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Chemical Reaction of Interaction of Hypocrellin B with Gelatin-Formation of Cysteine-Substituted Derivative

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The interactions between hypocrellin B (HB) and two types of gelatin (G) were studied in this work. The maximum absorption wavelength of hypocrellin B-Type II gelatin was red-shifted with appearance of an isosbestic point at around 480 nm after interacted with Type II gelatin at pH 7.4. At the same time, the fluorescence of hypocrellin B-Type II gelatin was almost quenched completely. On the other hand, the absorption spectral indicated that the interaction of hypocrellin B with the cysteine-deficient Type I gelatin resembled those for hypocrellin B binding to the hydrophobic area of biomolecules. After reacted with Type II gelatin, hypocrellin B could not be extracted by organic solvent, suggesting a covalent bonding to the protein framework. Furthermore, it was proved that the spectra of hypocrellin B-Type II gelatin was very similar to those of 5,8-2-cysteine-hypocrellin B and the reaction was inhibited by potassium-ferricyanide. All of these evidences confirm that a chemical derivative, cysteine-S-hypocrellin B, was formed *via* reaction of hypocrellin B with Type II gelatin. It was further proved that the Type II gelatin did not possess a hydrophobic area for binding of hypocrellin B but the Type I did. Accordingly, it implies that Type I gelatin is a proper choice as a drug carrier while Type II is not a proper drug carrier when a drug molecule has electrohphiles in the structure that can be attacked by cysteine in Type II gelatin. Furthermore, the spectral indicated that hypocrellin B may be used to recognize a denatured protein containing cysteine from the nature ones.

Keywords: Hypocrellin B-Type II gelatin, Cysteine, Chemical reaction, Hydrophobic interaction.

INTRODUCTION

Hypocrellin A (HA) and hypocrellin B (HB) (Fig. 1) are kinds of photosensitizers naturally present in a fungus named Hypocrella bambusae which grown mainly in Yunnan province of China. As a kind of potential photodynamic drugs, they are well known for the high photodynamic activity to tumors and other biological targets but very low dark-toxicity[1-4]. Recently, it was reported that fluorescence of hypocrellin was originated from the intramolecular excited-state proton or hydrogen transfer[5], so turn to the intramolecular hydrogen bonds, which was originated of the solvent-sensitive spectral properties of hypocrellins[6]. Recently, based on the environment-sensitive spectral responses, interactions of hypocrellins with various of bio-molecules have been studied. It was reported that hypocrellin A could be randomly bounded to some hydrophobic regions of human serum albumin (HSA)[7]. Later, it was clarified that hypocrellin B could specifically bound to site 1 in IIa subdomain of human serum albumin by taking the fluorescence responses of hypocrellin B and the tryptophan residue in human serum albumin as the dual probes[8]. Further, it was demonstrated that fluorescence emissions of hypocrellin B in human serum albumin, PBS solution and liposome were all sensitive to pH change in physiological range (pH 6-8)[9,10]. Generally, the absorbance and fluorescence are greatly strengthened when hypocrellins are solved in organic solvent or binding to hydrophobic areas of bio-molecules but weakened in aqueous solutions or a polar microenvironment. Interestingly, the absorbance of hypocrellin B was enhanced but the fluorescence was almost silenced when hypocrellin B was bound to hyaluronan, a polysaccharide molecule, which was ascribed to the hydrophobic interaction and formation of the specific intermolecular hydrogen bonds[11]. It was also reported that hypocrellin B fluorescence could be used not only to recognize various kinds of biomolecules, including proteins, polysaccharides and liposome, but also to distinguish a specific binding from nonspecific bindings[12]. Up to now, it is known that hypocrellin B molecules may weakly interact with biological molecules, which results in either fluorescence strengthening or weakening but without remarkable spectral shifting.

As a kind of denatured proteins derived from collagens, gelatins are widely-used biological materials for drug preparations

and drug deliveries. Specially, hypocrellin B-gelatin nanoparticles were prepared to improve the solubility of hypocrellins in aqueous solution[13]. Therefore, it is meaningful to get a better understanding of the interaction of hypocrellin B with gelatin. Furthermore, study on the interaction of hypocrellin B to gelatin may learn how to identify a nature protein from fully or partly denatured ones. In the current work, interaction of hypocrellin B with two types of gelatins, Type I and Type II, was studied. Interestingly, it was found that Type II gelatin could chemically react with hypocrellin B to produce a covalent derivative while Type I gelatin bind hypocrellin B only *via* a hydrophobic interaction. Based on experimental evidences, it was determined that a chemical derivative, R-cysteine-S-hypocrellin B, was formed *via* a reaction of hypocrellin B with the free -SH groups of cysteine residue in Type II gelatin.

