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# pH Effect on the spectroscopic behavior and photoinduced generation of semiquinone anion radical of hypocrellin B

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**Abstract**

The spectroscopic behavior and photoinduced generation of the semiquinone anion radical ($HB^{\cdot -}$) of hypocrellin B (HB), a naturally occurred pigment, have been investigated in ethanol-buffer (1:1 by volume) in a wide range of pH from −0.6 to 14. The absorption and emission properties of HB are strongly affected by pH. Protonation and deprotonation induce a hypsochromic and a bathochromic shift of the spectra, respectively, and a decrease in the quantum yield of fluorescence. The protonation and deprotonation pKa values of HB in the ground and excited singlet states have been determined by means of spectrophotometric and spectrofluorometric titrations and Förster Cycle calculation. The generation of $HB^{\cdot -}$ is influenced strongly by pH; $HB^{\cdot -}$ could not be detected either by electron paramagnetic resonance (EPR) or by spectrophotometric measurements in anaerobic acidic or strong alkaline media, and is stable in anaerobic neutral and weak alkaline environment. 

*Keywords:* Hypocrellin B (HB); Protonation; Deprotonation; Spectroscopic properties; Semiquinone anion radical; Hydroquinone

## 1. Introduction

Photodynamic therapy (PDT) is an innovative and attractive modality for the treatment of small and superficial tumors. PDT, as a multimodality treatment procedure, requires both a selective photosensitizer and a powerful light source, which matches the absorption spectrum of the photosensitizer. Following excitation of photosensitizers to long-lived excited singlet and/or triplet states, the tumor is destroyed either by reactive oxygen species (type II mechanism) and/or radical products (type I mechanism) [1]. Hypocrellins, including hypocrellin A (HA) and hypocrellin B (HB), are new photosensitizers, found in a parasitic fungus that is common in parts of the People's Republic of China and Sri Lanka [2]. These perylenequinonoid compounds are selected as potential photosensitizers for PDT owing to their high quantum yields of singlet oxygen [3], substantial absorption in the red spectral region, availability in pure monomeric form and facility for site-directed chemical modifications to optimize the properties of red light absorption, tissue biodistribution and toxicity [4].

Hypocrellin has been used as a photo-therapeutic agent for various skin diseases and superfacial tumors, and has been taken orally as a folk medicine for several centuries in China [2,5]. Recently, investigations have shown that hypocrellin A possesses a light-induced toxicity against the human immunodeficiency virus (HIV-1), the herpes simplex virus type I, the Sindbis virus and the vesicular stomatitis virus (VSV), with the

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involvement of singlet oxygen, i.e. a type II mechanism [6–8]. In addition, Fehr and coworkers [9] reported that hypocrellin A absolutely required oxygen for its antiviral activity, unlike hypericin whose virucidal activity in the absence of oxygen might be related to its ability to acidify its environment upon optical excitation [10–14]. It was not observed that hypocrellin acidified its surrounding medium in the presence of light [9].

Also the nature of the mechanism responsible for the selective biodistribution of hypocrellin is not clear. Several possible factors have been suggested [15]. Cells are known to display a strong heterogeneity in local pH value. Moreover, malignant tissues demonstrated lower pH than normal tissue [16]. The examination of various ionic species that predominate throughout the physiological pH range is of substantial interest because of their possible role in selective retention of neoplastic tissues.

Previous studies have suggested that the spectroscopic characteristics, photosensitization activity and self-photosensitized degradation were associated with pH values of the reaction media [17–20]. Zhang and Zhang [21,22] have made theoretical investigations of molecular thermodynamics on the intramolecular proton-transfer process and dissociation. However, experimental efforts have not yet to be made. Herein we report in detail the pH dependence of the absorption and fluorescence spectra and the photoinduced generation of the semiquinone anion radical of hypocrellin B (type I mechanism); the pKa of HB, and of HA, in the ground and excited singlet state, were also determined.

