**Intramolecular cross-linking in the native JHBP molecule**

Dominika Bystranowska1, Zbigniew Szewczuk2, Marek Lisowski2, Ewa Sitkiewicz3, Andrzej Ożyhar1, Marian Kochman1,\*

1Department of Biochemistry, Faculty of Chemistry, Wrocław University of Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland.

2Faculty of Chemistry, University of Wrocław, 50-383 Wrocław, Poland.

3Institute of Biochemistry and Biophysics, Department of Biophysics, Polish Academy of Sciences, Warszawa, Poland.

\*To whom correspondence should be addressed. Telephone: +48(71)3206332. Fax: +48(71)3206337. E-mail: marian.kochman@pwr.wroc.pl.

Short Running Title: Intramolecular JHBP cross-linking

**Abstract**

Juvenile hormone binding protein (JHBP) acts as a shuttle, carrying one of the most crucial hormones for insect development to target tissues. We have found that although the JHBP molecule does not contain tryptophan residues, it exhibits a weak fluorescence maximum near 420 nm upon excitation at 315 nm. Gel filtration experiments performed in denaturing conditions and ESI-MS analyses excluded the possibility that some low molecular ligand was bound to the protein molecules. Further UV and CD spectroscopy studies, as well as immunoblotting, showed that the unusual JHBP optical properties were due to dityrosine intramolecular cross-linking. These bridges were detected both in native and recombinant protein molecules. We believe that in Galleria mellonella hemolymph the DT generation occurs via ROS-mediated oxidation leading to the formation of cross-linked JHBP monomers. MS analyses of peptides generated after JHBP proteolysis indicated, that the dityrosine bridge occurs between the Y128 and Y130 residues.

Keywords: dityrosine, *Galleria mellonella*, JHBP, protein oxidation

The abbreviations used are: CD, circular dichroism; DT, dityrosine; ESI-MS, electrospray ionization mass spectrometry; JH, juvenile hormone; JHBP, juvenile hormone binding protein; MS/MS, tandem mass spectrometry; ROS, reactive oxygen species.

**1. Introduction**

Juvenile hormone (JH) is a key hormone that regulates embryogenesis, controls larval development and stimulates adult insect reproduction (reviewed in [[1](#_ENREF_1)]). During larval stages, the presence of JH maintains larval characteristics, whereas its disappearance when ecdysone is present stimulates the initiation of metamorphosis. Because of the lipophilic nature of juvenile hormone, JH displays a propensity for surface binding that makes its distribution and concentration difficult to control. For this reason, in insect hemolymphs, JH is always bound to a highly specific JH binding protein (JHBP) [[2](#_ENREF_2)]. The role of this protein is to transport hormone molecules from distant biosynthesis sites located in the *corpora allata* to the peripheral target cells, facilitating the passage of JH molecules into the circulatory system, reducing non-specific binding [[3](#_ENREF_3)], and protecting the hormone from enzymatic degradation [[4](#_ENREF_4)].

This low molecular weight carrier protein has been identified and characterized in different lepidopteran species [[5-9](#_ENREF_5)]. *Galleria mellonella* hemolymph JHBP consists of a single polypeptide chain with a molecular mass of about 30 kDa and a JH-JHBP dissociation constant of [[10](#_ENREF_10)]. It has been reported previously that this protein contains 245 amino acid residues, of which the first 20 are cleaved post-translationally [[11](#_ENREF_11)], followed by N-glycosylation of the N94 residue [[12-14](#_ENREF_12)]. An analysis of the three-dimensional structure of *G. mellonella* JHBP revealed that this molecule has an elongated shape with two hydrophobic cavities that are similar in size (named W and E), located at opposite poles with a flexible N-terminal arm situated at the opening to the W cavity [[15](#_ENREF_15)]. It has also been reported previously, that the JHBP molecule contains a single JH binding site per protein molecule [[10](#_ENREF_10)]. It has been further demonstrated that JH binding induces a profound conformational transition in the JHBP molecule [[16](#_ENREF_16), [17](#_ENREF_17)]. Such a pronounced structural alteration might have physiological importance for the transmission of hormone signals, as has been suggested in [[16](#_ENREF_16)]. There are no Met and Trp residues in the *G. mellonella* JHBP sequence [[11](#_ENREF_11)]. From out of the ten tyrosine residues present in the JHBP molecule seven are located near the W cavity, as shown by the high-resolution crystal structure, and two of them are located inside [[15](#_ENREF_15)]. Due to the absence of W residues in the JHBP molecule, the Y and F residues are the main contributors of the ultraviolet fluorescence of JHBP.