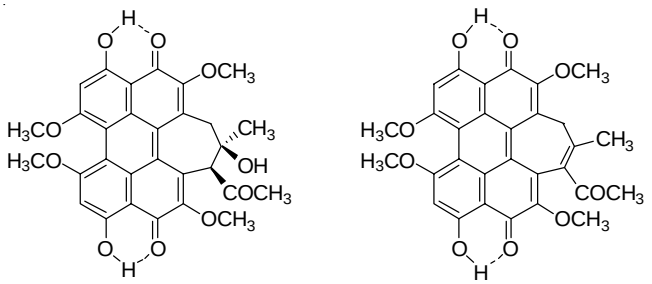

Fig.1. Molecular structure of hypocrellin A (HA) and hypocrellin B (HB)

EXPERIMENTAL

Hypocrellin B was derived as described previously[14]. Type I gelatin was purchased from Sigma Chemical Co and the Type II gelatin was provided by Institute of Biophysics, Chinese Academy of Sciences. Phosphate buffered saline (PBS) of pH 7.4 was prepared by 10 mM KH_2PO_4 and 10 mM K_2HPO_4. The working solutions were prepared immediately before use.

It was reported that the maximum solubility of hypocrellin B in aqueous solution was 8 μM/mL[12], therefore, 8 μM/mL of hypocrellin B was used for all of the experiments in this work. To get rid of any aggregation of hypocrellin B molecules in aqueous solution, a concentrated solution of hypocrellin B in dimethyl sulfoxide (DMSO) (2 mg/mL) was added slowly into the gelatin aqueous solution in micro liter quantities and mixed quickly by shaking until reaction completion. 5,8-2-cysteine-hypocrellin B derivative was synthesized by following the thermal method reported previously[15].

Spectral measurements: Absorption spectra were obtained on a UV-1601 UV-visible spectrophotometer (Shimadzu) and fluorescence emission spectra on an F-4500 spectrofluorimeter (Hitachi) with selective excitation of hypocrellin B at 470 nm at room temperature. Samples were dark-adapted at room temperature for 0.5 h prior to fluorescence measurement. All measurements were carried out at room temperature.

RESULTS AND DISCUSSION

Fluorescence emission spectra of hypocrellin B-Type II gelatin: Fluorescence emission spectra of hypocrellin B (8 μM) in various concentration (2.5-15 g/L.) of Type II gelatin were measured at a series of time with range of 0-500 min, from which the effects of gelatin concentration and reaction time on fluorescence intensity of hypocrellin B are shown in Fig. 2. Time-dependent fluorescence decaying suggested that more and more hypocrellin B molecules were binding to the gelatin. It shows fluorescence decay curves coincide when the concentration of Type II gelatin are equal to or higher than 12.5 g/L, suggested that all of the hypocrellin B molecules (8 μM) react completely with the gelatin. Therefore, 12.5 g/L of the gelatin was used for all of the experiments. Fig. 2 also shows that reaction of the gelatin with 12.5 g/L was complete within 3 h which was chose as the reaction time for the experiments.

