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# Addition of β-Lactoglobulin Produces Water-Soluble Shikonin

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Supporting Information

**ABSTRACT:** Shikonin and its ester derivatives belong to a group of secondary metabolites with a wide array of beneficial effects on human health. However, shikonin is principally used in oil-based preparations due to the low solubility of the pigment in aqueous media, and the positive properties of shikonin are not exploited to their full potential. Such low aqueous solubility often results in poor bioavailability, makes shikonin undesirable for oral administration, and restricts its broadened use in the food and pharmaceutical industries. The purpose of this study was to enhance the aqueous solubility of shikonin by the addition of β-lactoglobulin and to characterize the macromolecule−ligand binding interaction by means of spectrophotometry, spectrofluorometry, high-performance liquid chromatography, and mass spectrometry. In the presence of β-lactoglobulin the solubility of shikonin is increased up to 181-fold. One shikonin molecule binds covalently to β-lactoglobulin via $Cys^{121}$, whereas the remaining pigment molecules most probably bind to the protein via noncovalent interactions.

**KEYWORDS:** *shikonin, β-lactoglobulin, binding interaction, enhanced water solubility*

## INTRODUCTION

Shikonin (Figure 1) and its ester derivatives (shikonins) are representatives of 1,4-naphthoquinones or naphthazarins,

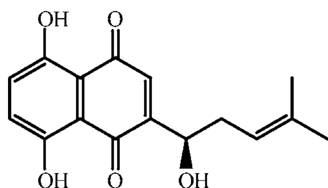


**Figure 1.** Shikonin.

respectively. Shikonins are a group of secondary metabolites that are often found in the root epidermis of at least 150 Boraginaceae plants, among which *Lithospermum*,[1] *Arnebia*,[2] *Alkanna*,[3] and also *Echium*[4] genera have been most frequently studied.

Shikonin has an absorbance maximum at ∼518 nm and has a characteristic red color. Shikonins possess anti-inflammatory,[5] wound-healing,[6] antifungal,[7] antioxidative,[1,3] antitumor,[8] antibacterial,[9] and antithrombotic properties.[10] Some of the latest research is focused on shikonin's beneficial effects in diabetes treatment and breast cancer, gastric cancer, prostate cancer, bladder cancer, and lung cancer treatment.[11−13] Due to their lipophilicity, shikonins currently predominate in oil-based preparations such as creams and ointments. The insolubility in water of shikonin is usually related to its poor bioavailability, which hinders its oral uptake and consequently prevents its widespread usage in the pharmaceutical and food industries.[14] Shikonins are also thermally labile[15] and are prone to photooxidation[16] and polymerization.[17]

Some approaches aimed at improvement of the stability of shikonin and especially at enhancement of its water solubility have been considered. With the addition of cyclodextrins, shikonin forms inclusion complexes, and as a result its water solubility can be greatly improved while at the same time the pigment is less prone to degradation. The drawback of this approach is a high excess (up to 50%) of the oligosaccharide needed for the solubilization of shikonin in aqueous media.[14,18] Shikonin-containing microcapsules can also increase the water solubility of these naphthoquinones, although the raw encapsulating material must be chosen with care to ensure that the final product is suitable for oral intake. In addition, release studies of shikonin-containing microcapsules are required to adequately express the pharmacological properties of the pigment.[19,20]

To our knowledge, no study has been published in which the water solubility of shikonin was enhanced by the addition of a protein. Although the pigment is supposed to form a complex with the human serum albumin, the published results are ambiguous and fail to provide any data on the increased water solubility of the naphthoquinone in its complexed form.[21]

Whey proteins are widely incorporated in the food and pharmaceutical industries and are generally recognized as safe. At least nine variants of the most abundant whey protein, β-lactoglobulin (β-Lg), are known (A, B, C, D, E, H, I, J, and W), and among these the A and B (β-LgA and β-LgB) variants, present in bovine milk, are the most common.[22] β-LgA and β-LgB comprise 162 amino acid residues and are nearly identical; $Asp^{64}$ and $Val^{118}$ in β-LgA (MM = 18363 Da) are replaced by $Gly^{64}$ and $Ala^{118}$ in β-LgB (MM = 18276 Da).[23] This property makes separation and isolation of LgA and β-LgB individually, and from other constituents of whey, a challenging task.[24] β-Lg contains five Cys amino acid residues, of which four form

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Table 1. Preparation of Working Solutions

| | β-Lg/shikonin | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1:1 | 1:2 | 1:3 | 1:5 | 1:10 | 1:15 | 1:20 | 1:25 | 1:30 | 1:50 | 1:75 | 1:100 |
| 0.17 mM β-Lg (μL) | 1000 | 1000 | 1000 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 4.2 mM shikonin (μL) | 40 | 80 | 120 | 20 | 40 | 60 | 80 | 100 | 120 | 200 | 300 | 400 |
| EtOH (μL) | 80 | 40 | 0 | 380 | 360 | 340 | 320 | 300 | 280 | 200 | 100 | 0 |
| 10 mM $NH_4OAc$ (μL) | 200 | 200 | 200 | 4000 | 4000 | 4000 | 4000 | 4000 | 4000 | 4000 | 4000 | 4000 |

disulfide bridges, that is, via $Cys^{106}$–$Cys^{119}$ and $Cys^{66}$–$Cys^{160}$.[25] This protein has one free Cys residue, $Cys^{121}$; the reactivity of this thiol functional group is associated with the gelation process, and it increases at pH >6.5. Despite the fact that there is much research available, the biological function of β-Lg is still unclear, but because the globular protein is a member of the lipocalin family, a transport function for β-Lg is usually assumed.[26] The amino acid sequence of lipocalins usually varies considerably, but their tertiary structure is similar as they all possess a structural element, a β-barrel, that is formed from eight antiparallel β-sheets.[26,27] β-Lg is a hydrophobic globular protein and has nine β-sheets, eight of which form the β-barrel, and a single α-helix.[28] Hydrophobic amino acid residues face the interior of the β-barrel, whereas the hydrophilic amino acid residues are exposed to the solvating medium on the outer surface of the protein. Thus, the β-barrel forms a hydrophobic calyx, which can act as a preferential binding site for a wide array of hydrophobic ligands.[29−31] Some studies suggest that there might also be an alternative binding site located on the outer surface of the protein.[30,32] β-Lg can also be used as an entrapment agent for some compounds of interest, such as (−)-epigallocatechin-3-gallate; an entrapped secondary metabolite shows higher stability in the form of produced nanoparticles, and consequently its activity is preserved.[33]

The binding of a ligand to the calyx is not only a function of protein structure and ligand size but also depends on extrinsic parameters such as the medium pH. For example, β-Lg undergoes a Tanford transition at pH ∼7,[28] a transition associated with the position of the EF loop ($Ala^{86}$–$Glu^{89}$) in the protein. This loop is in a closed position at pH <∼7 and prevents the ligand from binding into the protein core; at a pH higher than ∼7, however, it is in the open position and the steric hindrance at the calyx entrance is removed. The opening/closing of the EF loop is closely related to protonation or deprotonation of the $Glu^{89}$ carboxylic group.[28,34]

Our goal was to improve the aqueous solubility of shikonin by the addition of a whey protein (β-Lg) and to characterize the protein–ligand interaction in the light of the stoichiometry and mechanism. For this purpose we used spectrophotometry, spectrofluorometry, mass spectrometry (MS), and high-performance liquid chromatography (HPLC). The enhanced solubility of shikonin could potentially give rise to increased usage of this health beneficial pigment in the food industry.