We have investigated the spectroscopic properties of JHBP samples and found that they differ significantly from those reported for a number of simple proteins which do not contain W residues. The aim of our work was to gain deeper insight into the distinct JHBP optical features encompassing the appearance of a minor UV absorption peak in the range between 310 nm and 340 nm, as well as additional fluorescence with a maximum at 420 nm. Here, we present data that show two tyrosyl side chains that became oxidized in the JHBP molecule and contribute to the formation of Y-Y (dityrosine) bridges. Dityrosine (DT) bond formation either occurs spontaneously or it can by induced by oxidative stress. Two critical Y residues that participate in the DT cross-linking formation were defined.

**2. Materials and Methods**

*2.1. Materials*

Juvenile hormone III (10*R*,*S*-JH III) was purchased from Sigma (USA). Endoproteinase Glu-C (sequencing grade) was obtained from CalBiochem (USA). Dityrosine was synthesized at BioCentrum Ltd. (Kraków, Poland) from l-tyrosine as described by Lee et al. [2008], using Mn(III) as an oxidizing agent. Dityrosine-conjugated BSA was prepared as described by Kato et al. [[18](#_ENREF_18)]. Riboflavin was from Sigma (USA). All other chemicals were of analytical grade and commercially available.

*2.2. JHBP purification*

JHBP was isolated from the hemolymph of the 7th instar *G. mellonella* larvae according to a procedure described previously [[19](#_ENREF_19)]. The protein solution was dialyzed against a 0.1 M sodium phosphate buffer, pH 7.2 and concentrated to about 1.0 mg⋅ml-1 with an Amicon Ultra-10 centrifugal filter device (Millipore, USA). To estimate the concentration of hJHBP, the Bradford protein assay [[20](#_ENREF_20)] was applied and bovine serum albumin was applied as a standard. After purification protein samples were analyzed by 10% SDS-PAGE [[21](#_ENREF_21)] and stained with Coomassie Brilliant Blue [[22](#_ENREF_22)]. The JH-binding activity was determined with a charcoal assay [[23](#_ENREF_23)] in the presence of a 0.1% gelatin [[10](#_ENREF_10)].

*2.3. Photooxidation of JHBP samples*

Photooxidation of the protein samples was performed by irradiating 19-35 µM JHBP sample solutions (protein in a 0.1 M sodium phosphate buffer, pH 7.2) with 30 μM of riboflavin as the sensitizer in the sample cell of a Fluorolog-3 fluorometer (Spex, Jobin Yvon Inc., France). Solutions were irradiated at room temperature in 5 × 5 mm quartz cells (Hellma, Germany) using an excitation wavelength of λex= 445 nm for 5 min. Broad excitation bandwidths (20 nm) were used for irradiation. To substantiate inter- or intramolecular cross-linking of JHBP molecules, SDS-PAGE was performed. The irradiation reaction was stopped by rapidly desalting the protein samples on a PD-10 column (Amersham Biosciences, Germany) or performing separation by gel filtration on an HR 10/300 Superdex-75 size exclusion column (Amersham Biosciences) connected to the ÄKTAexplorer system and equilibrated with a 0.1 M of a sodium phosphate buffer, pH 7.2 at a flow rate of 0.5 ml⋅min-1. Finally, proteolytic digestion was performed.

### *2.4. Analysis of dityrosine content in JHBP samples*

The determination of the DT content in protein samples was performed at BioCentrum Ltd. (Kraków, Poland) as described below. Small aliquots of recombinant rJHBP samples (25 μl) were dried under a vacuum. Protein lyophilizates were dissolved in 100 μl of Milli-Q water (Millipore, USA), evaporated in a vacuum centrifuge and then hydrolyzed in the gas phase using 6 M HCl with the addition of phenol, for 24 h at 115 °C. Hydrolyses were carried out on two samples: one containing 24.8 μg of native JHBP and the other consisting of 22.8 μg of oxidized rJHBP. After drying, the hydrolysates were dissolved in 50 μl of an acetonitrile solution (water/acetonitrile 7:2 (v/v)). DT content was determined according to a procedure described by Smail et al. [[1995]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0125). Hydrolyzed samples were injected onto a LC-18-DB 5 μm 4.6 × 250 mm column (Supelco, USA). RP-HPLC was performed at a flow rate of 1.0 ml min−1 using a linear gradient of acetonitrile gradually increased from 0% to 30% solvent B for 20 min (solvent A: 0.1% CF3COOH; solvent B: 0.07% CF3COOH, 80% acetonitrile). The eluate was collected and analyzed spectrophotometrically using a UV–vis detector set at a wavelength of 210 nm. The DT content was determined for the analyzed JHBP samples from aliquots containing 0.4 nmol of protein hydrolysate.