## 2. Experimental

### 2.1. Chemicals

HA was extracted from the fungus of *Hypocrella bambusae* and recrystallized twice from acetone. HB was prepared by dehydration of HA [23]. Reduced glutathione (GSH) was purchased from Biotech Technology Corporation, Chinese Academy of Sciences. The solvents used were of analytical grades and were purchased from the Beijing Chemical Plant (China). Concentrations of HB in neutral solution were calculated from the absorbance at 458 nm ($\varepsilon$ $2.16 \times 10^4$ $M^{-1}$ $cm^{-1}$). For the experiments at different pH, the following buffers were used: HCl (pH-0.6–0.6), acetate buffer (pH 4–6), phosphate buffer (pH 6.5–8.6), carbohydrate buffer (pH 9–10.5), $K_2HPO_4$–NaOH (pH 11–12) and NaOH for higher pH. The ionic strength of each buffer was adjusted to 0.2 M with sodium chloride.

### 2.2. Methods

The UV-visible absorption spectra were recorded on a Hewlett–Packard 8541A diode array spectrophotometer. The dissociation constants of HB, and of HA, were determined at room temperature according to the experimental method reported by Albert and Sejeant [24]. Emission spectra were recorded on a Hitachi MPF-4 fluorescence spectrophotometer, corrected for $\lambda$ response for the detection system. The fluorescence quantum yield was obtained using quinine sulfate in 1 N $H_2SO_4$ solution as standard. In all cases the absorbance values at the excitation wavelength were lower than 0.1 for 1 cm pathlength.

EPR spectra were recorded at room temperature (22–24°C) on a Bruker ESP-300E spectrometer operating at 9.8 GHZ, X-band with 100 kHz field modulation. The following instrumental settings were employed: microwave power, 8 mW; modulation amplitude: 0.08 G; time constant 0.2 s; scan time, 4 min; scan width 20 G. Anaerobic ethanol-buffer (1:1 by volume) solutions of HB and GSH were made in cuvettes, which allowed purging the reactive volume with argon for 30 min in the dark. The sample was immediately transferred to a quartz capillary specially made for EPR analysis, and irradiated directly in the cavity with a 532 mm YAG-900 laser (Spectro-Physics Lasers, MT.VIEW, California, USA). EPR spectra were recorded, stored and manipulated by using an IBM/PC computer.

## 3. Results

The neutral HB is not soluble in water. However, in order to study the interaction of HB with polyions in aqueous media, we prefer to deal with

the experimental measurements in water-contained media. Therefore it is necessary to estimate the solubility of HB. The solubility of HB was evaluated in ethanol-buffer (1:1 by volume, pH 7), and estimated to be about $5 \times 10^{-5}$ M. The solubility of HB can reach $2 \times 10^{-3}$ M in aqueous solution at pH 11. In this case, most of the HB molecules are present as deprotonated species. These are enough to survey the pH effect on the absorption and emission spectra and the generation of the semiquionone anion radical of hypocrellin B.

### 3.1. Spectroscopic behavior during deprotonation process

There are two phenolic hydroxyl groups in the structure of hypocrellin B. Previous studies have proposed that these two phenolic hydroxyl groups participate in the formation of intramolecular H-bonding and that intramolecular proton transfer was responsible for the coexistence and equilibrium of two tautomeric structures in solution [22] (Fig. 1). Therefore, pH will influence the spectroscopic properties by inducing the dissociations of these phenolic hydroxyl groups.

The changes of the absorption spectra of HB in ethanol-buffer solution (1:1 by volume) are shown in Fig. 2 as pH increases up to 14 from 7. The absorption spectrum of HB at pH 7 shows two main bands centred at 458 and 548 nm. When the pH increases to 9 from 7, the short-wavelength band shifts red to 478 nm and a new peak at 638 nm appears, with three isobestic points at 475, 505 and 570 nm [Fig. 2(a)]. When the pH continues to increase from 9 to 14, the absorption at 638 nm increased, with four isobestic points at 316, 404,