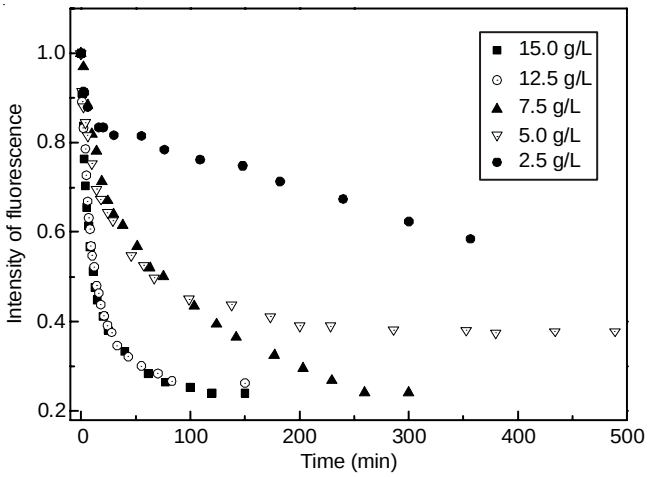

Fig. 2. Fluorescence intensity of hypocrellin B-Type II gelatin

Absorption and fluorescence spectra of hypocrellin B-gelatin: For probing the interaction of hypocrellin B (8 μM) with Type II gelatin (12.5 g/L) in an PBS solution (pH 7.4), the absorption and fluorescence spectra were measured at a series of reaction time with a range of 5-150 min, as shown in Fig. 3. It shows that one of the most remarkable features is red-shifting of the maximum absorption wavelength from 470 to 515 nm with an isosbestic point appeared at 480 nm (Fig. 3A). It is known that a general rule for weak interactions of hypocrellin B with biomolecules is either strengthening or weakening the spectra of hypocrellin B but never leads to such a typical spectral shifting. Therefore, it is suggested that a chemical reaction instead of a weak interaction takes place. Indeed, after reacted with the gelatin, hypocrellin B could not be extracted by chloroform, suggesting that hypocrellin B was covalently combined to the protein framework so isolatable. Another most remarkable feature is the fluorescence silence of binding of hypocrellin B to Type II gelatin, as shown in Fig. 3B. The fluorescence silence is quite uncommon for hypocrellin B binding to biomolecules except for hyaluronan. The significance of fluorescence silence will be further discussed below.

Compared the absorption spectrum of hypocrellin B-Type II gelatin to those of thiol-modified derivatives of hypocrellin B, the spectral similarity implies formation of a thiol-substituted derivative of hypocrellin B with Type II gelatin. Table-1 lists the absorption features of hypocrellin B, hypocrellin B-Type II gelatin and three derivatives with thiol-compounds.

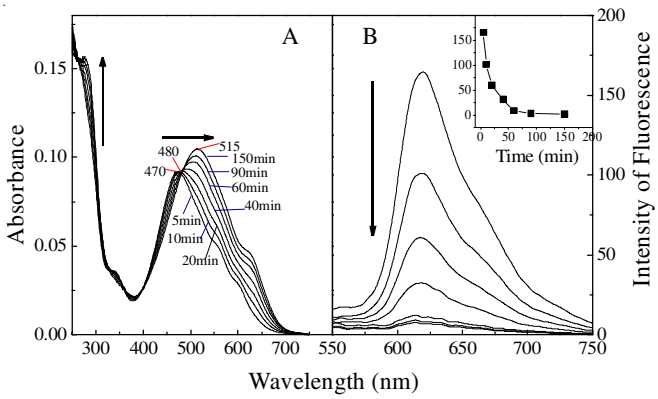

Fig. 3. Absorption (A) and fluorescence (B) spectra of hypocrellin-Type II gelatin

Among these, the cysteine residues are most likely the candidates for the chemical reaction with hypocrellin B. It is known that Type II procollagen, containing cysteine-rich domains[16,17] and is the predecessor of Type II gelatin. During the denaturation, breaking of a disulfide bond releases two free -SH groups of cysteine residues, which are chemically reactive to hypo-crellin B. On the other hand, Type I gelatin is cysteine-deficient[18], therefore, detecting the spectral responses of hypocrellin B-Type I gelatin could provide a further evidence.

TABLE-1
ABSORPTION MAXIMA OF HYPOCRELLIN B AND THE THIOL-MODIFIED DERIVATIVES

Derivative	Nm (ε)
HB	466 (4.46)
5,8-2-Cysteine-HB[15]	514 (4.28)
5,8-2-$HOCH_2CH_2S$-HB[15]	514 (4.28)
5,8-2-CO_2HCH_2S-HB[15]	514 (4.28)
HB-Type II gelatin	515

The absorption and fluorescence spectra for the interaction of hypocrellin B with Type I gelatin are shown in Fig. 4. Interestingly, the spectra are quite contrary to those in Fig. 3 but reproduce basic feature for hypocrellin B binding to the hydrophobic microenvironment in protein, polysaccharides or liposome[12], *etc*., strengthening absorbance and fluorescence but without spectral shift, suggesting a hydrophobic weak interaction rather than a chemical reaction.

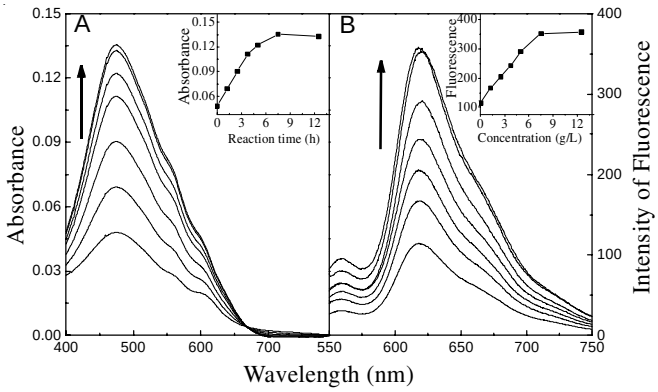

Fig. 4. Absorption (A) and fluorescence (B) spectra of hypocrellin B-Type II gelatin

