Riboflavin-Sensitized Photochemical Changes in β-Lactoglobulin in an Aqueous Buffer Solution as Affected by Ascorbic Acid

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The effects of ascorbic acid on the riboflavin-sensitized photochemical changes in β-lactoglobulin in an aqueous buffer solution as determined by high performance gel permeation liquid chromatography (HPGPLC), insoluble protein content, and individual amino acid content during fluorescent light illumination were studied. The riboflavin-sensitized photochemical degradation of β-lactoglobulin was effectively inhibited by ascorbic acid, and its inhibitory effectiveness was concentration dependent. The 0.1% ascorbic acid treatment showed 74.4% inhibition of β-lactoglobulin degradation as determined by a HPGPLC during 6 h light illumination. Insolubility of β-lactoglobulin in a buffer solution during light illumination was also effectively decreased by ascorbic acid treatment. The riboflavin-sensitized photochemical reduction of cysteine, histidine, lysine, methionine, and tryptophan in β-lactoglobulin was high during 6 h fluorescent light illumination. The 0.1% ascorbic acid treatment exhibited 20.8% inhibition of total amino acid degradation in β-lactoglobulin during 6 h light illumination, showing strong inhibitory activity against the degradation of arginine, aspartic acid, cystein, glycine, histidine, phenylalanine, proline, serine, and tryptophan.

Keywords: *β-lactoglobulin; riboflavin; ascorbic acid; insoluble protein; amino acids*

INTRODUCTION

The light-induced changes in milk proteins have been widely studied previously. Zittle et al. (1964) reported that β-casein was readily photooxidized at pH 8.6 with the reduction or disappearance of histidine, tryptophan, and tyrosine. Aurand et al. (1966) reported that tryptophan content in casein in the presence of riboflavin and in whole milk greatly decreased during fluorescent light illumination. Studies by Dimick (1976) on the major serum proteins of homogenized milk revealed significant changes in amino acid composition in a high molecular weight protein fraction as a result of exposure to fluorescent light. It has been reported that photooxidation of casein micelles from cow or buffalo milk reduced the release of glycomarcropeptide and turbidity development in micelles by rennet (Gupta and Ganguli, 1978). Riboflavin reportedly increases photodecomposition of α-lactoglobulin and β-lactoglobulin. Gilmore and Dimick (1979) reported that the insoluble protein contents in β-lactoglobulin increased as the fluorescent light illumination time increased and that there was direct chemical evidence for peptide bond hydrolysis in sunlight and fluorescent light exposed α-lactoalbumin and β-lactoalbumin in the presence of riboflavin. Riboflavin has been suggested to generate singlet oxygen in milk during exposure to light because a singlet oxygen trapper, 1,3-diphenylisobenzofuran, and a singlet oxygen quencher, 1,4-diazobicyclo-[2,2,2]octane (DABCO), decreased the oxidation of milk as measured by TBA values (Aurand et al., 1977). The singlet oxygen formation in milk during light exposure was also confirmed by employing ESR technique (Bradley and Min, 1992). Amino acids readily react with singlet oxygen. Methionine, histidine, tryptophan, tyrosine, and cysteine are especially reactive with a singlet oxygen, whether as free amino acid or in peptide (Korycka-Dahl and Richardson, 1978). The conformation of a protein may make some residues less susceptible to photooxidation than others. Ascorbic acid reportedly has strong quenching ability for active oxygen species (singlet oxygen and superoxide anion radical) and effectively prevents the light-activated off-flavor formation, the color changes, and the riboflavin and retinyl palmitate reductions in milks (Jung et al., 1998a,b; Lee et al., 1998). Based on these facts, we hypothesized that ascorbic acid could also reduce the riboflavin-sensitized photochemical changes of milk proteins in aqueous solution. However, the qualitative and quantitative effects of ascorbic acid on the riboflavin-sensitized photochemical changes of milk proteins have never been reported previously.

Thus, the objective of this research was to study the effects of ascorbic acid, an effective active oxygen quencher, on the riboflavin-sensitized photochemical changes in β-lactoglobulin in a aqueous buffer solution (pH 7.0) as determined by HPGPLC, insoluble protein content, and individual amino acid contents during fluorescent light illumination.

MATERIALS AND METHODS

Materials. Ascorbic acid, riboflavin, and β-lactoglobulin were purchased from Sigma Chemical Co. (St. Louis, Mo). Transparent glass serum bottles (35 mL capacity), aluminum caps, and rubber septa were purchased from Supelco Inc. (Bellefonte, PA).

