of two BSA monomer and 18 tyrosine. Amino acid analysis and LC-MS confirmed that tyrosine reacted with Lys-136, Lys-204, Lys-224, Lys-322 and Lys-537 in BSA, however the grafting of tyrosine on proteins by laccase occurs randomly. Tyrosine can be applied to other protein products as a new cross-linking agent, and the formed dityrosine or lysine-[tyrosine-tyrosine]$_n$-lysine can be used to modify the properties of other α-helix based proteins such as wool or hair.

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References

[1] T. Heck, G. Faccio, M. Richter, L. Thöny-Meyer, biotechnology, Enzyme-catalyzed protein crosslinking, Appl. Microbiol. Biotechnol. 97 (2013) 461-475. https://doi.org/10.1007/s00253-012-4569-z.

[2] C.M. Vaz, L.A. de Graaf, R.L. Reis, A.M. Cunha, Effect of crosslinking, thermal treatment and UV irradiation on the mechanical properties and in vitro degradation behavior of several natural proteins aimed to be used in the biomedical field, J. Mater. Sci. Mater. Med. 14 (2003) 789-796. https://doi.org/10.1023/A:1025040522513.

[3] J. Buchert, D. Ercili Cura, H. Ma, C. Gasparetti, E. Monogioudi, G. Faccio, M. Mattinen, H. Boer, R. Partanen, E. Selinheimo, Crosslinking food proteins for improved functionality, Annu. Rev. Food Sci. Techol. 1 (2010) 113-138. https://doi.org/10.1146/annurev.food.080708.100841.

[4] J. Cortez, P.L. Bonner, M. Griffin, Transglutaminase treatment of wool fabrics leads to resistance to detergent damage, J. Biotechnol. 116 (2005) 379-386. https://doi.org/10.1016/j.jbiotec.2004.12.007.

[5] J. Cortez, A. Anghieri, P.L. Bonner, M. Griffin, G. Freddi, Transglutaminase mediated grafting of silk proteins onto wool fabrics leading to improved physical and mechanical properties, Enzyme Microb. Technol. 40 (2007) 1698-1704. https://doi.org/10.1016/j.enzmictec.2006.10.013.

[6] H. Ma, P. Forssell, R. Partanen, J. Buchert, H. Boer, Improving laccase catalyzed cross-linking of whey protein isolate and their application as emulsifiers, J. Agric. Food Chem. 59 (2011) 1406-1414. https://doi.org/10.1021/jf103591p.

[7] E. Selinheimo, P. Lampila, M.-L. Mattinen, J. Buchert, Formation of protein−oligosaccharide conjugates by laccase and tyrosinase, J. Agric. Food Chem. 56 (2008) 3118-3128. https://doi.org/10.1021/jf0730791.

[8] M. Struch, D. Linke, A. Mokoonlal, J. Hinrichs, R.G. Berger, Laccase-catalysed cross-linking of a yoghurt-like model system made from skimmed milk with added food-grade mediators, Int. Dairy J. 49 (2015) 89-94. https://doi.org/10.1016/j.idairyj.2015.04.002.

[9] M. Færgemand, J. Otte, K.B. Qvist, Cross-linking of whey proteins by enzymatic oxidation, J. Agric. Food Chem. 46 (1998) 1326-1333. https://doi.org/10.1021/jf970743c.

[10] J. Cortez, P.L. Bonner, M. Griffin, Application of transglutaminases in the modification of wool textiles, Enzyme Microb. Technol. 34 (2004) 64-72. https://doi.org/10.1016/j.enzmictec.2003.08.004.

[11] J.P. McDevitt, J. Winkler, Method for enzymatic treatment of wool, US, US006051033A, 2000 (18 April).

[12] R. Lantto, C. Schönberg, J. Buchert, E. Heine, Effects of laccase-mediator combinations on wool, Text. Res. J. 74 (2004) 713-717. https://doi.org/10.1177/004051750407400809.

[13] M. Nasiriboroumand, V. Dutschk, M. Montazer, Laccase-mediated dyeing of wool fabric with Weld (Reseda Luteola L.), International Conference Tex Teh V-2012, Bucharest, Romania, Faculty of Engineering Technology, 2012.

[14] M. Griffin, R. Casadio, C.M. Bergamini, Transglutaminases: nature's biological glues, Biochem. J. 368 (2002) 377-396. https://doi.org/10.1042/bj20021234.

[15] J.A. Macedo, A.L.F. Cavallieri, R.L. Da Cunha, H.H. Sato, The effect of transglutaminase from Streptomyces sp. CBMAI 837 on the gelation of acidified sodium caseinate, Int. Dairy J. 20 (2010) 673-679. https://doi.org/10.1016/j.idairyj.2010.03.014.

[16] A.L.C. Gaspar, S.P. de Góes-Favoni, Action of microbial transglutaminase (MTGase) in the modification of food proteins: A review, Food Chem. 171 (2015) 315-322. https://doi.org/10.1016/j.foodchem.2014.09.019.

[17] J.M. Cardamone, J.G. Phillips, Enzyme-mediated crosslinking of wool. Part II: Keratin and transglutaminase, Text. Res. J. 77 (2007) 277-283. https://doi.org/10.1177/0040517507078788.