*2.5. Spectroscopic analysis*

JHBP solutions (2 μM) were prepared in buffers with various pH values indicated. The compositions of the buffers used were 0.1 M acetate/acetic acid, 0.1 M sodium phosphate and 0.1 M sodium carbonate, pH 3.0, 7.2 and 9.9, respectively. Borate quenching of the DT fluorescence [[24](#_ENREF_24)] was studied by monitoring fluorescence intensities of 2 μM protein samples dialyzed against a 0.5 M borate/boric acid buffer, pH 8.6. Steady state fluorescence spectra for the native JHBP solution before and after irradiation were recorded using the Fluorolog-3 fluorometer (Spex, Jobin Yvon Inc., France). Fluorescence emissions from tyrosine and DT residues were measured using excitation at 280 nm and 315 nm, respectively. Slits with bandwidths of 5 nm were used both for excitation and emission channels. Control baselines were subtracted from each spectrum for all fluorescence measurements.

*2.6. Western blot analysis*

Protein samples were analyzed by 15% SDS-PAGE according to Laemmli [[21](#_ENREF_21)]. Electrophoresis was run at 30 mA/gel slab (height: 7.0 cm, width: 8.3 cm, thickness: 1.5 mm). The separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany) [[25](#_ENREF_25)]. The membrane was blocked in a TBS buffer (10 mM Tris-base pH 7.5, 150 mM NaCl) containing 5% () low-fat milk, followed by incubation overnight at 4 °C with the primary mouse anti-DT monoclonal antibody (1C3; 1:1000) directed against dityrosine residues [[26](#_ENREF_26)]. The filters were then washed with TBS three times for 15 min. Afterwards, the membranes were incubated with HRP-conjugated rabbit anti-mouse IgG antibody (1:2000; Sigma, USA) for 2 h, washed three times with TBS for 15 min, and developed using the ECL Plus™ Western Blotting Detection Reagents (Amersham Biosciences, Germany) according to the manufacturer's instructions.

*2.7. JHBP proteolysis and protein digest fractionation*

A 25-35 µM JHBP solution was prepared in a buffer containing a 0.1 M of sodium phosphate, pH 7.2, 0.1% SDS. Endoproteinase Glu-C (i.e., V8 protease) from *S. aureus* V8 was used. JHBP samples were digested using a 50:1 () substrate-to-protease ratio for 24 h at 25 °C. The efficiency of the endoproteinase digestion was determined by analyzing a 2 μg sample with SDS-PAGE. To remove SDS, 4 M guanidine chloride was added to the samples to a final concentration of 0.5 M. A white precipitate of guanidine⋅DS salt formed immediately and was centrifuged at 14 000 × g for 10 min and discarded prior to injecting the final mixture directly onto a reversed-phase high-performance liquid chromatography (RP-HPLC) column. Then, separation of the digested peptides on the RP-HPLC column was performed using the ÄKTAexplorer system (Amersham Biosciences, Germany). Digested samples were injected onto a PepRPC HR 5/5, 5 × 50 mm, C2/C18 RP-HPLC column (Pharmacia, Uppsala, Sweden). Peptides were eluted using a flow rate of 0.5 ml⋅min-1 and the linear gradient gradually increased from 100% solvent A to 100% solvent B in 60 min (solvent A: 15 mM HCOONH4, 0.1% HCOOH; solvent B: 15 mM HCOONH4, 0.1% HCOOH, 70% acetonitrile). The eluate (fractions of 250 µl) was collected and monitored with a UV-visible detector set at 220 nm and 280 nm. Fractions were stored at -20 °C for further analyses.