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Sample Preparation. To study the effect of ascorbic acid on the riboflavin-sensitized photochemical changes in β-lactoglobulin, sample solutions containing 300 mg of β-lactoglobulin in 1000 mL of buffer solution (0.05 M KH_2PO_4 + 0.15 M Na_2SO_4, pH 7.0) with added 1.5 mg of riboflavin and 0, 0.25, 0.5, and 1.0 g of ascorbic acid were prepared. The concentration (300 mg/L) of β-lactoglobulin was selected to eliminate the further dilution step for the following analysis of HPGPLC, insoluble protein, and individual amino acid. The 1.5 mg of riboflavin content per liter of solution was selected in this research based on the riboflavin content in commercial milks.

Light Storage. Ten milliliters of the prepared samples were, in triplicate, transferred to 35 mL capacity serum bottles and the sample bottles were sealed with rubber septa and aluminum caps. Then the samples were randomly placed in the storage box as described in detail by Fakourelis et al. (1987) and Jung et al. (1991, 1995). The sample bottles were rearranged randomly in the light storage box every 30 min during light illumination. The light intensity at the sample level was 3300 lux. The light storage box was placed in a 4 °C cooler and the temperature inside the storage box was 7 ± 2 °C during illumination.

High-Performance Gel Permeation Liquid Chromatography. β-Lactoglobulin content in the sample solution was determined by a high performance gel permeation chromatography (Spectra-Physics, Fremont, CA). The column used was a Macrosphere GPC 60 A (7 μm, 300 × 7.5 mm, Alltech Associates Inc., Deerfield, IL). The flow rate of eluting solution (mobile phase, 0.05 M KH_2PO_4 + 0.15 M Na_2SO_4, pH 7.0) was 0.5 mL/min. The quantification was made at 280 nm by a UV 1000 Spectra system detector (Spectra-Physics, Fremont, CA).

Insoluble Protein Content of β-Lactoglobulin. To determine the insolubility of the β-lactoglobulin, 1 mL aliquots of the samples were centrifuged at 2000 rpm for 10 min (Gilmore and Dimick, 1979). The supernant was decanted, and the insoluble sediment portion was washed twice with acetone prior to analysis for protein content. The protein content was measured by the Lowry's method (Lowry et al., 1951).

Amino Acid Analysis. The analysis of individual amino acids in β-lactoglobulin before and after light illumination was done by Pico-Tag method (Waters) after hydrolysis in boiling HCl at 110 °C for 24 h. To determine the cysteine content, cysteine residues were oxidized to cysteic acid with a mixture of formic acid and hydrogen peroxide (19:1, v/v) (Tarr, 1986). To determine the tryptophan content, samples were directly digested with 20 μL of 4 M methanesulfonic acid according to the digestion conditions of Lui and Chan (1971).

Derivatization of the hydrolyzed samples were done by an initial drying step followed by redrying and coupling steps. The initial step removes solvents and volatile components such as HCl. The redrying step neutralized any residual acid that may cling to the glass tube, and the coupling step makes the phenylthiocarbamyl (PITC) derivatives that were actually analyzed. The PITC-derivatized free amino acids were applied to a 30 cm Pico-Tag free amino acid column (3.9 × 300 mm, 4 μM) equilibrated with buffer A equipped with a Waters HPLC system (510 HPLC pump, 717 automatic sampler, 996 photodiode array detector, and Millennium 2010 chromatography manager) and eluted with a linear gradient composed by buffer B (0, 31, 33, 52, and 100%) at a flow rate of 1 mL/min at 40 °C. The quantification of the individual amino acid was done at 254 nm. Buffer A 140 mM sodium acetate (6% acetonitrile) and buffer B was 140 mM sodium acetate (60% acetonitrile).

Statistical Analysis. All the analysis was done in triplicate. Statistical analysis was done by using the Statistical Analysis System (SAS, 1988). Duncan's multiple range test was used to ascertain the treatment effects.