[18] Z. Jiang, X. Yuan, K. Yao, X. Li, X. Zhang, Z. Mu, L. Jiang, J. Hou, Laccase-aided modification: Effects on structure, gel properties and antioxidant activities of α-lactalbumin,

LWT. 80 (2017) 355-363. https://doi.org/10.1016/j.lwt.2017.02.043.
[19] P. Wang, X. Zhu, J. Yuan, Y. Yu, L. Cui, Y. Duan, Q. Wang, X. Fan, Grafting of tyrosine-containing peptide onto silk fibroin membrane for improving enzymatic reactivity, Fibers Polym. 17 (2016) 1323-1329. https://doi.org/10.1007/s12221-016-6460-5.
[20] A. Aljawish, I. Chevalot, J. Jasniewski, C. Paris, J. Scher, L. Muniglia, Laccase-catalysed oxidation of ferulic acid and ethyl ferulate in aqueous medium: a green procedure for the synthesis of new compounds, Food Chem. 145 (2014) 1046-1054. https://doi.org/10.1016/j.foodchem.2013.07.119.
[21] M.L. Mattinen, M. Hellman, P. Permi, K. Autio, N. Kalkkinen, J.J.J.o.a. Buchert, f. chemistry, Effect of protein structure on laccase-catalyzed protein oligomerization, J. Agric Food Chem. 54 (2006) 8883-8890. https://doi.org/10.1021/jf062397h.
[22] S. Kim, A. Cavaco-Paulo, Laccase-catalysed protein–flavonoid conjugates for flax fibre modification, Appl. Microbiol. Biotechnol. 93 (2012) 585-600. https://doi.org/10.1007/s00253-011-3524-8.
[23] S. Riva, Laccases: blue enzymes for green chemistry, Trends Biotechnol. 24 (2006) 219-226. https://doi.org/10.1016/j.tibtech.2006.03.006.
[24] T. Kudanga, B. Nemadziva, M. Le Roes-Hill, Laccase catalysis for the synthesis of bioactive compounds, Appl. Microbiol. Biotechnol. 101 (2017) 13-33. https://doi.org/10.1007/s00253-016-7987-5.
[25] R.M. Desentis-Mendoza, H. Hernández-Sánchez, A. Moreno, E. Rojas del C, L. Chel-Guerrero, J. Tamariz, M.E. Jaramillo-Flores, Enzymatic polymerization of phenolic compounds using laccase and tyrosinase from ustilago maydis, Biomacromolecules. 7 (2006) 1845-1854. https://doi.org/10.1021/bm060159p.
[26] T. Tzanov, C.J. Silva, A. Zille, J. Oliveira, A. Cavaco-Paulo, Effect of some process parameters in enzymatic dyeing of wool, Appl. Biochem. Biotechnol. 111 (2003) 1-13. https://doi.org/10.1385/ABAB:111:1:1.
[27] C.D. Blanco, M.D. González, J.M.D. Monmany, T. Tzanov, Dyeing properties, synthesis, isolation and characterization of an in situ generated phenolic pigment, covalently bound to cotton, Enzyme Microb. Technol. 44 (2009) 380-385. https://doi.org/10.1016/j.enzymictec.2008.02.003.
[28] J. Su, J. Noro, J. Fu, Q. Wang, C. Silva, A. Cavaco-Paulo, Enzymatic polymerization of catechol under high-pressure homogenization for the green coloration of textiles, J. Clean. Prod. 202 (2018) 792-798. https://doi.org/10.1016/j.jclepro.2018.08.205.
[29] J. Su, T.G. Castro, J. Noro, J. Fu, Q. Wang, C. Silva, A. Cavaco-Paulo, The effect of high-energy environments on the structure of laccase-polymerized poly (catechol), Ultrason. Sonochem. 48 (2018) 275-280. https://doi.org/10.1016/j.ultsonch.2018.05.033.
[30] C. Pezzella, S. Giacobbe, V.G. Giacobelli, L. Guarino, S. Kylic, M. Sener, G. Sannia, A.J.J.o.M.C.B.E. Piscitelli, Green routes towards industrial textile dyeing: A laccase based approach, J. Mol. Catal., B Enzym. 134 (2016) 274-279. https://doi.org/10.1016/j.molcatb.2016.11.016.
[31] K.M.G. Hossain, M.D. González, J.M.D. Monmany, T. Tzanov, Effects of alkyl chain lengths of gallates upon enzymatic wool functionalisation, J. Mol. Catal., B Enzym. 67 (2010) 231-235. https://doi.org/10.1016/j.molcatb.2010.08.011.
[32] Y. Yu, Q. Wang, J. Yuan, X. Fan, P. Wang, A novel approach for grafting of β-cyclodextrin onto wool via laccase/TEMPO oxidation, Carbohydr. Polym. 153 (2016) 463-470. https://doi.org/10.1016/j.carbpol.2016.08.003.
[33] Q. Zhou, L. Cui, L. Ren, P. Wang, C. Deng, Q. Wang, X. Fan, Preparation of a multifunctional fibroin-based biomaterial via laccase-assisted grafting of chitooligosaccharide, Int. J. Biol. Macromol. 113 (2018) 1062-1072. https://doi.org/10.1016/j.ijbiomac.2018.03.042.
[34] W. Jia, Q. Wang, X. Fan, A. Dong, Y. Yu, P. Wang, J. Yuan, Laccase-mediated dye-free coloration of wool fabric, Indian J. Fibre Text. Res. 43 (2018) 224-230. http://nopr.niscair.res.in/handle/123456789/44522.
[35] W. Jia, Q. Wang, X. Fan, A. Dong, Y. Yu, P. Wang, Mechanism and analysis of