*2.7. Electrospray mass spectrometry*

The Bruker MicrOTOF-Q spectrometer (Bruker Daltonik, Germany) equipped with an Apollo II electrospray ionization source (Agilent Technologies Inc., Santa Clara, CA, USA) with an ion funnel was used for the acquisition of the high-resolution electrospray ionization (ESI) mass spectra and tandem (MS/MS) mass spectra. HPLC fractions were infused into the mass spectrometer at a flow rate of 3 μl⋅min-1 at room temperature. All experiments were performed in the positive ion mode. The mass resolution was 15 000 FWHM. Before each analysis the instrument was calibrated externally with the Tunemix mixture (Bruker Daltonik, Germany) in the quadratic regression mode. The potential between the spray needle and the orifice was set at 4.5 kV. The capillary temperature was 200 °C, and N2 was used as a nebulizing gas. Scans were performed in the range of 300-1500. The MS/MS measurements were based on collision induced dissociation (CID). The precursor ions were selected in the quadrupole collision cell with the collision energy between 25 eV and 40 eV. The obtained fragment ions were directed to the TOF mass analyzer and registered as an MS/MS spectrum. In this type of experiment, only ions resulting from the fragmentation of the selected parent ion were observed. Data were acquired with micrOTOFcontrol 4.0 and processed for calibration and quantification of the samples with DataAnalysis software from Daltonik GmbH (Bremen, Germany) and ChemSketch Freeware (Advanced Chemistry Development, Inc., Canada).

*2.8. Circular dichroism spectroscopy*

CD spectra were recorded in a nitrogen atmosphere on a Jasco J-600 spectropolarimeter (Jasco Co. Ltd., Tokyo, Japan) equipped with a thermostated cell holder. The measurements were carried out at room temperature in a 0.1-cm path length quartz cuvette at a 4 μM protein concentration in a 0.1 M sodium phosphate buffer, pH 7.2. The spectra were recorded in the 195 nm to 260 nm wavelength range with a bandwidth of 1 nm at a speed of 20 nm⋅min-1 and a resolution of 0.2 nm. Each spectrum obtained was an average of 5 scans. The optical activity of the buffer was subtracted from the protein spectra. The spectral units were expressed as the molar ellipticity per residue by utilizing the Jasco system software and using protein concentrations determined by the Bradford protein assay. The prediction of the secondary structure was determined using the CDPro software package [[27](#_ENREF_27)].

**3. Results**

*3.1. Fluorescence studies of JHBP samples*

Emission and excitation spectra of 2 μM JHBP in a 0.1 M of sodium phosphate, pH 7.2, recorded directly after purification are shown in Figure 1A. Protein fluorescence is generally excited at the absorption maximum near 280 nm. Excitation at 280 nm reveals maximum at 308 nm, which can be attributable to Tyr residues, and a broad shoulder at ca. 360-420 nm which is not typical for Tyr residues. Particularly because the emission of tyrosine is relatively insensitive to solvent polarity [[28](#_ENREF_28)]. Excitation at 295 nm enhances the emission (data not shown), despite the fact that JHBP molecules do not contain Trp residues. Moreover the JHBP fluorescence spectrum shows two excitation maxima at 278 nm and 315 nm.

As the JH-binding pocket is one of two cavities present in the JHBP molecule, we suspected that the observed protein emission spectrum arose from the presence of an unknown low molecular weight molecule (LMW) present in the second pocket. Therefore, we attempted to separate this hypothetical ligand from JHBP using size-exclusion chromatography in denaturing conditions. ESI-MS analysis revealed, however, that there were no LMW compounds in the eluate (data not shown).

*3.2. pH-dependent fluorescence spectra of JHBP*

The spectroscopic properties of the JHBP molecules were further characterized for each of the concentrations of hydrogen ions. Figure 1B demonstrates the considerable effect of pH on the fluorescence intensity occurring at both 420 nm and 315 nm. Changing the acidic pH value from 3.0 (acetate) to alkaline pH 9.9 (carbonate) induced an increase in the intensity of the fluorescence near 420 nm, with a corresponding increase in the intensity of the excitation spectra at 315 nm. However, a major feature of the excitation spectra was the shift in the maximum from 280 nm to ~315 nm when we varied the pH from an acidic to an alkaline buffer solution.

Recorded spectra suggested the presence of dityrosine (DT) cross-links, which might be responsible for the additional fluorescence in the protein samples [[29](#_ENREF_29)]. It has been previously demonstrated that the fluorescence of proteins that contain DT is quenched by borate [[24](#_ENREF_24)]. Therefore, the fluorescence emission and excitation spectra of JHBP, as well as recombinant rJHBP (data not shown), were recorded in the presence and absence of a 0.5 M borate/boric acid solution, pH 8.6 (Figure 1C). A distinct decrease in the fluorescence intensity was observed when applying the borate buffer, compared to the fluorescence spectrum recorded in the carbonate buffer along with a blue shift in the emission maxima to 380 nm from approx. 420 nm.