## MATERIALS AND METHODS

**Chemicals and Standards.** Methanol (MeOH) and acetonitrile (MeCN) were purchased from J. T. Baker (Deventer, The Netherlands); ethanol (EtOH) was from Sigma-Aldrich (Steinheim, Germany); sodium hydroxide (NaOH), urea, *n*-hexane, acetic acid (AcOH), ammonium acetate ($NH_4OAc$), ammonia ($NH_3$), and phosphoric acid were from Merck (Darmstadt, Germany); formic acid (HCOOH) and disodium hydrogen phosphate ($Na_2HPO_4$) were from Kemika (Zagreb, Croatia); citric acid, ammonium bicarbonate ($NH_4HCO_3$), and L-tryptophan were from Fluka (Steinheim, Germany); iodoacetamide was from Sigma (Steinheim, Germany); and modified trypsin (Promega Sequencing grade Modified Trypsin), with characteristically high specificity of peptide bond cleavage, was purchased from Promega (Madison, WI, USA). Water with 18.2 MΩcm resistivity at 25 °C was used. Shikonin was purchased from Biomol (Plymouth Meeting, PA, USA). β-Lg (from bovine milk, 90%; mixture of A and B variants), β-LgA (from bovine milk, 90%), and β-LgB (from bovine milk, 90%) were purchased from Sigma.

**Materials.** Glass GC vials were purchased from Thermo Finnigan (San Jose, CA, USA) and plastic Protein LoBind tubes from Eppendorf (Hamburg, Germany). Unless stated otherwise, glass GC vials were used.

**Spectrophotometry.** Absorbance and absorption spectra of the solutions were obtained with a Lambda 45 UV−vis spectrophotometer (PerkinElmer, Waltham, MA, USA), and the results were evaluated using UV WINLAB (2.85.04.) software. Quartz cuvettes with a 1 cm optical path length were used. Spectra were recorded at 240 nm/min in 0.5 nm intervals, the range was set to 210−750 nm, and the slit width was set to 0.5 nm. Absorbance of the solutions was read at $\lambda_{max}$.

In the experiments in which the solubility of shikonin in aqueous media was evaluated, the UV−vis spectra of β-Lg/shikonin solutions in molar ratios from 1:1 to 1:100 were recorded. Fresh stock solution of β-Lg (0.17 mM) was prepared in 10 mM $NH_4OAc$, pH 7.5, on a daily basis, and a stock solution of shikonin was prepared in EtOH (4.2 mM). The preparation of working solutions of different β-Lg/shikonin ratios is given in Table 1.

The EtOH portion never exceeded 9.1%. Samples prepared in this manner were incubated for 24 h at room temperature and subsequently lyophilized. After freeze-drying, the solutions of 1:1, 1:2, and 1:3 ratios were redissolved in 10 mM $NH_4OAc$, pH 7.5 (1 mL), and all others in 0.1 mL of the same buffer. After 24 h of incubation at room temperature, the 1:1, 1:2, and 1:3 samples were filtered through a 0.45 μm Millipore Millex-HV hydrophilic poly(vinyldiene difluoride) (PVDF) membrane filter (Middlesex, USA), and all other samples were centrifuged at 12100*g* for 10 min on a MiniSpin centrifuge (Eppendorf). The supernatant from the centrifuged samples (50 μL) was diluted 20-fold with 10 mM $NH_4OAc$, pH 7.5 (950 μL). Blank samples were prepared in a similar manner, but β-Lg was omitted. The UV−vis spectra were recorded after 0, 7, 18, and 31 days; samples were stored in the dark at 6 °C and were centrifuged for 10 min at 12100*g* before the measurement. The calculated *A* represents a value at which the dilutions of the solutions were already accounted for.

In the experiments in which the effect of pH on the absorbance and absorption maximum of shikonin in the presence of β-Lg was evaluated, UV−vis spectra of shikonin with and without β-Lg were recorded in the pH interval 4−8. The working solutions were prepared by adding shikonin in EtOH (20 μL, 4.2 mM) to 10 mM $NH_4OAc$, pH 7.5 (1 mL), and to freshly prepared β-Lg solution (1 mL, 0.17 mM), respectively. Prepared blank and sample solutions were filtered through a 0.45 μm PVDF filter after a 24 h incubation, and the UV−vis spectra were recorded. Sodium citrate (50 mM) was used in the pH region 4−5.5, and sodium phosphate (50 mM) was used for the pH range 6−8.

**Spectrofluorometry.** Fluorescence intensity and emission spectra of the solutions were obtained by means of an LS 55 Luminiscence spectrometer (PerkinElmer) equipped with a thermostated cell. The results were evaluated using FL WINLAB (4.00.02) software. Quartz cuvettes (3 mm × 3 mm) were used. The excitation wavelength was set to 295 nm, the emission spectra were recorded between 285 and 450 nm, the excitation and emission slits were set to 5 and 10 nm, respectively, the scanning speed was set to 250 nm/min, the response

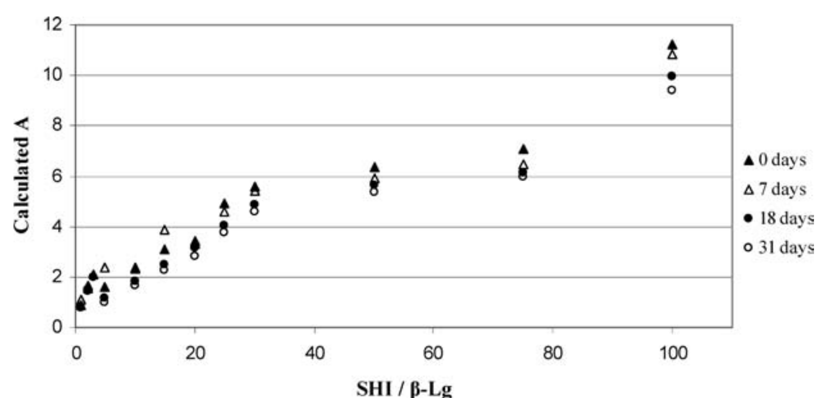


**Figure 2.** Effect of $\beta$-Lg on the solubility of shikonin in aqueous media. Spectra were recorded in 10 mM $NH_4OAc$, pH 7.5.

width was set to 15 nm, the detector voltage was set to 775 V, and the resulting spectrum was an average of three consecutive measurements. The fluorescence intensity of every individual scan was read at $\lambda_{max}$.