laccase-mediated coloration of silk fabrics, Fibers Polym. 19 (2018) 868-876. https://doi.org/10.1007/s12221-018-7553-0.
[36] V.E. Manhivi, E.O. Amonsou, T. Kudanga, Laccase-mediated crosslinking of gluten-free amadumbe flour improves rheological properties, Food Chem. 264 (2018) 157-163. https://doi.org/10.1016/j.foodchem.2018.05.017.
[37] X. Zhu, P. Wang, L. Cui, Y. Yu, Q. Wang, X. Fan, Enhancement reactivity of Bombyx mori silk fibroins via genipin-mediated grafting of a tyrosine-rich polypeptide, J. Text. Inst. 108 (2017) 2115-2122. https://doi.org/10.1080/00405000.2017.1315795.
[38] K.A. Majorek, P.J. Porebski, A. Dayal, M.D. Zimmerman, K. Jablonska, A.J. Stewart, M. Chruszcz, W. Minor, Structural and immunologic characterization of bovine, horse, and rabbit serum albumins, Mol. Immunol. 52 (2012) 174-182. https://doi.org/10.1016/j.molimm.2012.05.011.
[39] B.P. Partlow, M.B. Applegate, F.G. Omenetto, D.L. Kaplan, Engineering, Dityrosine cross-linking in designing biomaterials, ASC Biomater. Sci. Eng. 2 (2016) 2108-2121. https://doi.org/10.1021/acsbiomaterials.6b00454.
[40] D. Zaguri, T. Kreiser, S. Shaham-Niv, E. Gazit, Antibodies towards tyrosine amyloid-like fibrils allow toxicity modulation and cellular imaging of the assemblies, Molecules. 23 (2018) 1273. https://doi.org/10.3390/molecules23061273.
[41] H. Chen, A. Ji, S. Qiu, Y. Liu, Q. Zhu, L. Yin, Covalent conjugation of bovine serum album and sugar beet pectin through Maillard reaction/laccase catalysis to improve the emulsifying properties, Food Hydrocoll. 76 (2018) 173-183. https://doi.org/10.1016/j.foodhyd.2016.12.004.
[42] ExPASy: SIB Bioinformatics Resource Portal, PeptideMass, https://web.expasy.org/cgi-bin/peptide_mass/peptide-mass.pl, 2005.
[43] G. Qin, S. Lapidot, K. Numata, X. Hu, S. Meirovitch, M. Dekel, I. Podoler, O. Shoseyov, D.L. Kaplan, Expression, cross-linking, and characterization of recombinant chitin binding resilin, Biomacromolecules. 10 (2009) 3227-3234. https://doi.org/10.1021/bm900735g.
[44] G. Huang, J. Hou, X. Zhou, A measurement method for atmospheric ammonia and primary amines based on aqueous sampling, OPA derivatization and HPLC analysis, Environ. Sci. Technol. 43 (2009) 5851-5856. https://doi.org/10.1021/es900988q.
[45] A. Blainski, G.C. Lopes, J.C.P. De Mello, Application and analysis of the Folin Ciocalteu method for the determination of the total phenolic content from Limonium Brasiliense L, Molecules. 18 (2013) 6852-6865. https://doi.org/10.3390/molecules18066852.
[46] N. Sreerama, R.W. Woody, Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set, Anal. Biochem. 287 (2000) 252-260. https://doi.org/10.1006/abio.2000.4880.
[47] S.C. Fry, Isodityrosine, a diphenyl ether cross-link in plant cell wall glycoprotein: Identification, assay, and chemical synthesis, Meth. Enzymol. 107 (1984) 388-397. https://doi.org/10.1016/0076-6879(84)07027-0.
[48] R. Amadò, R. Aeschbach, H. Neukom, Dityrosine: in vitro production and characterization, Meth. Enzymol. 107 (1984) 377-388. https://doi.org/10.1016/0076-6879(84)07026-9.
[49] R. Aeschbach, R. Amadò, H. Neukom, Formation of dityrosine cross-links in proteins by oxidation of tyrosine residues, Biochim. Biophys. Acta. 439 (1976) 292-301. https://doi.org/10.1016/0005-2795(76)90064-7
[50] G.S. Harms, S.W. Pauls, J.F. Hedstrom, C.K.J.J.o.F. Johnson, Fluorescence and rotational dynamics of dityrosine, J. Fluoresc. 7 (1997) 283-292. https://doi.org/10.1023/A:1022525909128.
[51] C.S. Atwood, G. Perry, H. Zeng, Y. Kato, W.D. Jones, K.-Q. Ling, X. Huang, R.D. Moir, D. Wang, L.M. Sayre, M.A. Smith, S.G. Chen, A.I. Bush, Copper mediates dityrosine cross-linking of Alzheimer's amyloid-β, Biochemistry. 43 (2004) 560-568. https://doi.org/10.1021/bi0358824.
[52] A. Bouhekka, T. Bürgi, In situ ATR-IR spectroscopy study of adsorbed protein: Visible

light denaturation of bovine serum albumin on TiO2, Appl. Surf. Sci. 261 (2012) 369-374. https://doi.org/10.1016/j.apsusc.2012.08.017.
[53] A. Baral, L. Satish, D.P. Das, H. Sahoo, M.K. Ghosh, Construing the interactions between MnO2 nanoparticle and bovine serum albumin: insight into the structure and stability of a protein–nanoparticle complex, New J. Chem. 41 (2017) 8130-8139. https://doi.org/10.1039/C7NJ01227F.
[54] H.A. Alhazmi, FT-IR spectroscopy for the identification of binding sites and measurements of the binding interactions of important metal ions with bovine serum albumin, Sci. Pharm. 87 (2019) 5. https://doi.org/10.3390/scipharm87010005.
[55] R. Itri, W. Caetano, L.R. Barbosa, M.S. Baptista, Effect of urea on bovine serum albumin in aqueous and reverse micelle environments investigated by small angle X-ray scattering, fluorescence and circular dichroism, Braz. J. Phys. 34 (2004) 58-63. https://doi.org/10.1590/S0103-97332004000100009
[56] K.P. Fears, C.T. Love, D.E. Day, Albumin conformational change and aggregation induced by nanostructured apatites, Biointerphases. 12 (2017) 02D403. https://doi.org/10.1116/1.4982641.
[57] X.J. Ma, Y.J. Zhang, C.-M. Zeng, Inhibition of amyloid aggregation of bovine serum albumin by sodium dodecyl sulfate at submicellar concentrations, Biochemistry. 83 (2018) 60-68. https://doi.org/10.1134/S000629791801008X.
[58] K. Abrosimova, O. Shulenina, S. Paston, FTIR study of secondary structure of bovine serum albumin and ovalbumin, J. Phys.: Conf. Ser. 769 (2016) 1-6. https://doi.org/10.1088/1742-6596/769/1/012016.
[59] T. Hubbard, T. Blundell, Comparison of solvent-inaccessible cores of homologous proteins: definitions useful for protein modelling, Protein Eng. Des. Sel. 1 (1987) 159-171. https://doi.org/10.1093/protein/1.3.159.