Absorption and fluorescence spectra of hypocrellin B-Type II gelatin with different potassium ferricyanide concentration pretreatment: Based on these results, it is reasonable to propose that a covalent derivative is formed *via* the reaction of hypocrellin B with free -SH group of the cysteine residue in Type II gelatin. It is known that free -SH groups of the cysteine residues are readily oxidized by potassium ferricyanide or reactive oxygen species[19-21]. To provide further evidence, the Type II gelatin was pretreated by various concentration of potassium ferricyanide (0-3.2 mM/L) before reacted with hypocrellin B. The absorption spectra and fluorescence spectra of hypocrellin B reacted with Type II gelatin pretreated with 0.0 (a), 0.8(b), 1.6(c) and 3.2 mM (d) of potassium ferricyanide are shown in Figs. 5 and 6, respectively with those to the untreated gelatin as references. It is demonstrated that the spectra of free hypocrellin B are recovered with increase of concentration of potassium ferricyanide, suggesting that the reaction of hypocrellin B to the free -SH groups is inhibited. This result provides a confirmation for formation of the chemical derivative of hypocrellin B with the cysteine residues in Type II gelatin. Furthermore, it can be seen from Fig. 6 that the hypocrellin B fluorescence almost recover to, but never higher than the level of free hypocrellin B in PBS solution, suggesting that the Type II gelatin does not contain a hydrophobic area for binding of hypocrellin B but the Type I gelatin does.

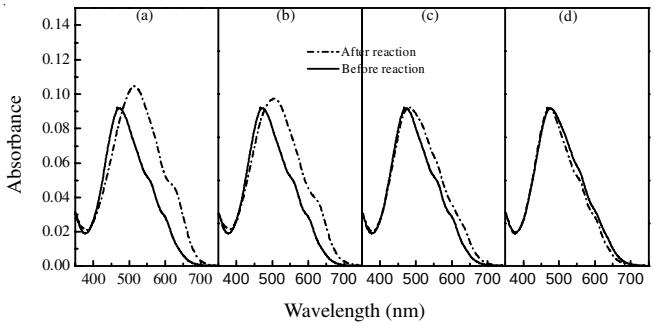

Fig. 5. Absorption spectra of hypocrellin B-Type II gelatin with different potassium ferricyanide pretreatment

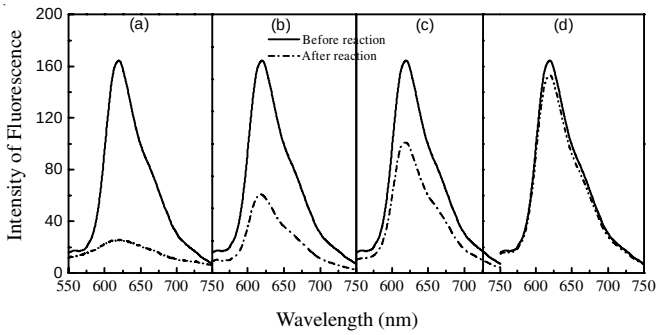

Fig. 6. Fluorescence spectra of hypocrellin B-Type II gelatin with different potassium ferricyanide pretreatment

It was reported that hypocrellin B could react with mercaptoacetic acid, mercaptooctanol, cysteine or cysteamine to form thiolated hypocrellin B *via* Michael addition of the nucleophilic anion RS^- attacking hypocrellin B in the presence of air[22,23]. To provide more direct confirmation, a 5,8-2-cysteine-hypocrellin B derivative was re-synthesized *via* thermal

reaction and characterized by following the reported procedures[15]. The absorption and fluorescence spectra of 5,8-2-cysteine-hypocrellin B, free hypocrellin B and of hypocrellin B-Type II gelatin complex in PBS solution are shown in Fig. 7. The absorption and fluorescence spectra of the hypocrellin B-gelatine complex resemble those of 5,8-2-cysteine-hypocrellin B derivative but apparently different from those of free hypocrellin B. It is known that hypocrellin B fluorescence is originated from the intramolecular hydrogen bonds between the peri-hydroxyl groups and the quinonoid carbonyl groups[5]. The stronger is the hydrogen bonds, the higher the fluorescence intensity is, thus the fluorescence is greatly strengthened in an organic solvent or a hydrophobic microenvironment. Although hyaluronan could also provide a hydrophobic area to hypocrellin B, formation of the specific intermolecular hydrogen bonds between one hypocrellin B and two hyaluronan molecules greatly weakens the intramolecular hydrogen bonds so quenches hypocrellin B fluorescence[11]. Due to formation of the derivative of hypocrellin B with the cysteine residues in Type II gelatin, two negatively charged S atoms adding to the 5 and 8 sites of hypocrellin B would strongly attract the nearby hydrogen ions on the hydroxyl groups, which weakens the intramolecular hydrogen bonds so quenches hypocrellin B fluorescence.