In the $\beta$-Lg fluorescence quenching experiments with shikonin, six solutions of $\beta$-Lg in 10 mM phosphate buffer, pH 7.5 (1 mL, 3.4 $\mu$M), were prepared from $\beta$-Lg stock solution (0.17 mM) by 50-fold dilution. To these solutions were added 0, 1, 2, 5, 10, and 15 $\mu$L of shikonin solution in EtOH (4.2 mM), which resulted in $\beta$-Lg/shikonin solutions of molar ratios of 1:0, 1:1.2, 1:2.5, 1:6.2, 1:12.4, and 1:18.5, respectively. The emission spectra were recorded after 30 min, 3.5 h, and 3 days of incubation at room temperature. The fluorescence of the buffer and that of free shikonin in the buffer were recorded at each interval at the highest concentration. Cuvettes were washed three times with deionized water and once with the sample solution and then thermostated with the sample for 5 min at 25 °C prior to the measurements. The apparent fluorescence intensities were corrected for dilution and inner-filter effect according to the equation

$$F_{corr} = F_{app}\left(\frac{V_0 + V_i}{V_0}\right)10^{(A_{iex}d_{ex}/2 + A_{iem}d_{em}/2)}$$

where $F_{corr}$ is the corrected value of fluorescence intensity at a given point $i$, $F_{app}$ is the apparent fluorescence intensity, $V_0$ is the initial volume of the sample, $V_i$ is the volume of the sample after the $i$th addition of shikonin solution, $d_{ex}$ and $d_{em}$ are excitation and emission path lengths in the cuvette, respectively, and $A_{iex}$ and $A_{iem}$ are the absorbances of the sample at excitation and emission wavelengths, respectively. The extent of photobleaching was determined by a continuous 15 min irradiation of $\beta$-Lg in 10 mM phosphate buffer, pH 7.5 (3.4 $\mu$M), at 295 nm. The influence of EtOH on the protein fluorescence was determined by the addition of EtOH (1, 2, 5, 10, and 15 $\mu$L) to the $\beta$-Lg solution in 10 mM phosphate buffer, pH 7.5 (3.4 $\mu$M).

In the experiments in which Eppendorf LoBind tubes were used to evaluate the extent of protein adsorption to the glass surface, three $\beta$-Lg solutions in 10 mM phosphate buffer, pH 7.5 (1 mL, 3.4 $\mu$M), were prepared to which 0, 1, and 5 $\mu$L of shikonin in EtOH (4.2 mM) were added; this produced $\beta$-Lg/shikonin solutions of molar ratios 1:0, 1:1.2, and 1:6.2, respectively. The emission spectra were recorded after 2.5 and 18 h of incubation at room temperature.

In the experiments in which the intrinsic fluorescence of L-Trp was quenched by shikonin, five L-Trp solutions in 10 mM phosphate buffer, pH 7.5 (1 mL, 9.8 $\mu$M), were prepared in Eppendorf tubes to which 0, 1, 2, 5, or 10 $\mu$L of shikonin in EtOH (4.2 mM) was added; this produced $\beta$-Lg/shikonin solutions of molar ratios 1:0, 1:0.43, 1:0.85, 1:2.13, and 1:4.25, and the emission spectra were recorded after 1 and 24 h of incubation at room temperature. The apparent fluorescence intensities were corrected for the dilution and inner-filter effect.

**Mass Spectrometry.** Mass spectra were obtained with the LTQ Velos system (Thermo Finnigan) equipped with two linear ion traps operating at two different pressure stages. The results were evaluated using Xcalibur (2.1) software. Samples were prepared in the following manner: $\beta$-Lg solutions in 10 mM $NH_4OAc$, pH 7.5 (0.1 mL, 0.17 mM), were diluted 10-fold with the same buffer, and afterward 0 and 4 $\mu$L of shikonin in EtOH (4.2 mM) were added, respectively; solutions of molar ratio 1:0 (blank) and 1:1 were obtained. Solutions were incubated at room temperature for 1 h and 3 days, respectively. They were subsequently diluted 50-fold with 0.02% HCOOH and injected into the MS system at 5 $\mu$L/min. A heated ESI (HESI) ion source in positive mode was used for the ionization of the analytes. The scan range was set to $m/z$ 1000−3000, capillary and HESI probe temperatures were maintained at 275 and 55 °C, respectively, the sheath gas was set to 30 ae, auxiliary gas was set to 5 a.u., sweep gas was set to 0 a.u., the automatic gain control (AGC) was on, and the source voltage was set to 2.5 kV.

**High-Performance Liquid Chromatography−Mass Spectrometry.** To obtain the tryptic peptide map of the product from $\beta$-Lg and shikonin ($\beta$-Lg−SHI), a solution of $\beta$-LgA in 50 mM $NH_4HCO_3$, pH 7.9 (1 mL, 5.6 $\mu$M), was prepared in an Eppendorf LoBind tube, then shikonin in EtOH (20 $\mu$L, 4.2 mM) was added, and the solution was incubated for 24 h at room temperature. To an aliquot of that solution (500 $\mu$L) trypsin (10 $\mu$L, 0.1 $\mu$g/$\mu$L) was added after the 24 h period so that the protein/enzyme mass ratio equaled 50:1. The solution was incubated for 3 h at 37 °C, and then the reaction was quenched by the addition of HCOOH (5 $\mu$L). A blank sample was prepared in a similar manner with the shikonin omitted and EtOH (20 $\mu$L) added in its place. The samples were analyzed by an Accela U-HPLC system (Thermo Finnigan) coupled with the LTQ Velos system (Thermo Finnigan).

The Accela U-HPLC system was equipped with a thermostated autosampler Accela autosampler (Thermo Finnigan) with a 25 $\mu$L loop, a quaternary pump Accela pump (Thermo Finnigan), and a diode array detector Accela PDA detector (Thermo Finnigan). Xcalibur (2.1) software was used for evaluation of the collected data. A HESI ion source in positive mode was used for the ionization of analytes. The scan range was set to $m/z$ 250−1500, capillary and HESI probe temperatures were maintained at 275 and 200 °C, respectively, the sheath gas was set to 35 ae, the auxiliary gas was set to 10 a.u., the sweep gas was set to 0 a.u., AGC was on, and the source voltage was set to 2.5 kV.

The mobile phase consisted of buffer A, which was 0.1% HCOOH in water (v/v), and buffer B, which was 0.1% HCOOH in MeCN (v/v). The following gradient was used: 5−22% B (0−7 min), 22−45% B (7−28 min), 45−80% B (28−29 min), 80% B (29−30 min), 80−5% B (30−31 min), 5% B (31−45 min). The separations were carried out on an ACE Excel 2 $\mu$m C18 column (100 mm × 2.1 mm i.d.) from Advanced Chromatography Technologies (Aberdeen, Scotland). The mobile phase flow rate was set to 150 $\mu$L/min, the column temperature was maintained at 30 °C, the tray temperature was set at 20 °C, and 15 $\mu$L of sample was injected onto the HPLC column with each run.