Graphical abstract

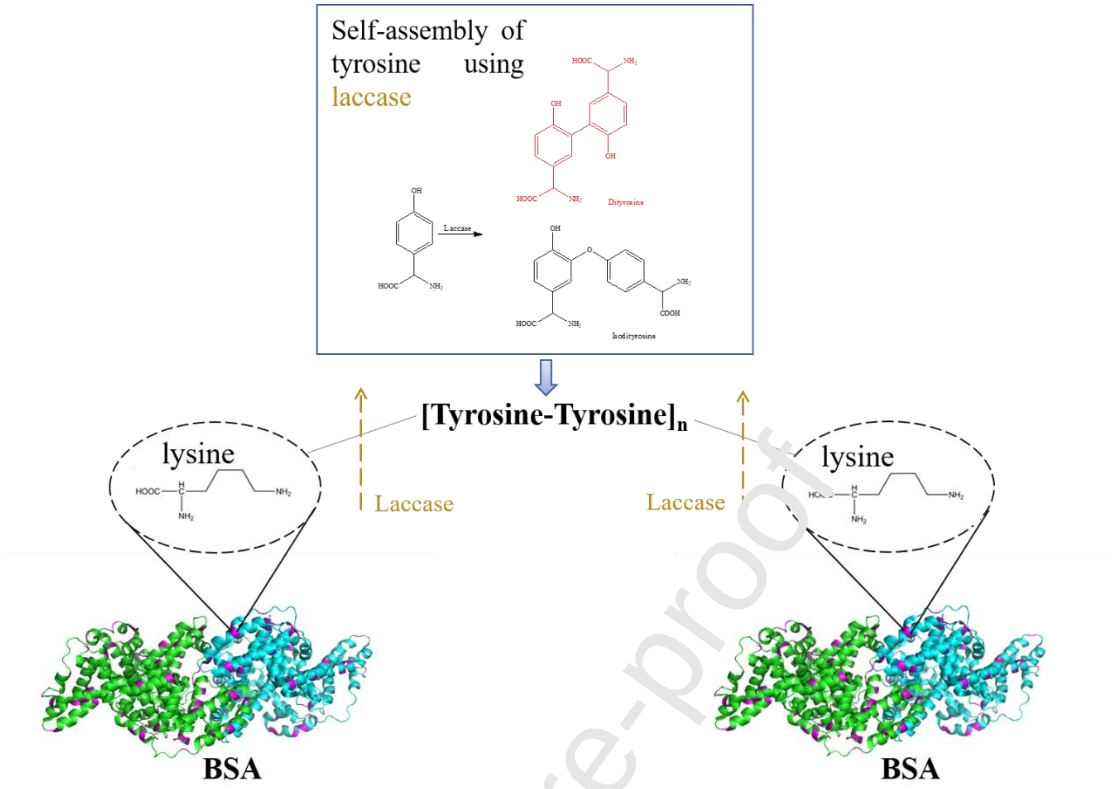


Figure legends

Fig. 1 LC-MS (a) and FTIR (b) spectra of tyrosine treated with or without laccase

Fig. 2 UV-Vis of tyrosine (a) and fluorescence spectra of tyrosine and down layer of tyrosine with BSA mixture solution by ultrafiltration (b)

Fig.3 FTIR (a) and CD (b) spectra of BSA complexes

Fig.4 Molecular weight analysis of BSA complexes (a) SDS-PAGE, (b) SEC spectra, (c) MALDI-TOF: 27000~147000 m/z, (d) MALDI-TOF: 128000~147000 m/z

Fig. 5 Proposed reaction process and structure of BSA-tyrosine conjugate and cross-linked BSA

Fig. 6 LC-MS of peptide T1 obtained by trypsin hydrolysis of pure BSA and BSA treated with tyrosine and laccase

Fig. 7 Reactive lysine in BSA showed as sticks (a) and chemical reactions between tyrosine and lysine (b)

Fig. S1 LC-MS of peptides obtained by trypsin hydrolysis of pure BSA and BSA treated with tyrosine and laccase: FPK (T1), HKPK (T2), FWCK (T3), CASIQK (T4), NYQEAK (T5)

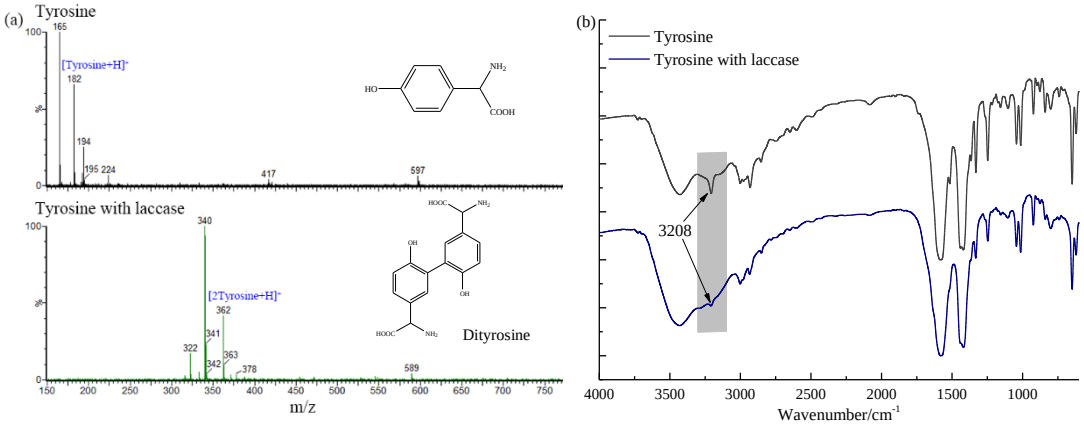


Fig. 1 LC-MS (a) and FTIR (b) spectra of tyrosine treated with or without laccase