*3.3. Enhanced DT formation after photosensitization of JHBP*

The reaction that creates dityrosine residues first requires the formation of tyrosine radicals. Then radical isomerization is followed by enolization in the final stage [[30](#_ENREF_30)]. It has been established that the photosensitization of proteins with riboflavin resulted in the Tyr radicals appearance leading to tyrosine-tyrosine cross-links [[31](#_ENREF_31)]. Given that JHBP molecules contain Tyr residues that are available for DT formation, it is possible to further stimulate DT formation with photooxidation. Therefore, samples of JHBP were irradiated for a short period of time (5 min) at the absorption maximum of the photosensitizer at 445 nm. Figure 2A illustrates that the riboflavin-treated protein (dashed line) exhibited an increase in the emission spectrum around 315 nm and 420 nm when exposed to UV light with an excitation wavelength of 280 nm. Simultaneously, a corresponding increase in the intensity of fluorescence at 420 nm was observed upon excitation at 315 nm (Figure 2B) with a small blue shift in the emission maximum. We then did SDS-PAGE, however, we were not able to observe any higher molecular mass protein molecules that corresponded to JHBP oligomers (data not shown).

*3.4. Immunochemical detection of dityrosine in the JHBP samples*

JHBP samples were further analyzed by Western blot to determine the presence of possible dityrosine cross-links in the protein molecules. As shown using the primary mouse anti-DT monoclonal antibody, both native JHBP and the dityrosine-conjugated BSA (132 kDa) used as a control demonstrated the presence of DT cross-linking in the immunoblot analysis (Figure 3). In contrast, no adducts were detected when the wild-type BSA (66 kDa) was used. The reduced SDS-PAGE migration of dityrosine-conjugated BSA clearly suggests that this protein forms a dimeric species. On the other hand, there was no evidences of a high level of JHBP aggregation (Figure 3). Taken together, the results of electrophoresis and the immunoblot analysis suggest that the intramolecularly cross-linked JHBP molecules are the product of the reaction of the coupling of tyrosyl residues.

It is noteworthy, that the amount of JHBP loaded into the well (Figure 3) was two-fold greater than the amount of dityrosine-conjugated BSA loaded (10 and 5 µg, respectively). Thus, the amount of spontaneously formed DT in the JHBP molecules is at a significantly lower level compared to the amount of induced DT within the BSA molecules.

*3.5. Structural characterization of oxidized JHBP*

In order to investigate whether the oxidation of JHBP molecules brings about any alterations in protein conformation, CD spectra in the far-UV region (195-260 nm) were carried out. Spectra were recorded on native JHBP samples, as well as on photooxidized protein under identical conditions in terms of buffer, pH and temperature. The far-UV CD spectrum of the native protein revealed a large positive maximum below 200 nm and a small local minima at 208 nm and 221 nm indicative of the α-helical character (Figure 4). However, the magnitude of the helicity was estimated to be in the range of only 15% to 19%, depending on the algorithm used for calculation (Table 1). The oxidation of JHBP leads to an increase in the molar ellipticity of the sample below 200 nm and a decrease in the molar ellipticity over the wavelength range 205-235 nm. The CD spectrum after photosensitization revealed a deeper minimum at around 221 nm and a shoulder at 208 nm. This indicates that riboflavin-mediated oxidation might result in an increase in the amount of protein α-helices. The three algorithms predicted an increase from 9% to 20% in the α-helices compared to the native protein (Table 1).

*3.6. Mass spectrometry analysis*

We attempted to identify the location of the putative oxidation sites within the JHBP molecules with the mass spectrometry method. Protein samples were subjected to V8 protease digestion. After JHBP proteolysis, the peptides were separated by RP-HPLC and characterized by tandem mass spectrometry (MS/MS). In total, seven of the eight generated peptides, which contained the Tyr residue, were identified from the ESI-MS spectra (data not shown). Figure 5A shows the protein sequence with the peptide that supposedly contained DT. A typical mass spectrum of the eluate which contained this peptide is shown in Figure 6. One high intensity signal was found in the ESI-MS spectrum at 873.93 (+2), and there were a series of isotopic signals corresponding to the mass of V8 peptide containing G124-E140 residues, which has a theoretical monoisotopic mass of 1745.81 Da (Figure 6A). The MS/MS spectrum clearly confirms a peak which was identified as GGAAYSYSVKTDDKGVE. This major signal at 873.93 (+2) was accompanied by a minor signal ca. 2 or 3 mass units lower ( 872.51 (+2)) when the protein samples were photooxidized (Figure 6B). MS/MS analysis was used in order to confirm whether this decrease in mass of about 2 Da was indicative of DT formation or whether the signal was derived from another peptide which also happened to have a similar mass. Because of the low intensity of the peak, we were not able to observed sufficient fragmentation of this peptide. However, there were no other signals of co-eluted peptide pairs differing in mass by 2 Da. Therefore, we conclude that the amino acids which were modified in the G124-E140 peptide are two Tyr residues present in close proximity to each other.