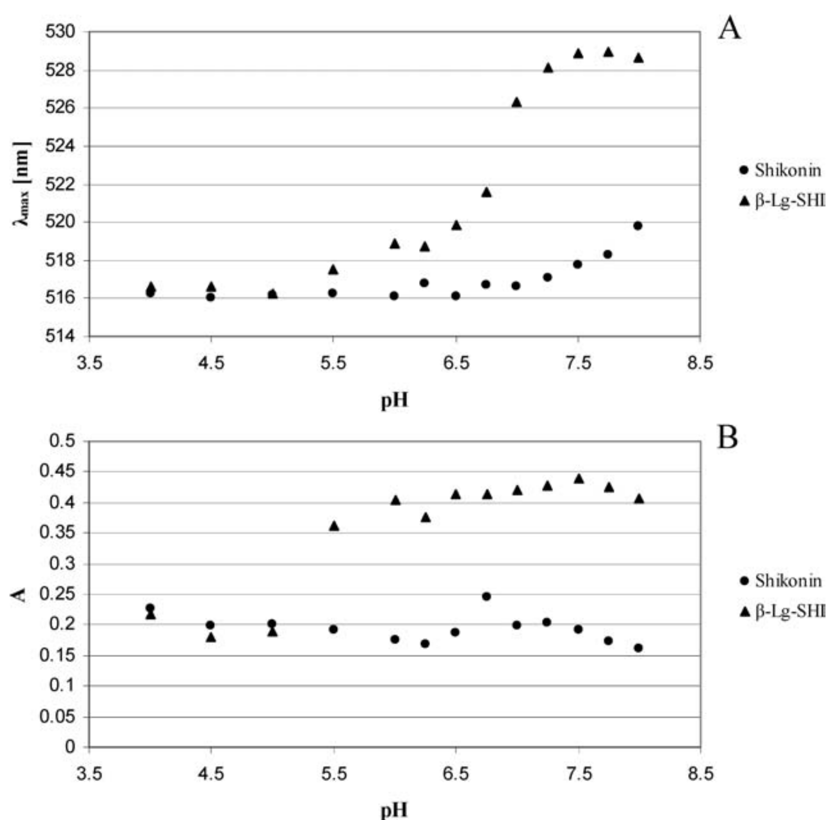


**Figure 3.** Effect of pH on $\lambda_{max}$ of shikonin (A), and on the absorbance of shikonin (at $\lambda_{max}$) (B), without and in the presence of $\beta$-Lg, respectively. Spectra were recorded in citric and phosphate buffer, respectively.

## RESULTS AND DISCUSSION

**Solubility of Shikonin in Aqueous Media in the Presence of $\beta$-Lactoglobulin.** Shikonin is a highly hydrophobic molecule, practically insoluble in aqueous solutions. Spectrophotometrically, we tested the effect of added $\beta$-Lg on the solubility of shikonin in an appropriate buffer by preparing different $\beta$-Lg/shikonin solutions; however, the concentration of the protein was held constant (Figure 2). The solutions were formed in a manner in which the EtOH portion did not exceed 9.1% (v/v); structural changes in the protein occur at values >20% EtOH, and these might influence the ligand−protein interaction.[35]

Blank samples (shikonin solutions without $\beta$-Lg) failed to show an increase in absorbance with the increased amount of shikonin; the absorbance of these solutions was nominally constant, averaging 0.062. The absorbance was even lower when we attempted to dissolve solid shikonin in the buffer. On the other hand, in the presence of $\beta$-Lg the amount of shikonin in solution increased continuously with the addition of shikonin. The calculated absorbances, when the dilutions were accounted for, were 7.06 and 11.24 in $\beta$-Lg/shikonin solutions prepared at molar ratios 1:75 and 1:100, respectively. In comparison with the blank samples this translates to 114- and 181-fold increases in solubility of shikonin at 3.1 mg/mL $\beta$-Lg concentration. The solubility of this pigment in aqueous media could be increased even further by an increase in protein concentration. The product formed between the pigment and the protein seemed to be relatively stable in the solution as the concentration of shikonin dropped only slightly after 7, 18, and 31 days in storage, respectively (Figure 2).

In comparison, the solubility of shikonin is significantly enhanced at relatively low protein concentrations, whereas a high carbohydrate excess, compared to shikonin, is a requisite for the formation of 1:1 shikonin inclusion complexes in cyclodextrins.[18] The product between $\beta$-Lg and shikonin ($\beta$-Lg−SHI) is apparently stable and may potentially be appropriate for oral intake as $\beta$-Lg is beneficial to human health.

**Characterization of the Interaction between $\beta$-Lg and Shikonin: Spectrophotometry.** The binding of a ligand to a macromolecule is frequently accompanied by solvatochromism, a phenomenon that occurs when the properties (mainly polarity and polarizability) of the immediate surrounding medium of a chromophore change. In these situations, the wavelength of the chromophore's absorption maximum generally shifts to higher or lower values, respectively. The absorption maximum ($\lambda_{max}$) of the ligand in the UV−vis spectra is usually red-shifted when the molecule surrounds itself with highly polarizable amino acid residues.[36−38] A relatively high bathochromic shift was noted in the UV−vis spectra of shikonin when $\beta$-Lg was added to the buffer solution at pH 7.5 (protein and ligand were mixed in molar ratio $\beta$-Lg/shikonin = 2:1) and $\lambda_{max}$ shifted from ∼518 to ∼529 nm (Supporting Information, Figure S1). The shift could also be observed visually as the color of the solution changed from red to slightly purple. This effect could not be attributed to the potential changes that $\beta$-Lg might have on the medium.[39] Relatively high $\lambda_{max}$ values corresponded to the highly polarizable and nonpolar surrounding area of shikonin, respectively, which indicated that the preferential binding site may lie inside the calyx that is formed by the $\beta$-barrel.