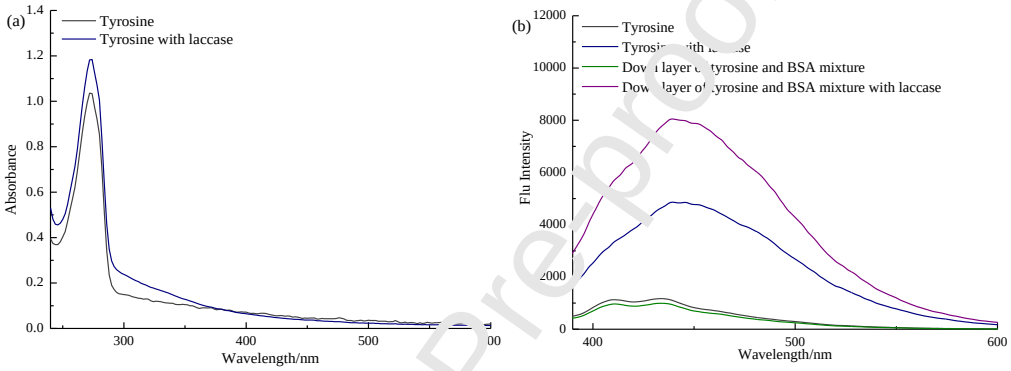


Fig. 2 UV-Vis of tyrosine (a) and fluorescence spectra of tyrosine and down layer of tyrosine with BSA mixture solution by ultrafiltration (b)

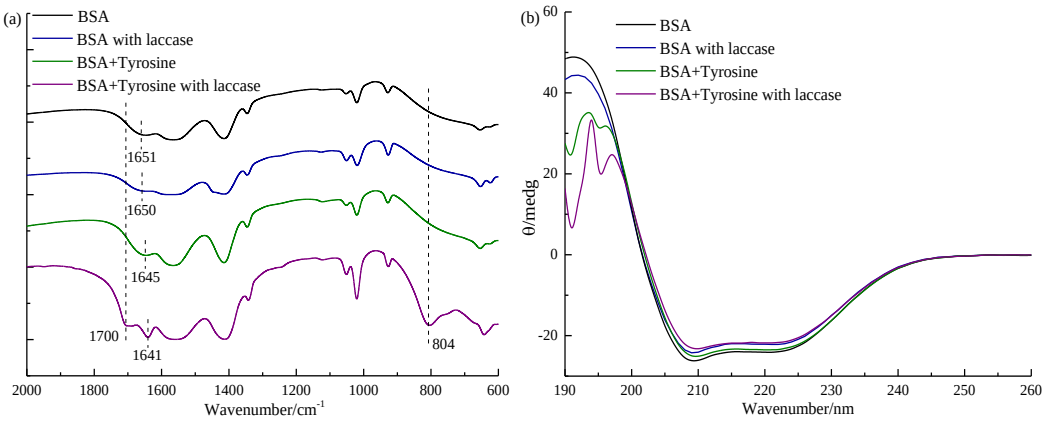


Fig.3 FTIR (a) and CD (b) spectra of BSA complexes

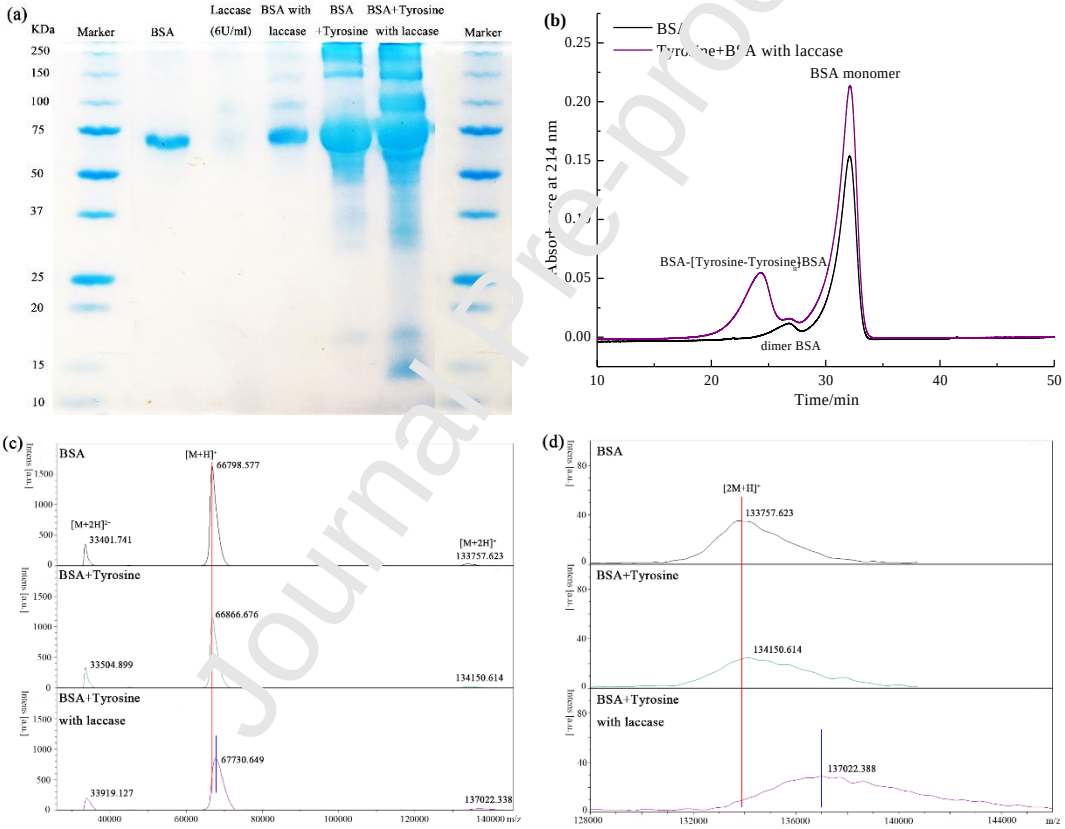


Fig.4 Molecular weight analysis of BSA complexes (a) SDS-PAGE, (b) SEC spectra, (c) MALDI-TOF: 27000~147000 m/z, (d) MALDI-TOF: 128000~147000 m/z