RESULTS AND DISCUSSION

***β*-Lactoglobulin Degradation as Determined by a HPGPLC.** The HPGPLC chromatograms for the β-lactogrobulin before and after fluorescent light exposure are shown in Figure 1. The peaks 1, 2, 3, and 4 represent β-lactoglobulin, ascorbic acid, breakdown product of β-lactoglobulin, and riboflavin, respectively. Peaks 1, 2, and 4 were identified by injecting each of β-lactoglobulin, ascorbic acid, and riboflavin in solution into HPGPLC. We also carried out a light illumination experiment with a blank (β-lactoglobulin-free sample) to identify peak 3. Peak 3 was not produced in the blank sample during light illumination. The result indicated the peak 3 was not generated from either ascorbic acid nor riboflavin but was derived from β-lactoglobulin. That is, riboflavin-sensitized photochemical reaction induced the formation of low molecular breakdown products of β-lactoglobulin. The breakdown products of β-lactoglobulin increased as the illumination time increased. A similar result was previously reported in the literature (Gilmore and Dimick, 1979). Gilmore and Dimick (1979) reported that α-lactoalbumin and β-lactoalbumin were hydrolyzed to produce low molecular compounds in the presence of riboflavin under sunlight or fluorescent light.

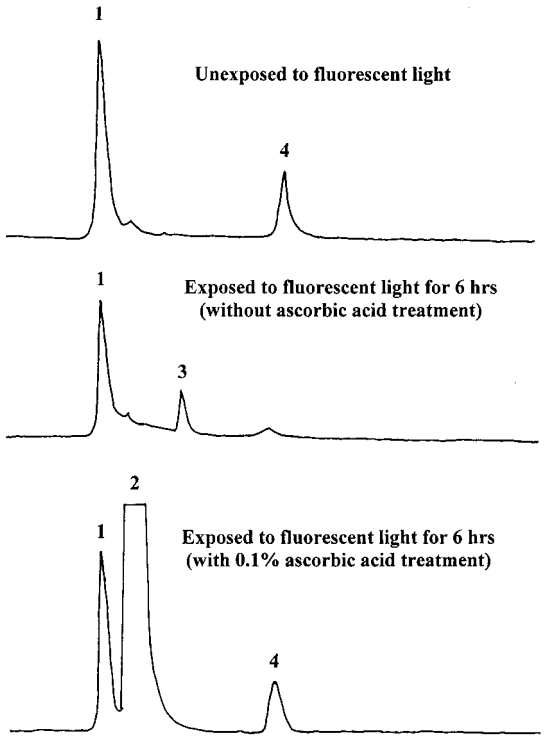

Figure 1. High performance gel permeation liquid chromatograms of β-lactoglobulin in an aqueous buffer solution (pH 7.0) containing riboflavin (1.5 μg/mL) before and after fluorescent light illumination. Peaks 1, 2, 3, and 4 represent β-lactoglobulin, ascorbic acid, breakdown product of β-lactoglobulin, and riboflavin, respectively.

The effects of ascorbic acid on the degradation of β-lactoglobulin in a buffer solution (pH 7.0) containing riboflavin during fluorescent light are shown in Figure 2. The β-lactoglobulin content in the solution containing riboflavin greatly decreased during fluorescent light illumination, resulting in 23.7% reduction after 6 h light illumination. However, in the sample solution wrapped with aluminum foil, there was no decrease in β-lactoglobulin in the solution containing riboflavin even after 6 h storage under the same conditions.

The β-lactoglobulin degradation in the buffer solution containing riboflavin was greatly decreased by the addition ascorbic acid and the protective property of ascorbic acid was concentration dependent. The contents of β-lactoglobulin in the solution treated with 0, 0.025,

Table 1. Effects of Ascorbic Acid on the Insoluble Protein Content in β-Lactoglobulin during Fluorescent Light Illumination

	insoluble protein content, %					
ascorbic acid, %	0 h	1 h	2 h	3 h	4 h	6 h
0	0	2.0 ± 0.32[a]	2.3 ± 0.18[a]	2.9 ± 0.44[a]	4.6 ± 0.18[a]	8.1 ± 0.06[a]
0.025	0	1.8 ± 0.24[a]	2.2 ± 0.25[a]	2.6 ± 0.31[a]	3.6 ± 0.20[b]	5.7 ± 0.29[b]
0.05	0	1.7 ± 0.28[a]	1.8 ± 0.13[b]	2.0 ± 0.27[b]	3.1 ± 0.26[c]	4.6 ± 0.34[c]
0.1	0	1.8 ± 0.25[a]	1.6 ± 0.33[b]	2.1 ± 0.22[b]	2.8 ± 0.31[c]	4.1 ± 0.10[d]
light protected[e]	0	0	0	0	0	0

[a–d] Mean values within each column with different italicized superscript letters were significantly different ($p < 0.05$). [e] Light protected: sample (containing no added ascorbic acid) protected from light by wrapping with aluminum foil.