The temperature and different buffers in various concentration ranges had no effect on the bathochromic shift, and the

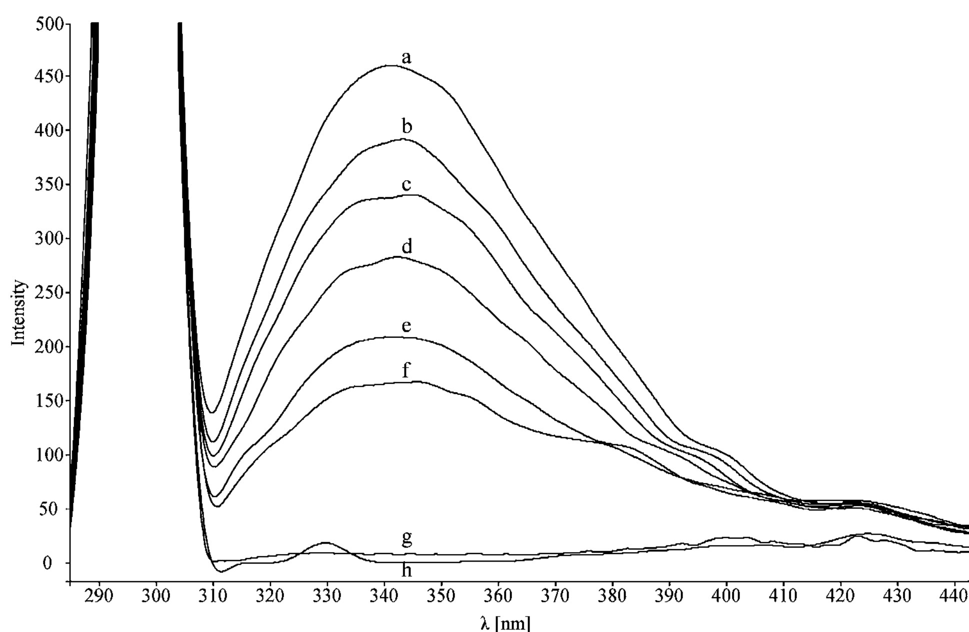


**Figure 4.** $\beta$-Lg fluorescence quenching by shikonin after 30 min of incubation at room temperature; molar ratio $\beta$-Lg/shikonin was (a) 1:0, (b) 1:1.2, (c) 1:2.5, (d) 1:6.2, (e) 1:12.4, (f) 1:18.5; (g) shikonin (63 $\mu$M) in 10 mM phosphate buffer, pH 7.5; (h) 10 mM phosphate buffer, pH 7.5. $\beta$-Lg concentration was equal to 3.4 $\mu$M throughout.

results were identical when $\beta$-LgA or $\beta$-LgB was used. In the solution preparation process a concentrated solution of shikonin in EtOH was added to that of protein so that the final solution contained ~2% of alcohol. Therefore, in another experiment the prepared samples were lyophilized and redissolved in the aqueous buffer prior to the analysis to discard the contribution EtOH might have made to the red shift. As a result, an even higher $\lambda_{max}$ was observed at 531 nm, and this excludes the alcohol as a source of the observed red shift (data not shown), although some local conformational changes in the protein could occur even at low alcohol concentrations. Removal of EtOH from the solution increased the dielectric constant of the medium, which possibly results in an even higher binding tendency of shikonin.

The red shift of $\lambda_{max}$ was also a function of the solution pH. The UV−vis spectra of shikonin (blank sample) and $\beta$-Lg−SHI (protein and ligand mixed in molar ratio $\beta$-Lg/shikonin = 2:1) were recorded in the pH range 4−8, and differences in $\lambda_{max}$ were observed (Figure 3A). The results show that for the blank samples, $\lambda_{max}$ was constant up to pH 7.25 at a value of ~516 nm, and in the pH range 7.25−8 the value slightly rose to 520 nm. Phenol hydroxyl functional groups in the naphthazarin ring are deprotonated in the alkaline environment, and consequently the UV−vis spectrum of shikonin in alkaline media differs from that in acidic media.[14] The data points in Figure 3A from $\beta$-Lg−SHI samples could be divided into three regions; $\lambda_{max}$ was comparable with $\lambda_{max}$ for the blank samples in the pH range 4−5, $\lambda_{max}$ slightly rose to ~518 nm in the pH range 5.5−6.25, and from pH 6.25 onward $\lambda_{max}$ steeply increased and reached 528.7 nm at pH 8.

The absorbance of the prepared solutions at different pH values was also monitored (Figure 3B). The absorbance is relatively constant, scattered around 0.2, in blank samples throughout the entire pH range. The data points from $\beta$-Lg−SHI samples could be divided into two regions; the absorbance is practically identical as in the blank samples in pH region 4−5 but is roughly twice as high in the pH region 5.5−8.

These results show that shikonin and $\beta$-Lg probably fail to interact at low pH. With the increase of the medium pH, the protein affinity toward the ligand increases, but the type of the interaction or the binding site is a function of pH. The EF loop of $\beta$-Lg is closed at pH 5.5 − 6.25 and, apart from that, a small bathochromic shift of $\lambda_{max}$ suggests a poorly polarizable or polar environment around shikonin molecule, therefore shikonin most likely binds to the outer surface of $\beta$-Lg at these conditions. In the upper pH range the EF loop is opened and the ligand is able to bind to the hydrophobic core of the protein, as could be confirmed by the large red shifts of $\lambda_{max}$ at slightly alkaline conditions. These spectrophotometric data implied that at least two binding sites for shikonin exist on $\beta$-Lg.

The calculated absorbances in Figure 2, however, failed to reach a certain plateau even when $\beta$-Lg and shikonin were mixed at the molar ratio 1:100, which means that even more than two molecules of shikonin might bind to the protein at pH 7.5. A similar conclusion was drawn when $\lambda_{max}$ was plotted against increasing shikonin concentration in the presence of $\beta$-Lg (Supporting Information, Figure S2). At low shikonin concentrations $\lambda_{max}$ shifted to 532.6 nm, but at higher concentrations the shift decreased until $\lambda_{max}$ reached a plateau at ~526 nm. Shikonin probably binds preferentially into the calyx of the $\beta$-barrel, and after the hydrophobic core is saturated, the pigment molecules occupy the binding site(s) on the $\beta$-Lg exterior. This is probably why $\lambda_{max}$ falls and the absorbance (solubility) of the solutions rises with the addition of shikonin.

**Characterization of the Interaction between $\beta$-Lg and Shikonin: Spectrofluorometry.** We showed that the solubility of shikonin increases in aqueous media in the presence of $\beta$-Lg, but the data obtained by means of spectrophotometry provided limited information regarding the interaction between the pigment and the macromolecule. An attempt was made to obtain some additional thermodynamic information about the interaction by spectrofluorometry.