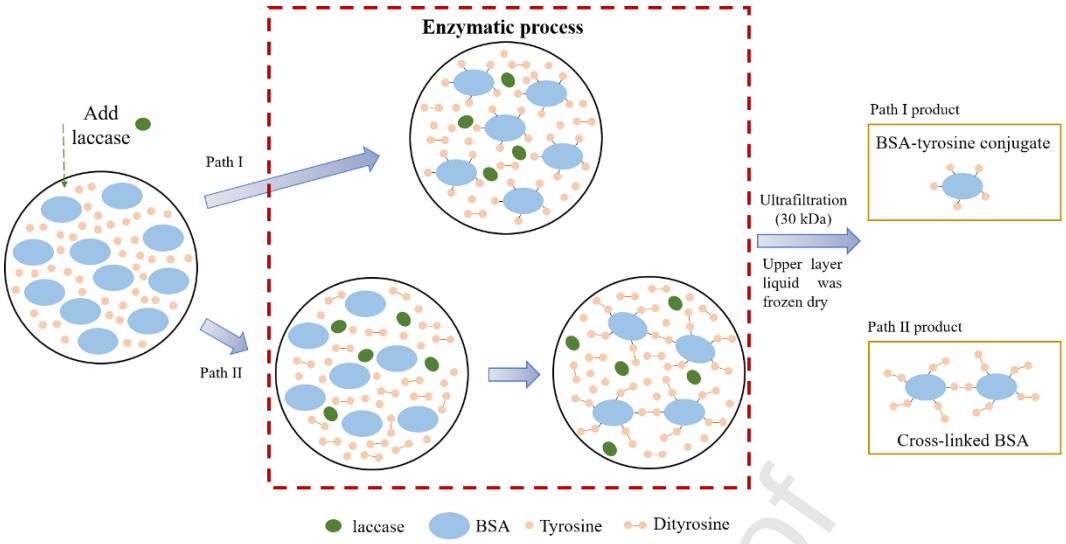


Fig. 5 Proposed reaction process and structure of BSA-tyrosine conjugate and cross-linked BSA

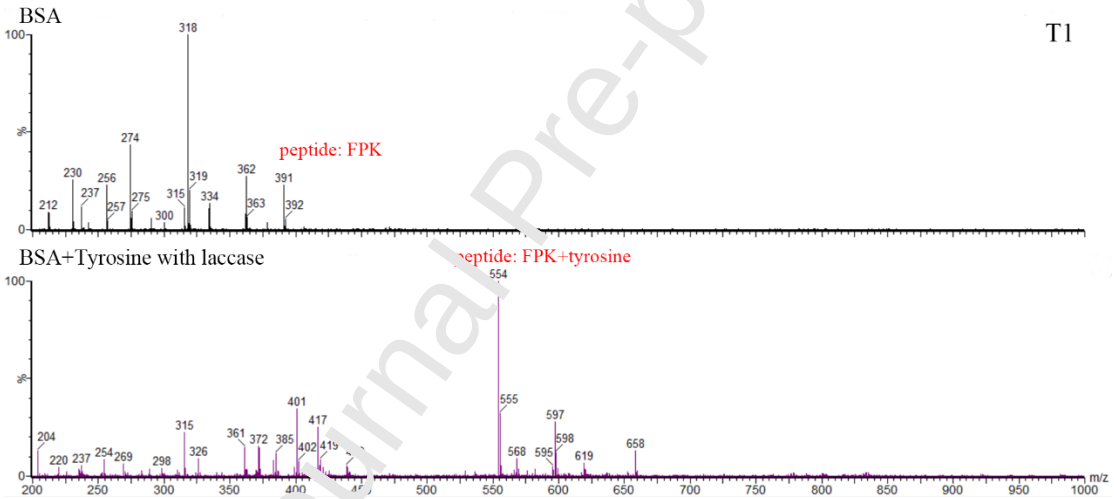


Fig. 6 LC-MS of peptide T1 obtained by trypsin hydrolysis of pure BSA and BSA treated with tyrosine and laccase