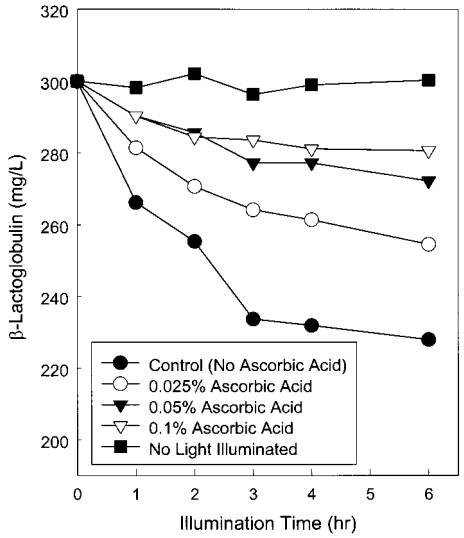

Figure 2. Effects of ascorbic acid on the riboflavin-sensitized photochemical degradation of β-lactoglobulin in a buffer solution (pH 7.0) during fluorescent light illumination as determined by HPGPLC.

0.05, and 0.1% ascorbic acid were 227.9, 254.6, 272.2, and 280.6 mg/L after 6 h fluorescent light illumination, respectively. That is, 0.025, 0.05, and 0.1% ascorbic acid treatments showed 37.7, 62.5, and 74.4% inhibition of β-lactoglobulin degradation in the buffer solution containing riboflavin after 6 h fluorescent light illumination. Duncan's multiple range test showed that the 0.025% ascorbic acid treatment was significantly effective in the inhibition of β-lactoglobulin degradation after 6 h light illumination ($p < 0.05$). There were also significant differences in the inhibition of β-lactoglobulin degradation after 6 h light illumination, among each of the 0.025, 0.05, and 0.1% ascorbic acid treatments ($p < 0.05$). Addition of ascorbic acid also greatly decreased the formation of low molecular breakdown product of β-lactoglobulin. The sample treated with 0.1% ascorbic acid produced only trace amounts of the low molecular compound after 6 h light illumination (as shown in Figure 1).

Solubility. The solubility changes of β-lactoglobulin in a buffer solution (pH 7.0) containing riboflavin during light illumination are shown in Table 1. As the light illumination increased, the insoluble protein content of β-lactoglobulin increased from 0% before illumination to 8.1% after 6 h illumination. The sample solution protected from light with aluminum foil did not increase the insoluble protein content of the β-lactoglobulin after 6 h storage under same condition. This result indicated that light illumination induced the protein denaturation of β-lactoglobulin in a buffer solution (pH 7.0) containing riboflavin, resulting in the significant increase in insolubility of the β-lactoglobulin. Gilmore and Dimick (1979) also reported previously that the insoluble protein contents in β-lactoglobulin increased in the presence of riboflavin as the fluorescent light illumination time increased.

Table 2. Effects of Ascorbic Acid on the Contents of Individual Amino Acids of β-Lactoglobulin in an Aqueous Buffer Solution during Fluorescent Light Illumination

	amino acid content, pM		
amino acid	0 h	6 h (0% AA[d])	6 h (0.1% AA)
Ala	2261 ± 94.9[a]	1862 ± 74[b]	1958 ± 82.4[b]
Arg	488 ± 16.3[a]	384 ± 24[c]	440 ± 17.9[b]
Asp	264 ± 13.0[a]	171 ± 15[c]	227 ± 29.6[b]
Cys	307 ± 10.5[a]	175 ± 7.1[c]	215 ± 20.5[b]
Glu	4024 ± 58.6[a]	3407 ± 54.9[b]	3394 ± 34.2[b]
Gly	600 ± 17.5[a]	446 ± 10.2[c]	558 ± 22.8[b]
His	328 ± 21.1[a]	203 ± 12.9[c]	297 ± 10.8[b]
Ile	1451 ± 40.3[a]	1310 ± 38.9[b]	1313 ± 39.0[b]
Leu	3478 ± 83.5[a]	3072 ± 128.6[b]	3155 ± 145.8[b]
Lys	2135 ± 35.8[a]	1402 ± 89.2[b]	1364 ± 96.6[b]
Met	597 ± 22.3[a]	441 ± 15.8[b]	440 ± 20.0[b]
Phe	652 ± 14.0[a]	571 ± 12.6[c]	611 ± 16.7[b]
Pro	1496 ± 44.4[a]	1398 ± 42.5[b]	1492 ± 39.2[a]
Ser	886 ± 16.4[a]	690 ± 16.1[c]	766 ± 10.4[b]
Thr	1343 ± 39.3[a]	1172 ± 41.9[b]	1211 ± 45.4[b]
Trp	329 ± 14.0[a]	152 ± 8.4[c]	241 ± 17.4[b]
Tyr	401 ± 13.9[a]	314 ± 11.2[c]	334 ± 3.7[b]
Val	1514 ± 44.6[a]	1381 ± 34.4[b]	1373 ± 48.5[b]
total	22554	18550	19389