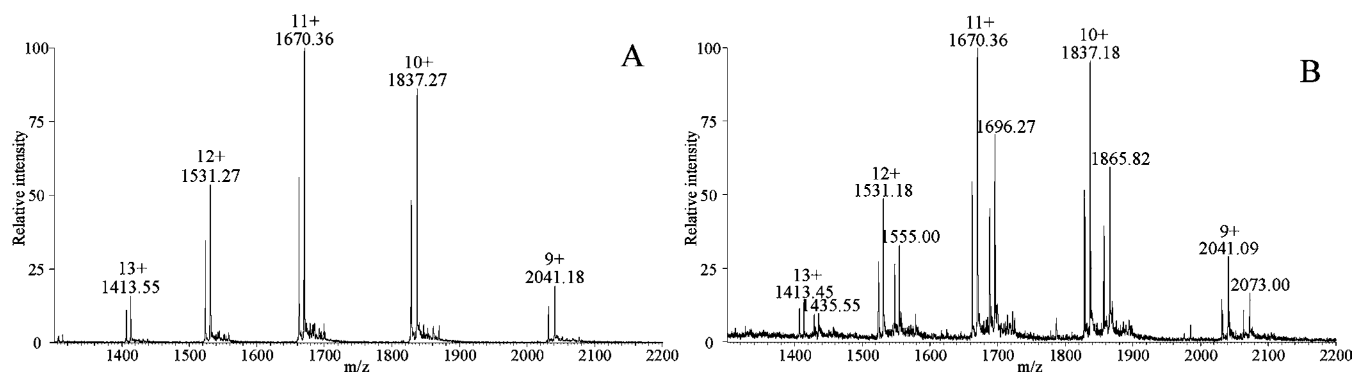


**Figure 5.** Mass spectra of $\beta$-Lg solution (A) and $\beta$-Lg/shikonin = 1:1 solution (B) after a 3 day incubation period at room temperature. $\beta$-Lg standard was a mixture of $\beta$-LgA and $\beta$-LgB, which reflects in double signals in the spectra.

By exciting $\beta$-Lg at $\lambda_{ex}$ = 295 nm the major contribution to the intrinsic protein fluorescence stems predominantly from $Trp^{19}$ and less from $Trp^{61}$, which is quenched by the neighboring disulfide bond.[30] Care should be taken in the interpretation of fluorescence results as erroneous conclusions can easily be drawn.[40,41] To ensure the reliability of the fluorescence data, some other parameters were examined. The photobleaching of $\beta$-Lg was negligible in a 3 × 3 mm cuvette after 15 min of constant irradiation at 295 nm. Ethanol had no observable effect on the fluorescence intensity at the concentrations used in this study. All raw data were also corrected for inner-filter effect at excitation and emission wavelengths.

After the initial evaluation of these critical parameters, the fluorescence quenching experiments with shikonin were conducted (Figure 4). It can be seen from the emission spectra that shikonin quenches the Trp fluorescence of $\beta$-Lg, although the inner-filter effect was not taken into consideration at that point. Shikonin did not fluoresce under any conditions. The drop in the fluorescence intensity of $\beta$-Lg indicated that an interaction between the protein and the pigment takes place. The position of the emission maximum wavelength did not change with the addition of the ligand, indicating that an energy transfer between a shikonin molecule and Trp in the excited state might occur. Static quenching could not be excluded on those grounds as more than one shikonin molecule probably binds to the protein (spectrophotometry data), and the drop in the fluorescence intensity of $\beta$-Lg could stem from a combination of static and dynamic quenching.

Due to the poor reproducibility of the fluorescence measurements, additional experiments were conducted in which the fluorescence of $\beta$-Lg was again quenched by shikonin, but the emission spectra were recorded after different incubation periods, after 30 min, 3.5 h, and 3 days (Supporting Information, Figure S3). The fluorescence of $\beta$-Lg was continuously dropping over time despite different $\beta$-Lg/shikonin molar ratios, but the fluorescence intensity of a solution at a certain $\beta$-Lg/shikonin ratio stabilizes after 24 h (data not shown). The dynamic equilibrium in noncovalent complexation processes is usually established sooner, and it seemed that the fluorescence drop of $\beta$-Lg was not caused exclusively by shikonin quenching. Different potential scenarios were possible: (i) EtOH can act as a ligand itself; thus, the alcohol might form a kinetic complex with the protein and EtOH is being only slowly driven out of the binding site by shikonin, which forms the thermodynamic product with the macromolecule; (ii) shikonin can precipitate upon its addition to aqueous media and is only slowly dissolved as $\beta$-Lg−SHI is formed; (iii) $\beta$-Lg can adsorb to the glass vial walls, which were being used, and the amount and the kinetics of the adsorbed protein can be a function of shikonin concentration; (iv) shikonin can induce Trp or $\beta$-Lg degradation; and (v) at least one shikonin molecule can covalently bind to $\beta$-Lg, causing the chemical reaction to proceed slowly.

The first two possibilities were quickly eliminated because the use of MeOH instead of EtOH to prepare shikonin working solution and filtering the solutions containing $\beta$-Lg and shikonin immediately after preparation did not produce different results; the fluorescence still dropped continuously over time. However, when plastic Eppendorf LoBind tubes were used instead of glass vials in sample preparation, the drop in the $\beta$-Lg fluorescence over time was significantly lower, indicating that $\beta$-Lg adsorption to the glass surface is more pronounced (Supporting Information, Figure S4). Accordingly, Eppendorf plastic tubes were used subsequently.

MS data revealed that shikonin did not cause Trp or $\beta$-Lg degradation over time (data not shown). Although no degradation products of $\beta$-Lg were observed, some additional signals appeared in the MS spectrum, which indicated the formation of $\beta$-Lg−SHI (Figure 5), although protein−ligand noncovalent complexes are uncommon in MS spectra, especially if they are based on a nonionic type of interaction. That was consistent with a covalent bond forming between $\beta$-Lg and at least one shikonin molecule, an event that could also contribute to the continuous drop in $\beta$-Lg fluorescence over time. The calculated relative difference in $m/z$ of signals corresponding to $\beta$-Lg and that of $\beta$-Lg−SHI was 287.4 Da, which corresponds closely to the monoisotopic mass of shikonin (288.1 Da). The signals for $\beta$-Lg−SHI were not observed at pH 6, at which the EF loop of $\beta$-Lg is closed. This finding reveals that one of the binding sites or reaction sites of shikonin might be located in the calyx of $\beta$-Lg, because the MS signals for $\beta$-Lg−SHI were obtained only at pH >7 when the EF loop is open. Most probably, the covalent and noncovalent bonds form at pH >7, but below this pH only noncovalent interaction between $\beta$-Lg and shikonin is possible.

To confirm that the fluorescence quenching of $\beta$-Lg was not a consequence of collisional quenching but was in fact an outcome of $\beta$-Lg−SHI formation, we decided to use the Stern−Volmer approach: quenching the fluorescence intensity of L-Trp with shikonin (Supporting Information, Figure S5). In contrast to the fluorescence experiments done with $\beta$-Lg, the continuous fluorescence drop over time was not observed here. This indicates that with $\beta$-Lg a covalent bond does not form between shikonin and Trp amino acid residue, but that another

amino acid residue, which is in the vicinity of Trp, was probably involved in bond formation. This claim was substantiated by ESI and APCI MS analyses as only signals for L-Trp and shikonin were observed and not those that would indicate a covalent product L-Trp−shikonin. Collisional quenching of fluorescence is described by the equation

$$\frac{F_0}{F} = 1 + k_Q \tau_0 [Q]$$

where $F_0$ and $F$ are the fluorescence intensities in the absence and presence of quencher, respectively, $k_Q$ is the bimolecular quenching constant, $\tau_0$ is the lifetime of the fluorophore in the absence of quencher, and $[Q]$ is the free concentration of quencher. Diffusion-controlled quenching typically results in values of $k_Q$ of $\sim 2 \times 10^{10}$ $M^{-1}$ $s^{-1}$, and larger apparent values of $k_Q$ usually indicate some type of binding interaction.[35,42] The relationship between $F_0/F$ and shikonin concentration is depicted in Supporting Information, Figure S6.