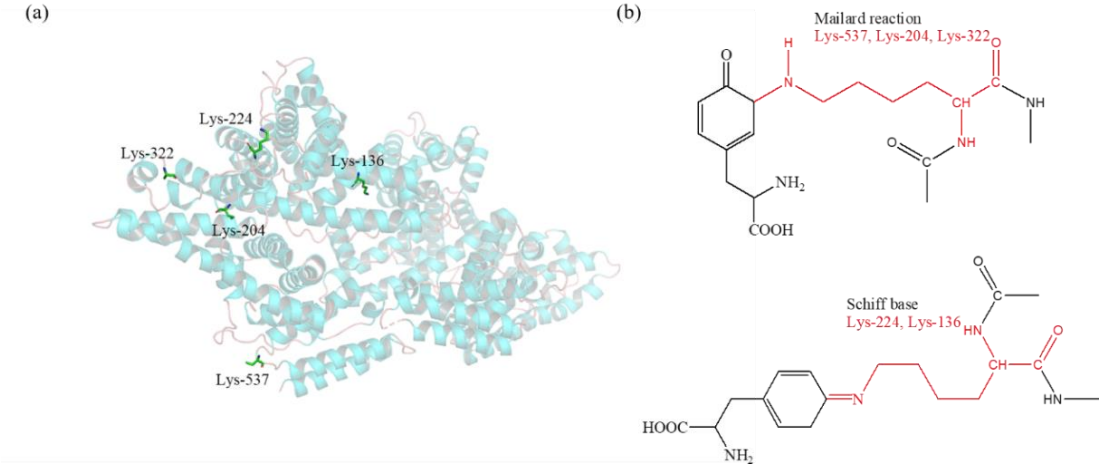
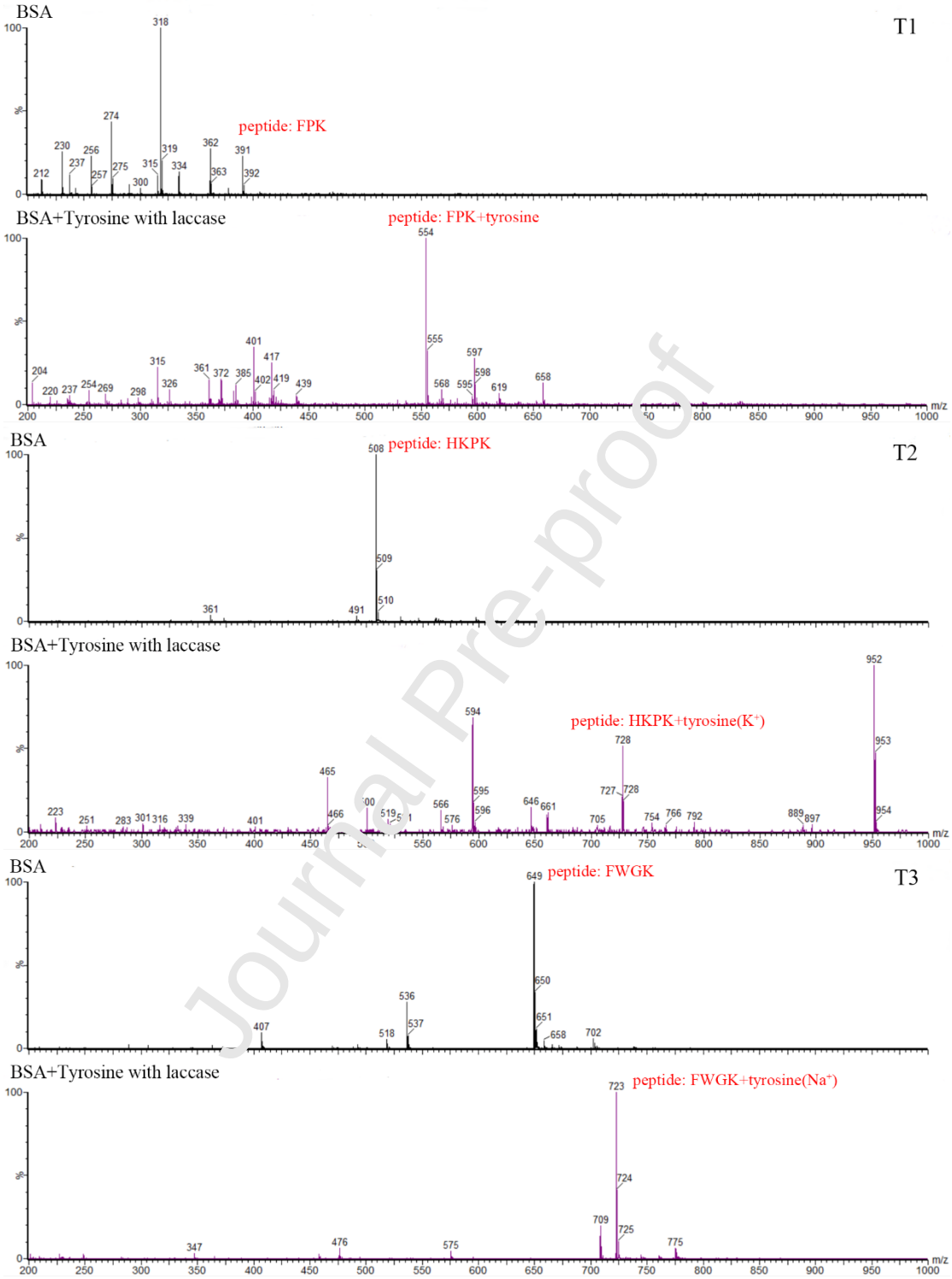


Fig. 7 Reactive lysine residues in BSA showed as sticks (a) and possible chemical reactions between tyrosine and lysine (b)