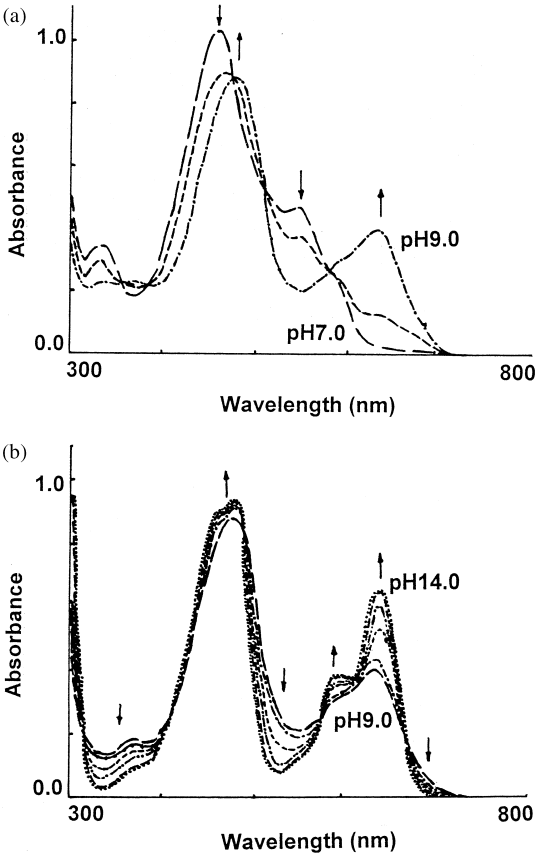



Fig. 2. pH dependence of the absorption spectra of hypocrellin B (49 $\mu$). (a) pH 7, 8, 9; (b) pH 9, 10, 11, 12, 13, 14. The arrows indicate the change directions.

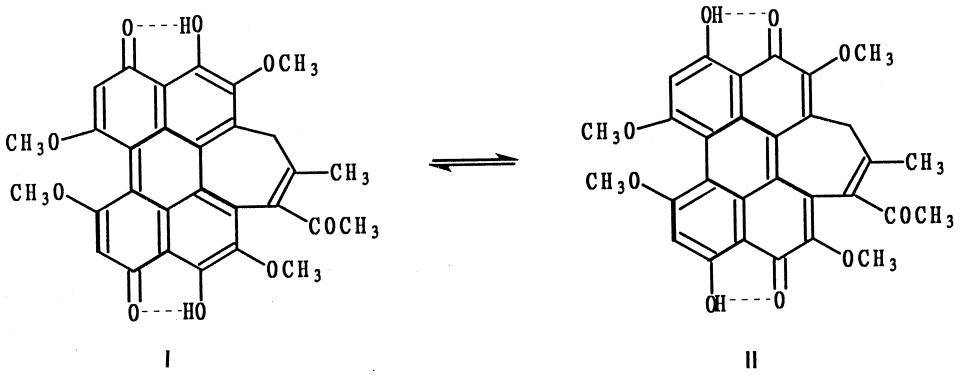



Fig. 1. The tautomerization equilibrium of hypocrellin B.

488 and 570 nm. Meanwhile, the short-wavelength absorption band (~480 nm) split into two peaks (470 and 490 nm). This is due to the fact that the dissociations of two phenolic hydroxyls prevent tautomerization of I and II and destroy the tautomeric equilibrium shown in Fig. 1. Therefore, two absorption peaks for the deprotonated species for II and I [2], respectively, were observed.

The UV-visible absorption titration at 638 nm is shown in Fig. 3. Two equilibrium constants were calculated, corresponding to $pKa_1 = 8.4 \pm 0.2$ and $pKa_2 = 11 \pm 0.1$. They are attributed to the primary and secondary dissociations of the two phenolic hydroxyl groups. As a consequence of these equilibria in the pH range investigated, HB is present in three different forms viz., HB ($YH_2$), monoanionic form ($YH^-$) and dianionic form ($Y^{2-}$); their absorption properties are listed in Table 1.

pH strongly influences the emission spectra of HB. In Fig. 4, the corrected fluorescence spectra of HB at different pH taken by excitation at 570 nm (an isobestic point in the absorbance variations),

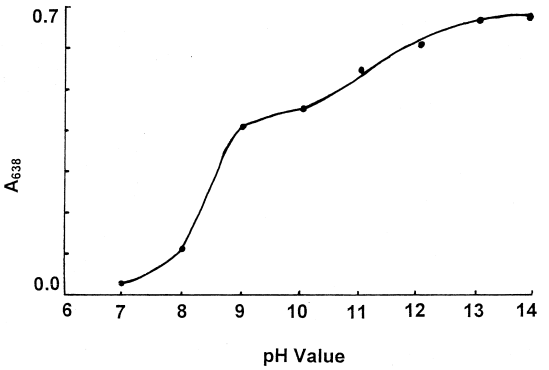



Fig. 3. Effect of pH on the absorbance at 638 nm ($A_{638}$) of hypocrellin B obtained from Fig. 2.