The bimolecular quenching constant was calculated from the slopes and amounted in both instances to $\sim 3 \times 10^{12}$ $M^{-1}$ $s^{-1}$, considering that the $\tau_0$ of L-Trp was approximately identical to that of Trp in β-Lg and equals 1.28 ns. It can be concluded that the L-Trp and β-Lg fluorescence drop did not arise from collisional quenching because $k_Q$ is too large by a factor of >100.

The spectrofluorometric data were complex due to various factors influencing the fluorescence of β-Lg, and additional thermodynamic information concerning the interaction between β-Lg and shikonin could not be established, but the majority of the obtained spectroscopic data implied a covalent bond formation.

**Characterization of the Interaction between β-Lg and Shikonin: Covalent Bonding.** Apart from spectrofluorometric and MS results, some additional observations made the formation of at least one covalent bond between the protein and pigment more probable. In noncovalent interactions we expected the MS signals for the product β-Lg−SHI to disappear if the product was exposed to high concentrations of urea (7 M), but the opposite was observed. The product β-Lg−SHI formed more rapidly when urea was added to the mixture of β-Lg and shikonin. This suggested that the protein unfolding did not cause a perturbation of the shikonin binding site, but that the reactive site of β-Lg was made even more accessible to shikonin (data not shown). After the solution had been washed with *n*-hexane, the organic fraction retained a reddish color and the aqueous fraction still gave strong MS signals for β-Lg−SHI; therefore, a portion of shikonin molecules most probably bind to the protein via a noncovalent interaction and dissociate in the presence of urea.

The naphthoquinone moiety is electron deficient, and if a covalent bond is formed with β-Lg, a nucleophilic attack from the protein side can be expected. The signals for β-Lg−SHI in the MS spectrum suggest that only one molecule of shikonin binds covalently to β-Lg (Figure 5), so an individual reactive site within the protein was contemplated. When the amino acid sequence of β-Lg was examined, it was found that not one nucleophilic amino acid appears only once; the exception was $Cys^{121}$, which is the only Cys amino acid residue with a free thiol functional group; the remaining four form disulfide bridges. Cys is known to be a strong nucleophile within various proteins, although its nucleophilicity depends on the pH and on the immediate area surrounding the amino acid residue.[43] To confirm the covalent bond formation between β-Lg and shikonin, a tryptic digest of β-LgA and β-LgA−SHI was performed, and the two resulting peptide maps, which were obtained by means of HPLC-MS analysis, were compared.

First, β-LgA was digested with trypsin under nonreducing conditions and an HPLC-MS method was developed to acquire a complete β-LgA peptide map (Figure 6A). β-Lg (β-LgA + β-LgB) was not used in these analyses because additional peptide peaks in the chromatograms would make their interpretation more difficult. Individual peaks (peptides) in the chromatogram were identified on the basis of their MS spectra (Table 2). Second, β-LgA/shikonin solution in a molar ratio 1:15 was prepared and incubated for 24 h. This solution was subsequently subjected to tryptic digest under nonreducing conditions, and after HPLC-MS analysis a tryptic peptide map of β-LgA-SHI was obtained (Figure 6B). This peptide map was different from the peptide map of β-LgA.

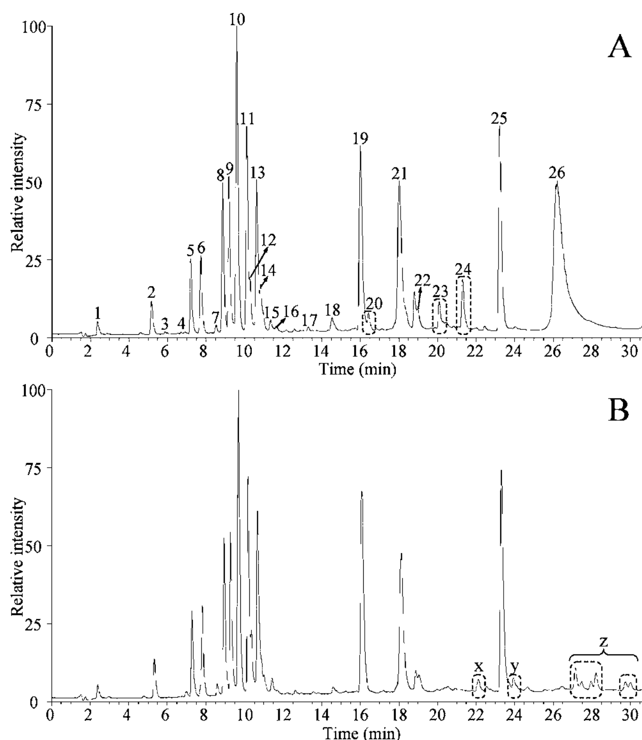


Figure 6. Tryptic peptide maps of β-LgA (A) and β-LgA−SHI (B). Peak assignation is presented in Table 2, and the peptide map differences are emphasized by frames.

With the addition of shikonin to β-LgA solution peaks 20, 23, and 24 disappeared from the chromatogram, and at the same time peaks *x* and *y* and peak group *z* were observed, which were not present in the peptide map of β-LgA. All other peaks are common to both peptide maps. Peak 20 was identified as $Leu^{149}$−$Ile^{162}$, and peaks 23 and 24 as $Tyr^{102}$−$Arg^{124}$. Peptide $Tyr^{102}$−$Arg^{124}$ contains three Cys amino acid residues, and peaks 23 and 24 are most probably a result of disulfide bond formation between different Cys residues ($Cys^{106}$ and $Cys^{121}$ in peptide 23 and $Cys^{106}$ and $Cys^{119}$ in peptide 24), which was confirmed by fragmentation analyses of those peaks (MS/MS data not shown). It is known that intramolecular disulfide cross-linking reactions can occur within the protein, and the result is the formation of free thiol functional groups, which are not normally present in the native protein.[44] In the peptide map of β-LgA−SHI peak *x* (most intense ion at *m*/*z* 909.0; 3+