### *3.7. Identification of DT in JHBP hydrolysates*

Both native and photooxidized rJHBP samples were hydrolyzed as described in Materials and methods. The hydrolysates were separated by RP-HPLC. The retention time of the fluorescent peak from the oxidized JHBP hydrolysate was identical to that of the synthesized dityrosine, while for the native protein hydrolysate the peak characteristic of DT was practically invisible (data not shown). Based on the measurement of the peak area and the calibration curve for dityrosine standard, the amount of DT in the photooxidized protein hydrolysate was 2.15 × 10−3 mol of DT/mol of rJHBP. To confirm that the observed peak represents DT, the pooled chromatographic fractions were additionally examined by ESI-MS and MS/MS methods. The resulting mass spectra showed the presence of DT in the protein hydrolysates (results not shown).

**4. Discussion**

The JHBP molecule contains 10 Y residues with different accessibilities ([Fig. 5](http://www.sciencedirect.com/science/article/pii/S0003986111003584#f0025)), which represent 4.4% of the total amino acid composition. The abundance of Y residues increased the opportunity for the formation of cross-linking bonds between residues located in close proximity, as the reaction was dependent on the close positioning of a pair of Y residues.

DT is a covalently bound diphenol, produced by the reaction of two tyrosyl radicals. It was identified by intense fluorescence with a maximum near 420 nm that was measurable upon excitation at either 315 nm (alkaline solutions, pH > 7) or 284 nm (acidic solutions, pH ⩽ 7) [[30]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0160), [[32]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0170) . In this work, we provide a range of evidence for the generation of the protein o,o′-dityrosine within JHBP molecules. (i) All the observed spectroscopic changes indicated that although there is some variability in the fluorescence maxima of JHBP, in general, the protein spectra seem to be similar to those observed for DT ( [Fig. 1](http://www.sciencedirect.com/science/article/pii/S0003986111003584#f0005) and [Fig. 2](http://www.sciencedirect.com/science/article/pii/S0003986111003584#f0010)). Two peaks appearing in the JHBP excitation band, one at around 285 nm and another at 315 nm were presumably associated with the protonated and deprotonated forms of DT [[33]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0180). In contrast, DT emission spectra contain one peak which may differ only in the position of its λmax. (ii) We have established that the JHBP samples displayed an almost identical DT signal to Y–Y itself and showed exactly the same pH-dependent variations in the excitation bands (reviewed in [[34]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0185)). (iii) Furthermore, borate/boric acid solutions have a quenching effect on the excitation and fluorescence emission spectra of JHBP. (iv) Consequently, after riboflavin-sensitized photooxidation we observed a fivefold increase of protein fluorescence upon excitation at 315 nm ([Fig. 2](http://www.sciencedirect.com/science/article/pii/S0003986111003584#f0010)B). (v) Additionally, the formation of DT cross-links in the JHBP samples was verified using the monoclonal antibody directed against DT residues ([Fig. 3](http://www.sciencedirect.com/science/article/pii/S0003986111003584#f0015)).

Although under physiological conditions all amino acid residues can be oxidized by reactive oxygen species (ROS), Y residues are particularly easily oxidized. They are converted mainly to 3,4-dihydroxyphenylalanine (DOPA) and Y–Y cross-linked derivatives [[35]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0190). DT formation has been linked to a number of serious human disease (reviewed in [[36]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0195)). It is known to occur, inter alia, in amyloid fibril formation [[37]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0200), Parkinson’s disease [[38]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0205), cerebrospinal fluid damage [[39]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0210), lipofuscin granules in the brain [[18]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0095) or epidermoid carcinoma [[40]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0215). DT also plays important physiological roles in invertebrate proteins, where it has been identified as a product of normal post-translational modifications. DT contributes to the elasticity and integrity of such structural proteins as collagen [[41]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0220) and elastin [[42]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0225), silk fibroin and keratin [[43]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0230) or insect protein resilin [[44]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0235).