Table 1
Absorption (A) and emission (F) properties of protonation and deprotonation species of hypocrellin B

| Species | $\lambda_A$ (nm) (log$\varepsilon$) | $\lambda_F$ (nm) | $\phi_F \times 10^3$ |
|---|---|---|---|
| $YH_2$ (HB) | 458(4.33), 540(3.98), 580(3.14) | 617 | 58 |
| $YH^-$ | 478(4.27), 638(394) | 680 | 0.34 |
| $Y^{2-}$ | 480(4.30), 590(3.91), 638(4.13) | 680 | 13 |
| $HBH^+$ | 440(4.26) | 600 | 1.2 |

are shown. At pH 7–9, the emission spectra have a maximum at 617 nm. As pH increases, the intensity at 617 nm decreases drastically, up to nearly zero at pH 9 and 10, and a new emission peak at 680 nm appears concomitantly with an increasing intensity. The dependences of the fluorescence intensity on pH at 617 and 680 nm are illustrated in Fig. 5. The maximum at 617 nm at pH ~7.0 is

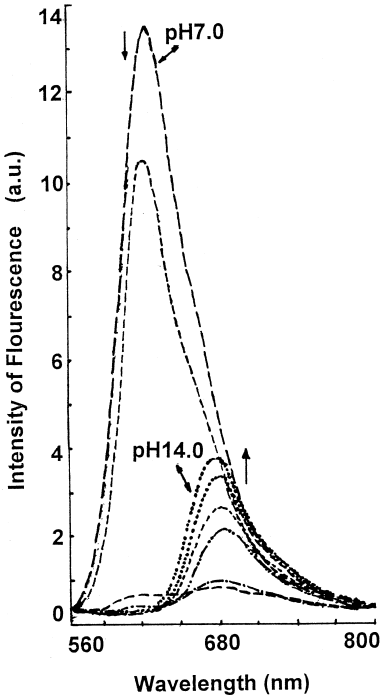



Fig. 4. pH dependence of fluorescence spectra of hypocrellin B in the range of pH between 7 and 14 (a.u., arbitrary unit). The arrows indicate the change directions.

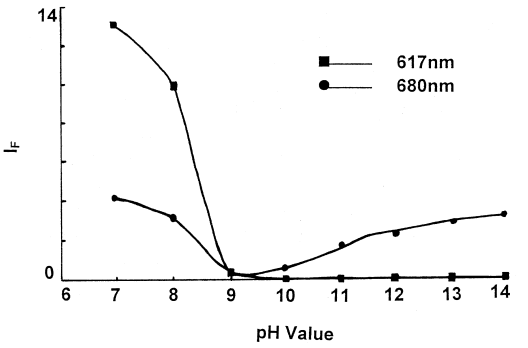



Fig. 5. Effect of pH on fluorescence intensity ($I_F$) at 617 and 680 nm obtained from Fig. 4.

obtained where the compound is present almost exclusively as the parent form of HB; the maximum at 680 nm is observed at pH 14, where the compound is present almost exclusively as the dianionic form ($Y^{2-}$).

The variation in the fluorescence intensity with pH closely parallels with the titration curve of the absorption spectra (Fig. 3). This fact indicates that, during the life of the emitting excited states, no different protropic equilibria are established with respect to those existing in the corresponding ground states [25]. Emission properties of these three HB forms are listed in Table 1.

The different absorption properties of these three HB forms suggest that the pKa of their excited states (pKa*) may be different from the pKa of the respective ground state [26]. The red shifts of the absorption and emission are indicative of a lowering of pKa in the excited state [27]. The difference in pKa (ΔpKa) between the ground state and excited states can be calculated by the Förster Cycle [28].