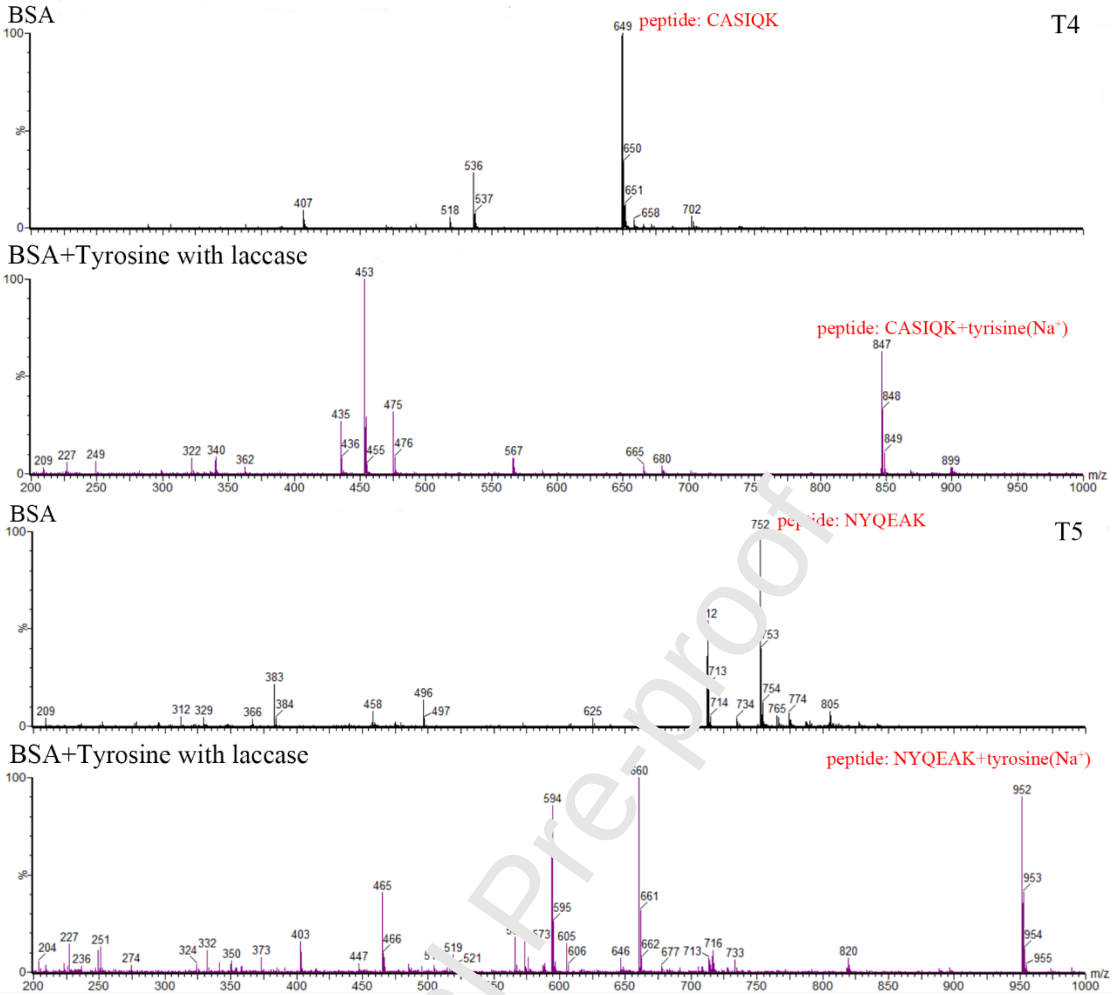


Fig. S1 LC-MS of peptides obtained by tryptic hydrolysis of pure BSA and BSA treated with tyrosine and laccase: FPK (T1), HKPK (T2), FWGK (T3), CASIQK (T4), NYQEAK (T5)

Tables legends

Table 1 Free phenolic hydroxyl contents of tyrosine solution untreated and treated with laccase

Table 2 Free amino contents of upper layer of tyrosine and BSA mixture untreated and treated with laccase

Table 3 The secondary structure of BSA complexes estimated by Circular dichroism (CD) spectra

Table 4 Amino acid content of pure BSA and BSA treated with tyrosine and laccase

Table S1 Relative solvent accessibility (RSA) of lysine residues in BSA (PDB ID: 3V03, chain A)

Table 1 Free phenolic hydroxyl contents of tyrosine solution untreated and treated with laccase

Sample	Free phenolic hydroxyl content (mg/L)
Tyrosine	260.72±1.41
Tyrosine with laccase	248.61±3.38

Table 2 Free amino contents of upper layer of tyrosine and BSA mixture untreated and treated with laccase

Sample	Free amino content (mmol/L)
Tyrosine + BSA	3.10±0.11
Tyrosine + BSA with laccase	2.12±0.17

Table 3 The secondary structures of BSA complexes estimated by circular dichroism (CD) spectra

Sample	α-helix (%)	β-sheet (%)	β-turn (%)	Random coil (%)
BSA	64.7	25.9	4.8	5.4
BSA with laccase	63.7	25.4	4.3	7.7
BSA + Tyrosine	60.9	23.5	5.8	10.1
BSA + Tyrosine with laccase	58.3	22.3	5.2	14.2

Table 4 Amino acid contents of pure BSA and BSA treated with tyrosine and laccase

Sample	Arg (%)	Lys (%)
BSA	6.01±0.19	12.36±0.26
Tyrosine + BSA with laccase	6.11±0.62	11.43±0.45

Table S1 Relative solvent accessibility (RSA) of lysine residues in BSA

Residues	RSA (%)	Residues	RSA (%)	Residues	RSA (%)	Residues	RSA (%)
Lys-4	70	Lys-159	41	Lys-285	22	Lys-471	18
Lys-12	60	Lys-173	21	Lys-294	50	Lys-474	55
Lys-20	35	Lys-180	41	Lys-312	82	Lys-499	45
Lys-41	50	Lys-187	49	Lys-316	39	Lys-504	77
Lys-51	33	Lys-204	74	Lys-322	33	Lys-520	29
Lys-64	49	Lys-211	40	Lys-350	43	Lys-523	61
Lys-76	70	Lys-221	8	Lys-362	49	Lys-524	32
Lys-93	52	Lys-224	43	Lys-375	52	Lys-533	6
Lys-106	8	Lys-232	38	Lys-377	41	Lys-535	18
Lys-114	84	Lys-239	46	Lys-388	49	Lys-537	91
Lys-116	89	Lys-242	28	Lys-396	89	Lys-544	53
Lys-127	80	Lys-261	41	Lys-413	5	Lys-556	54
Lys-131	59	Lys-273	36	Lys-431	29	Lys-563	20
Lys-132	49	Lys-275	52	Lys-439	52	Lys-573	44
Lys-136	44	Lys-280	36	Lys-4[illegible]	76		

Data obtained by PYMOL software using the amino acid sequence based on crystal structure of bovine serum albumin (PDB ID: 3V03, Chain A).

Highlights

1. Tyrosine was oxidized into dityrosine using laccase as a catalyst.

2. Tyrosine promotes the formation of tyrosine-BSA conjugate and cross-linked BSA.

3. Five lysine residues (Lys-537, Lys-136, Lys-204, Lys-224 and Lys-322) in BSA reacted with tyrosine by the Schiff base or Maillard reactions in the presence of laccase.