**Table 2. Characterization of Peaks in the Chromatogram of β-LgA after Tryptic Digest**

| peak | $t_r$ (min) | peptide | peptide mass (Da) |
|---|---|---|---|
| 1 | 2.4 | Ala[139]–Lys[141] | 330.4 |
| 2 | 5.2 | Ile[71]–Lys[75] | 572.7 |
| 3 | 5.9 | Trp[61]–Lys[70] | 1250.4 |
| 4 | 6.7 | Trp[61]–Lys[69] | 1122.2 |
| 5 | 7.2 | Ile[84]–Lys[91] | 916.0 |
| 6 | 7.7 | Gly[9]–Lys[14] | 672.8 |
| 7 | 8.5 | Thr[125]–Lys[135] | 1245.3 |
| 8 | 8.9 | Ala[142]–Arg[148] | 837.1 |
| 9 | 9.2 | Leu[1]–Lys[8] | 933.2 |
| 10 | 9.6 | Thr[76]–Lys[83] and Val[92]–Lys[101] | 903.1 and 1193.4 |
| 11 | 10.1 | Thr[125]–Lys[138] | 1635.8 |
| 12 | 10.3 | Ile[78]–Lys[83] | 673.9 |
| 13 | 10.6 | (Trp[61]–Lys[70])S–S(Leu[149]–Ile[162]) | 2907.2 |
| 14 | 10.8 | Ile[84]–Lys[101] | 2091.4 |
| 15 | 11.4 | (Trp[61]–Lys[69])S–S(Leu[149]–Ile[162]) | 2979.0 |
| 16 | 11.6 | Thr[76]–Lys[91] | 1801.1 |
| 17 | 13.3 | Thr[76]–Lys[101] | 2976.6 |
| 18 | 14.5 | (Trp[61]–Lys[69])S–S(Tyr[102]–Arg[124]) | 3780.3 |
| 19 | 16.0 | Val[41]–Lys[60] | 2313.7 |
| 20 | 16.4 | Leu[149]–Ile[162] | 1658.8 |
| 21 | 18.0 | (Val[41]–Lys[69])S–S(Leu[149]–Ile[162]) | 5074.7 |
| 22 | 19.0 | Val[41]–Lys[69] | 3417.9 |
| 23 | 20.1 | Tyr[102]–Arg[124] | 2676.1 |
| 24 | 21.3 | Tyr[102]–Arg[124] | 2676.1 |
| 25 | 23.2 | Val[15]–Arg[40] | 2708.1 |
| 26 | 26.2 | β-LgA | 18363 |

charge) was tentatively assigned as Val[15]–Arg[40], with an oxidized Met; shikonin molecules could readily play the role of oxidizing agent. The mass of peak *y* (most intense ion at $m/z$ 1035.0; 4+ charge) corresponds to the peptide Val[92]–Arg[124], which has a shikonin molecule attached to it. Peaks *z* (most intense ion at $m/z$ 987.8; 3+ charge) had identical MS and MS/MS spectra, and they all correspond to the peptide Tyr[102]–Arg[124], which also has a single shikonin molecule attached to it. The reason for the appearance of six *z* peaks was not established. The only peaks representing peptides with a free thiol group were not present in the peptide map of β-LgA–SHI, and peaks that did not appear in the peptide map of β-LgA were mainly attributed to the peptide Tyr[102]–Arg[124]–shikonin. It can be concluded that a covalent bond forms between shikonin and the free thiol group Cys[121], which is found in the hydrophobic calyx of native β-Lg. The reactivity of β-Lg is not novel, and the reaction between β-Lg and a specific electrophile does not always proceed via Cys attack;[45] other amino acid residues such as lysine, hydroxylysine, or arginine can also take part in the covalent bond formation. Therefore, to further confirm our previous observation, Cys[121] within the native protein was treated with iodoacetamide, and shikonin was added to the protected protein solution only subsequently; in this instance, the covalent bond between the macromolecule and the pigment failed to form.

The reaction mechanism most probably proceeds through a nucleophilic addition in which the thiol lone electron pair of Cys[121] attacks the electrophilic C2 site of the naphthazarin ring of shikonin and, consequently, a thioether bond is formed (Figure 7). The addition is likely to proceed via 1,4-reductive Michael addition.[46] The resulting naphthoquinol is reoxidized to naphthoquinone by the $O_2$ in solution or by the unreacted

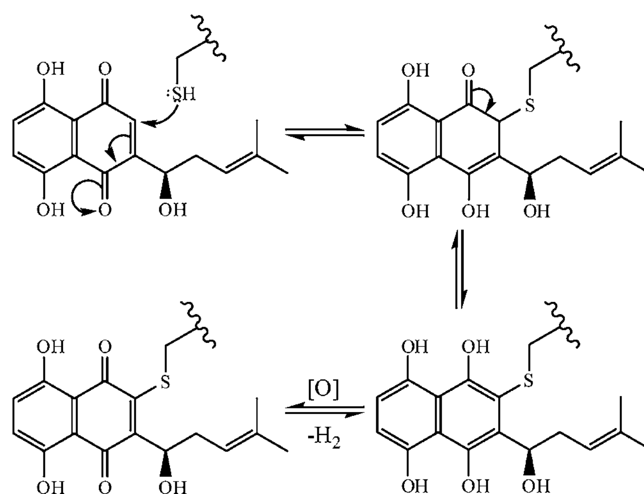


**Figure 7.** Proposed reaction mechanism between shikonin and Cys[121] within β-Lg.

shikonin molecules. This is also confirmed by the MS spectra ($\Delta m/z$ (Tyr[102]–Arg[124]; Tyr[102]–Arg[124]–shikonin) ~ 288 Da) and by the fact that shikonin is fluorescent in the reduced form,[47] but shikonin failed to fluoresce under any circumstance even after its addition to the β-Lg.

In conclusion, the interaction between shikonin and β-Lg was studied by spectrophotometry, spectrofluorometry, HPLC, and MS. We showed that the solubility of shikonin in aqueous media is increased up to 181-fold in the presence of β-Lg at a concentration of 3.1 mg/mL. One shikonin molecule binds to the protein covalently via Cys[121] in a 1,4-reductive addition of a Michael type, which is followed by a reoxidation of the resulting naphthoquinol. This was confirmed by comparing β-Lg and β-Lg–SHI tryptic maps obtained by HPLC-MS. Other shikonin molecules in all likelihood bind to the outer surface of the protein through weaker noncovalent interactions. The complexity of the analytical results rendered the determination of detailed thermodynamic parameters difficult. In combination with the whey protein β-Lg, the enhanced water solubility of shikonin could potentially increase the usage of this pigment, which is beneficial to human health. Further research is needed to assess the stability, toxicity, and bioavailability of the product.

## ■ ASSOCIATED CONTENT

### Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

*β*-Lg, *β*-lactoglobulin; *β*-LgA, *β*-lactoglobulin A; *β*-LgB, *β*-lactoglobulin B; SHI, shikonin; *β*-Lg(A)−SHI, product of the interaction between *β*-lactoglobulin(A) and shikonin; HPLC, high-performance liquid chromatography; MS, mass spectrometry; PVDF, poly(vinyldiene difluoride); AGC, automatic gain control; APCI, atmospheric pressure chemical ionization; HESI, heated electrospray ionization.

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