The pKa and pKa* of HA can also be obtained by using the same method (Table 2). These results in Table 2 indicate that the acidity of HB, as well as of HA, was enchanced in the excited state as compared with that in ground state. But the lowering of pKa (ΔpKa) for hypocellins between the excited state and the ground state is less than those of hypericin [27]. This might be responsible for the absence of an acidification effect on the surrounding medium by hypocrellin and the requirements of oxygen for its antiviral activity [9], which is different from the case of hypericin [7].

### 3.2. Absorption and emission properties during the protonation process

When the pH decreases from 0.6 to −0.6, the absorption of HB shifts blue from 458 to 440 nm, with isobestic points at 350 and 428 nm (Fig. 6). The protonation of HB may disrupt its chromophore structure, with substantial intramolecular H-bonding between the carbonyl group and its neighboring hydroxyl protons, which accounts for the blue shift of the absorption maxima of HB by HCl. The inset illustrates the absorbance at 548 mn as a function of pH; similarly, the pKa ($HBH^+/HB$) = 0.3 was obtained. Protonation influences the emission spectra of HB significantly. The fluorescence peak shifts blue to 600 nm and the fluorescence at 620 nm decreases drastically (Fig. 7). Table 1 lists the absorption and emission spectra properties of $HBH^+$. In the same way, pKa* can be calculated according to the Förster Cycle [28], i.e. −1.8. The corresponding pKa and pKa* of HA ($HAH^+/HA$) can be determined similarly (Table 2).

Table 2
pKa and pKa* of hypocrellin A and B

| Compound | HA | | | HB | | |
|---|---|---|---|---|---|---|
| pKa | –0.3 | 8.1 | 10.8 | –0.3 | 8.4 | 11.0 |
| pKa* | –1.7 | 5.8 | 10.7 | –1.8 | 6.0 | 10.9 |

### 3.3. pH dependence of photoinduced generation of semiquinone anion radical of HB (HB)

#### 3.3.1. EPR detection

Irradiation of an argon-saturated ethanol-buffer (1:1 by volume, pH 8.5) containing HB (50 μM) and GSH (5 mM) generates a 24-line hyperfine EPR signal (Fig. 8). This stable spectrum is

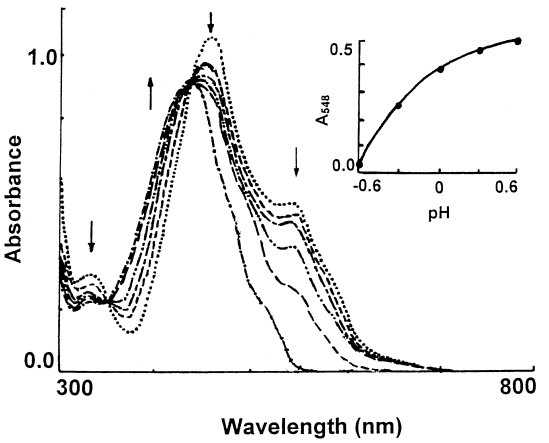



Fig. 6. Absorption spectra of hypocrellin B (49 μM) in ethanol-buffer (1:1 by volume). The concentrations of hydrochloric acid ([HCl) are as follows: 0, 0.25, 0.5, 1, 2 and 4 M. The inset shows the absorbance at 548 nm ($A_{548}$) as a function of pH. The arrows indicate the change directions.

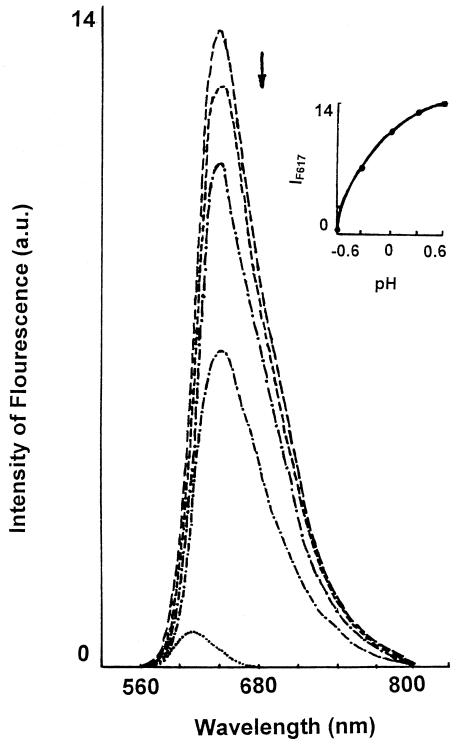



Fig. 7. Fluorescence spectra of hypocrellin B in ethanol-buffer (1:1 by volume). The concentrations of hydrochloric acid ([HCl]) are as follows: 0.25, 0.5, 1, 2 and 4 M. The inset shows the intensity of fluorescence at 617 nm ($I_{F617}$) as a function of pH (a.u., arbitrary unit). The arrows indicate the change directions.