The identification of the presence of DT in JHBP led us to analyze the possible structural changes in the protein molecules under riboflavin-mediated oxidative stress conditions ([Fig. 4](http://www.sciencedirect.com/science/article/pii/S0003986111003584#f0020)). The CD spectra of JHBP, recorded in the far-UV region (195–260 nm), combined with structural analysis using the CDPro package revealed that native JHBP contains as little as about 15–19% α-helices and 32–37% β-sheets, whereas the rest of the protein assumes β-turns and a random conformation. The calculated values are not in agreement with the data obtained from the crystal structure (α-helices: 33%, β-sheets: 39%, other: 28%) [[15]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0075). This may be due to the limitations of the applied algorithm and/or may indicate, that ammonium sulfate is able to induce conformational transitions within the JHBP molecule during crystallization. It has been previously shown that protein helicity varies greatly depending on the concentration of ammonium sulfate [[45]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0240). The CD spectrum of oxidized protein was different. This indicates that the photooxidation of the JHBP molecule can induce structural alteration or stabilize one conformation state that appears during the breathing of the protein molecule. The measurement of the oxidized JHBP CD spectra, followed by structural analysis, revealed that the fraction of α-helices had substantially increased with a concomitant decrease in the β-structure.

In the JHBP molecule the closest pair of Y residues are Y128 and Y130, which are situated in one of the β-sheets ([Fig. 5](http://www.sciencedirect.com/science/article/pii/S0003986111003584#f0025)). Although the presence of DT was not observed by crystallographic studies (most likely due to the insignificant number of oxidized protein molecules), the probability that there is cross-linking between Y128 and Y130 is quite high. Although side chains of these Y residues are buried inside a hydrophobic cavity, they exhibit at least some freedom of movement and degree of solvent accessibility. The ortho carbons of their rings are separated by 4.07 Å in the crystal structure ([Fig. 5](http://www.sciencedirect.com/science/article/pii/S0003986111003584#f0025)B). The distance between the other Y–Y pairs ranges from 6.89 to 15.50 Å in the native JHBP molecule. Given that we did not find any other cross-linked peptides in the MS analyses ([Fig. 6](http://www.sciencedirect.com/science/article/pii/S0003986111003584#f0030)), and that formation of DT bridges occurs intramolecularly, the data strongly suggest that Y coupling occurs only between the Y128 and Y130 residues in the JHBP molecule. These results, together with the estimation of the DT content of only 2.15 × 10−3 mol per mole of photooxidized rJHBP hydrolysates, also seems to provide evidence that most JHBP molecules are found in an unmodified form. We could not detect any substantial decrease in tyrosine residues in the JHBP molecules after irradiation. However, it cannot be excluded that the large increase in CD was also caused by the oxidative degradation of other amino acid residues. The approximate value of 3 × 10−2 mol of DT per one mole of JHBP molecule can be estimated from fluorescence spectra. In light of our experiments the profound conformational transition of JHBP molecules observed from CD analyses is difficult to explain. One of the possibilities is that the energy of the excited state of riboflavin was directly used to relocate the amino acid backbone in the protein molecule. We are fully aware that the above explanation is highly speculative. Although it has been previously found that the structure of the JHBP molecule is flexible and exhibits an intrinsic ability for conformational transition [[16]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0080) and [[22]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0115). Another explanation which might give rise to the difference in the spectra of native and oxidized JHBP, is the strong coupling of aromatic transitions that occurs if two or more aromatic rings are in close proximity. This could significantly influence the far-UV CD [[Sreerama 1999]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0245).

The question remains as to when DT is formed in JHBP molecules. Interestingly, the background level of superoxide radicals was detected even in the intact hemolymph of G. mellonella [[46]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0250). Formation of the potential ROS is accompanied by the process of melanization during insect cellular immune reactions. Melanization reactions associated with cuticular wounding and defense responses in insects have been attributed to the action of phenol oxidase (tyrosinase, E.C.1.14.18.1). In the larvae of Drosophila melanogaster, for instance, View the MathML sourceand H2O2 have been found to be generated in association with melanotic encapsulation reactions (reviewed in [[47]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0255)). However, a contradictory report concerning the role of tyrosinase was published by Kim and Han [[48]](http://www.sciencedirect.com/science/article/pii/S0003986111003584#b0260), showing that under appropriate conditions this enzyme can function as a scavenger which reduces the tyrosyl radical back to Y residue.

In this report we presented the results of several experiments that demonstrated that the unusual fluorescence of JHBP was due to the formation of DT molecules. It still remains to be discovered what type of small molecules there are that can bind within the protein E cavity. The search for the second JHBP ligand is of great interest and is currently under investigation.

In conclusion, the data presented in this report show that DT is formed in the native JHBP molecule and that this cross-linked bond occurs between the Y128 and Y130 residues.