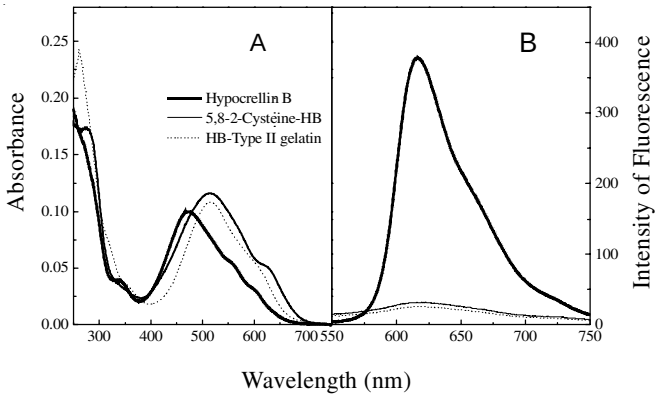

Fig. 7. Absorption spectra (A) and fluorescence spectra (B) of hypocrellin B, 5,8-2-cysteine-HB and HB-Type II gelatin

Absorption and fluorescence spectra of hypocrellin B, 5,8-2-cysteine-hypocrellin B and hypocrellin B-Type II gelatin: It was indicated that that rate and productivity of the reaction of hypocrellin B with cysteine were affected by pH values[23]. For pKa value of RS-H of cysteine[24] is 8.3, there will be certain amount of RS-H groups being dissociated at pH 7.4. Therefore, the reaction of hypocrellin B to the free -SH groups in cysteine residues of Type II gelatin at pH 7.4 is expectable, however, the reaction rate should be much slower than those at higher pH values. Indeed, it took hours for the reaction at pH 7.4 in the current work but only minutes at a pH higher[23] than 9.

Briefly, it can be concluded that hypocrellin B can react with Type II gelatin to form a chemical derivative based on observation in this work. Specifically, the derivative is most likely 5- or (and) 8-cysteine-S-hypocrellin B formed by two-cysteine-S-groups attacking the site 5 or (and) 8 of hypocrellin B. In this case, hypocrellin B is firmly combined to the gelatin framework. On the other hand, photodynamic therapy requires immediate drug releases at the target tissues. Therefore, Type II gelatin is not a suitable drug carrier when a drug molecule has electrohphiles in the structure that can be attacked by cysteine in type II gelatin but the Type I gelatin is, because the formation would seriously depress the photodynamic activity. But drugs that do not have such electrophiles would not affected by the cysteine conjugation reaction and thus can be carried either by type II or type I gelatin. Furthermore, it is known that protein denaturation is commonly companied by breaking the disulfide bonds so will release the -SH groups of cysteine residues, therefore, the current work also provides a method to distinguish a natural protein containing cysteine residues from the partially or completely denatured ones.

Conclusion

In this current work, it is concluded that hypocrellin B undergoes a chemical reaction with Type II gelatin based on a series of experimental evidences. First of all, appearance of an isosbestic point is a feature of strongly chemical reaction instead of the weak interactions; Secondly, failure to extract hypocrellin B from the reacted samples by organic solvent suggests that a covalent binding of hypocrellin B to the protein framework. Thirdly, the spectral similarity to the thiylated derivatives of hypocrellin B suggests formation of R-S-substituted derivative of hypocrellin B with Type II gelatin. Fourthly, distinctive spectral response of hypocrellin B to cysteine-rich Type II gelatine from that to the cysteine-deficient Type I gelatine implies that a reaction takes place between hypocrellin B and the free thiol groups of cysteine residus in Type II gelatin to form singly or doubly substituted R-cysteine-S-hypocrellin B derivatives. Fifthly, the reaction is further confirmed by the inhibition effect of potassium ferricyanide which oxidizes the reactive R-S- into the inactive R-S form. On contrary, hypocrellin B could only interact with Type I gelatin *via* weakly hydrophobic interaction. Therefore, it can be concluded that Type II gelatin is not a proper drug carrier for drugs which has electrohphiles in the structure that can be attacked by cysteine in Type II gelatin, but the Type I is due to that the covalently combined drugs would be not releasable. Furthermore, currently reported methodology could also be used to distinguish a nature protein containing cystaine residues from the denatured ones.

ACKNOWLEDGEMENTS

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