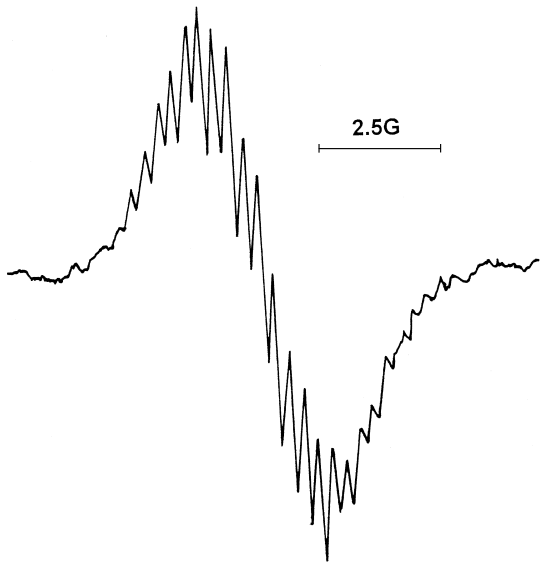



Fig. 8. EPR spectrum obtained on photolysis of deoxygenated ethanol-buffer (1:1 by volume, pH 8.5) containing HB (50 $\mu$M) and GSH (5 mM).

consistent with that ascribed to the semiquinone anion radical of HB ($HB^{\cdot -}$) [29,30]. GSH, as a typical electron donor (D), transfers one electron to excited HB, to form $HB^{\cdot -}$[Eq. (1)].

$$HB^* + D \rightarrow HB^{\cdot -} + D^{\cdot +} \quad (1)$$

When the pH of the reaction medium is lower than 6 or higher than 11, the EPR signal of $HB^{-}$ is weakened drastically and cannot be detected.

### 3.3.2. Spectrophotometric measurements

When an argon-saturated ethanol-buffer (1:1 by volume, pH 8.5) solution of HB (40 $\mu$M) and GSH (5 mM) was irradiated, the changes of absorption spectra are recorded in Fig. 9(a) and (b). It can be seen that the absorbance of HB at 470 nm (for HB) decreases, and a new band of an intermediate at 623 nm appears, accompanied by an isobestic point at 573 nm [Fig. 9(a)]. On further irradiation of the intermediate, another new absorption band at 507 nm is formed, while the absorbance at 623 nm decreases, with an isobestic point at 538 nm [Fig. 9(b)]. These results are in good agreement with previous studies [18,31–33]. The absorption bands centred at 623 and 507 nm can be assigned to the semiquinone anion radical of HB ($HB^{\cdot -}$) and the hydroquinone of HB ($HBH_2$) [32].

When pH decreases to 5.2, keeping the other conditions constant, the absorption of HB (44 $\mu$M) at 458 nm decreases and a new band at 486 nm is formed with three isobestic points at 322, 478 and 496 nm [Fig. 9(c)]. Like HA, the new intermediate at 486 nm is assigned to $HBH_2$ [32]. $HB^{-}$ cannot be observed.

When the pH increases up to 11.3, keeping the other conditions constant, the absorbance of HB (40 $\mu$M) at 480 nm also decreases and a new absorption band at 507 nm appears with isobestic points at 320, 406, 506 and 552 nm [Fig. 9(d)]. As in the case of pH 8.5, the intermediate of 507 nm can be assigned to $HBH_2$ [32]. $HB^{\cdot -}$ cannot be observed.