[a–c] Mean values within each row with different italicized superscript letters were significantly different ($p < 0.05$). [d] AA: ascorbic acid.

The ascorbic acid treatment greatly inhibited the increase of insolubility of β-lactoglobulin in a buffer solution containing riboflavin during light illumination. After 6 h of light illumination, the insoluble protein contents of the β-lactoglobulin treated with 0, 0.025, 0.05, and 0.1% ascorbic acid were 8.1, 5.7, 4.6, and 4.1%, respectively (Table 1). That is, the 0.025, 0.05, and 0.1% ascorbic acid treatment inhibited 29.6, 43.3, and 49.4% of insolubility development in β-lactoglobulin solution containing riboflavin after 6 h light illumination. Duncan's multiple range test showed that 0.025% ascorbic acid treatment was significantly effective in the reduction of insolubility of β-lactoglobulin after 6 h of illumination ($p < 0.05$). There were also significant differences among each of the 0.025, 0.05, and 0.1% ascorbic acid treatments ($p < 0.05$).

Amino Acid Contents. Amino acid contents in the β-lactoglobulin solution (300 mg/L) are shown in Table 2. Glutamic acid content (4024 pM) was highest in the β-lactoglobulin, followed by leucine (3478 pM), alanine (2261 pM), lysine (2135 pM), valine (1514 pM), proline (1496 pM), isoleucine (1451 pM), and threonine (1343 pM) in decreasing order. The total amino acid content in the β-lactoglobulin solution decreased from 22554 to 18550 pM after 6 h fluorescent light illumination, resulting in 17.8% reduction of total amino acids. All of

the amino acid decreased after 6 h light illumination. However, the degree of loss of amino acids during light illumination was greatly dependent on the types of individual amino acid. Proline and valine did not greatly decreased during 6 h light storage, resulting only 6.6% and 8.8% reduction, respectively. However, the light-induced reductions of cysteine, histidine, lysine, methinone, and tryptophan were especially high after 6 h fluorescent light illumination. Among these amino acids, the light-induced loss of tryptophan was highest, resulting in 53.8% loss. This result was similar to the previous report (Finley and Shipe, 1971). Finley and Shipe (1971) reported that the protein portion of milk exposed to light resulted in losses of tryptophan, tryosine, lysine, cystein, and methionine. The reduction of these amino acids may be explained by photosensitized production of singlet oxygen, since these amino acids are reportedly very reactive with a singlet oxygen, whether as the free amino acid or in a peptide (Korycka-Dahl and Richardson, 1978).

The addition of ascorbic acid greatly decreased the amino acid degradation in the solution containing riboflavin. The 0.1% ascorbic acid showed 20.82% inhibition of total amino acid degradation. The protective activity with 0.1% ascorbic acid on the individual amino acid degradation was greatly dependent on the kind of individual amino acid. For example, 0.1% ascorbic acid did not show significant inhibitory activity against the degradation of lysine, isolecine, glutamic acid, valine, and methionine in the β-lactoglobulin. However, 0.1% ascorbic acid treatment was significantly effective in preventing the degradation of cysteine, histidine, serine, histidine, glycine, aspartic acid, arginine, proline, phenylalanine, and tryptophan. The protective activity of ascorbic acid on the riboflavin-sensitized photochemical changes of milk protein has never been previously reported in the literature. Since milks are sold mostly in plastic packages and displayed under light illuminated conditions, photochemical degradation of nutrients in milks is inevitable during storage, distribution, and marketing. This present result suggested that the milk proteins in milks might be possibly protected from the light-induced changes by addition of ascorbic acid. To confirm this idea, however, additional experiments in real milk system should be followed.

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