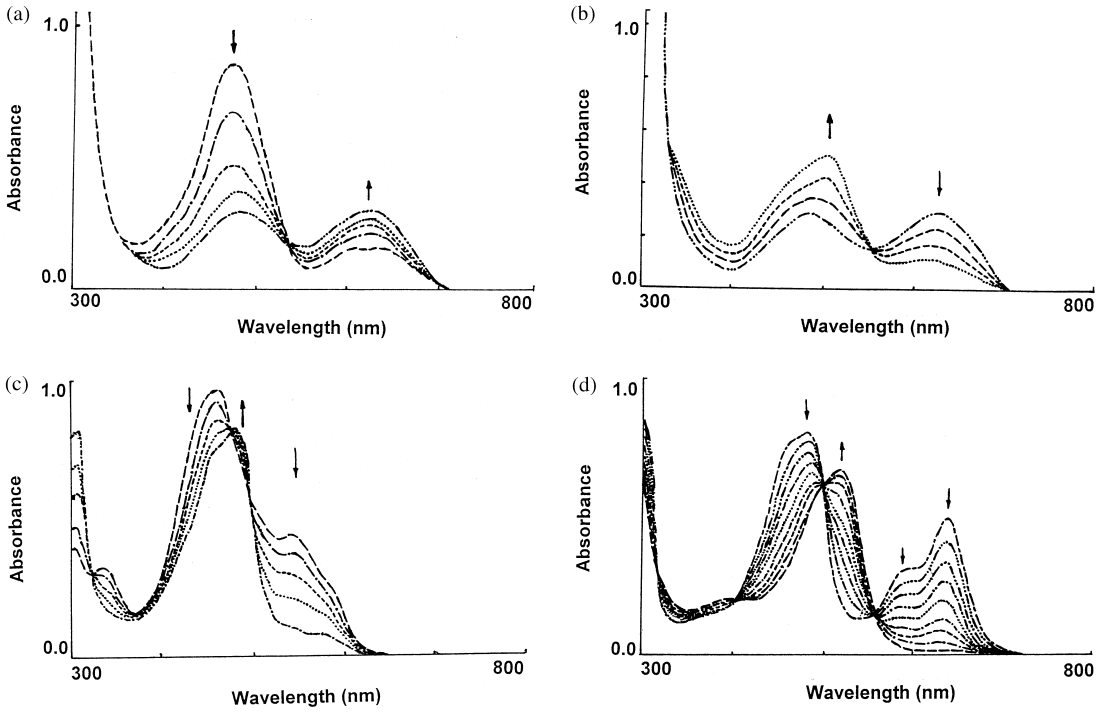



Fig. 9. Absorption spectrum changes recorded when a deoxygenated sample containing hypocrellin B and GSH (5 mM) in ethanol-buffer (1:1 by volume) was irradiated for (a) 0, 5, 15, 60, 105 s at pH 8.5 with 40 $\mu$M HB: (b) 105, 225, 405, 705 s at pH 8.5 with 40 pLM HB; (c) 0, 40, 80, 120, 160 s at pH 5.21 with 44 $\mu$M HB; and (d) 0, 5, 15, 25, 45, 75, 120, 240 s at pH 11.3 with 40 $\mu$M HB. The arrows indicate the change directions.

### 3.4. Discussion

Hypocrellins, natural pigments, possess light-induced antiviral activity against some viruses [6–9], and it has been suggested that this antiviral activity absolutely required oxygen [7] and that hypocrellin did not acidify the surroundings [9]. There are two phenolic hydroxyl groups which participate in the formation of intramolecular H-bonding with their peri-carbonyl groups. Protonation of the carbonyl groups or deprotonation of the phenolic hydroxyl groups [Eq. (2)] will change the electronic distribution in the chromophore and can be observed in the dependence of absorption and emission on the pH value:

$$HBH^{+} \rightleftharpoons HB(YH_2) \rightleftharpoons YH^{-} \rightleftharpoons Y^{2-} \quad (2)$$

Protonation induces a blue shift of the spectra and a decrease in the fluorescence quantum yield (Table 1). This is attributed to the lowered electron conjugation of $=OH^{+}$, and the destruction of intramolecular H-bonding in $HBH^{+}$, respectively. Deprotonation results in a red shift of the spectra, and also a decrease of fluorescence quantum yield (Table 1). This is because $-O^{-}$ in $YH^{-}$ or $Y^{2-}$ have more electronic conjugation than $-OH$ with the aromatic ring, and the destruction of intramolecular H-bonding in HB (or $YH_2$) induced from deprotonation decreases the plane rigidity, respectively. In terms of absorption spectra, the red-shift effect is derived from the enhanced electron conjugation of $-O^{-}$ in anionic forms prevailing over the blue-shift effect from the destruction of intramolecular H-bonding.

In addition, the proton-donating ability of solvents ($\alpha$) influences the absorption and emission properties of HB (Table 3). As the solvent proton-donating ability ($\alpha$) decreases, the absorption and emission spectra shift red. This indicates that the

Table 3
Solvent proton-donating ability effect on the absorption (A) and emission (F) maximum peaks of hypocrellin B

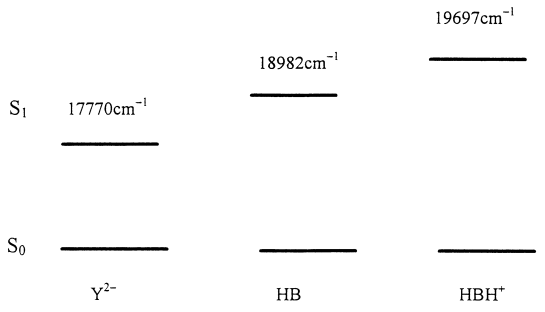
| Solvent | $\lambda_A$ (nm) | $\lambda_F$ (nm) | $\alpha$ |
|---|---|---|---|
| Methanol | 458 | 615 | 0.93 |
| Ethanol | 460 | 617 | 0.83 |
| Acetonitrile | 460 | 620 | 0.19 |
| Acetone | 462 | 622 | 0.08 |
| Chloroform | 464 | 625 | 0 |
| *N,N*-dimethylformamide | 468 | 628 | 0 |
| Dimethylsulfoxide | 470 | 629 | 0 |

$\alpha$: A measure of solvent ability to donate a proton [34].

intermolecular H-bonding formed between HB and solvent will compete with the intramolecular H-bonding, and show similar results with the protonation and deprotonation processes. The results in Table 3 illustrate the effect of the proton-donating ability of solvents on the spectroscopic behavior of HB, which further suggests the importance of the intramolecular H-bonding.

Ground and excited singlet state deprotonation and protonation pKa values of HB and HA have been determined by means of spectrophotometric and spectrofluorometric titrations and Förster Cycle calculation (Table 2). It is evident that pKa* values are lower than those of pKa, indicating that the acidities of HA and HB in the excited state increase compared with those in the ground state. Also, HA is a slightly more easily deprotonated than HB under the same conditions.

The energy levels of the deprotonated ($Y^{2-}$) and protonated species ($HBH^+$) as well as of HB, are illustrated as follows, calculated from the average of absorption and emission maximum peaks. It can be seen that protonation results in an energy increase, while deprotonation induces an energy decrease.




The pH value influences the photoinduced generation of the semiquinone anion radical of HB ($HB^{\cdot-}$) which is the precursor of superoxide anion radical and then of the hydroxyl radical [33]. The EPR observations are fully consistent with the spectrophotometric measurements. In acidic media, transfer of the second electron, as well as the disproportionation process, may be catalysed by protonation of the radical anion ($HBH^{\cdot}$) as the concentration of free $H^+$ ion increases [35] [Eqs. (3–5)] and reduces the stability of $HB^{\cdot-}$ significantly.

$$HB^{\cdot-} + H^+ \rightarrow HBH^{\cdot} \quad (3)$$

$$HBH^{\cdot} + D \rightarrow HBH^- + D^{\cdot+} \quad (4)$$

$$HBH^{\cdot} + HBH^{\cdot} \rightarrow HBH_2 + HB \quad (5)$$

When the pH is higher than $pKa_2$ (i.e. 11 for HB), the dianionic species ($Y^{2-}$) is predominant and its intramolecular H-bonding is completely destroyed. Thus the absence of intramolecular H-bonding, which can stabilize HB, accelerates the formation of the hydroquinone of HB ($HBH_2$), and shortens the lifetime of the formed semiquinone anion radical. $HB^{\cdot-}$ can reach high stability only in the pH range between 7 and 9.5 and can be detected easily either by the EPR method or by the spectrophotometric method, owing to the presence of intramolecular H-bonding.

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