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**References**

[1] W.G. Goodman, N.A. Granger, in: L.I. Gilbert (Ed.), Insect development: morphogenesis, molting and metamorphosis, Academic Press, London, 2009, pp. 305-394.

[2] P. Hidayat, W.G. Goodman, Insect Biochem. Molec. Biol. 24 (1994) 709-715.

[3] B. Hammock, J. Nowock, W. Goodman, V. Stamoudis, L.I. Gilbert, Mol. Cell. Endocrinol. 3 (1975) 167-184.

[4] L.L. Sanburg, K.J. Kramer, F.J. Kezdy, J.H. Law, H. Oberlander, Nature 253 (1975) 266-267.

[5] E. Whitmore, L.I. Gilbert, J. Insect Physiol. 18 (1972) 1153-1167.

[6] W.G. Goodman, E.S. Chang, in: G.A. Kerkut, L.I. Gilbert (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, Pergamon Press, Oxford, 1985, pp. 491-510.

[7] S.C. Trowell, Comp. Biochem. Physiol. 103B (1992) 795-807.

[8] G.D. Prestwich, K. Touhara, L.M. Riddiford, B.D. Hammock, Insect Biochem. Mol. Biol. 24 (1994) 747-761.

[9] R.P. Braun, G.R. Wyatt, J. Biol. Chem. 271 (1996) 31756-31762.

[10] A. Ożyhar, M. Kochman, Eur. J. Biochem. 162 (1987) 675-682.

[11] J.M. Rodriguez Parkitna, A. Ożyhar, J.R. Wiśniewski, M. Kochman, Biol. Chem. 383 (2002) 1343-1355.

[12] M. Duk, H. Krotkiewski, E. Forest, J.M. Rodriguez Parkitna, M. Kochman, E. Lisowska, Eur. J. Biochem. 242 (1996) 741-746.

[13] J. Dębski, A. Wysłouch-Cieszyńska, M. Dadlez, K. Grzelak, B. Kłudkiewicz, R. Kołodziejczyk, A. Lalik, A. Ożyhar, M. Kochman, Arch. Biochem. Bophys. 421 (2004) 260-266.

[14] B. Winiarska, A. Dwornik, J. Dębski, K. Grzelak, D. Bystranowska, M. Zalewska, M. Dadlez, A. Ożyhar, M. Kochman, Biochim. Biophys. Acta (2011) doi:10.1016/j.bbapap.2011.1002.1002.

[15] R. Kolodziejczyk, G. Bujacz, M. Jakób, A. Ożyhar, M. Jaskolski, M. Kochman, J. Mol. Biol. 377 (2008) 870-881.

[16] E. Wieczorek, M. Kochman, Eur. J. Biochem. 201 (1991) 347-353.

[17] D. Krzyzanowska, M. Lisowski, M. Kochman, J. Pept. Res. 51 (1998) 96-102.

[18] Y. Kato, W. Maruyama, M. Naoi, Y. Hashizume, T. Osawa, FEBS Lett. 439 (1998) 231-234.

[19] E. Wieczorek, J.M. Rodriguez Parkitna, J. Szkudlarek, A. Ożyhar, M. Kochman, Acta Biochim. Pol. 43 (1996) 603-610.

[20] M.M. Bradford, Anal. Biochem. 72 (1976) 248-254.

[21] U.K. Laemmli, Nature 227 (1970) 680-685.

[22] G. Fairbanks, T.L. Steck, D.F. Wallach, Biochemistry 10 (1971) 2606-2617.

[23] W. Goodman, P.A. O'Hern, R.H. Zaugg, L.I. Gilbert, Mol. Cell. Endocrinol. 11 (1978) 225-242.

[24] D.A. Malencik, S.R. Anderson, Biochem. Biophys. Res. Commun. 178 (1991) 60-67.

[25] H. Towbin, T. Staehelin, J. Gordon, Proc. Natl. Acad. Sci. USA 76 (1979) 4350-4354.

[26] Y. Kato, X. Wu, M. Naito, H. Nomura, N. Kitamoto, T. Osawa, Biochem. Biophys. Res. Commun. 275 (2000) 11-15.

[27] N. Sreerama, R.W. Woody, Anal. Biochem. 287 (2000) 252-260.

[28] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, 3rd ed, Springer Science+Business Media, LLC, New York, USA, 2006.

[29] R. Aeschbach, R. Amadò, H. Neukom, Biochim. Biophys. Acta. 439 (1976) 292-301.

[30] C. Giulivi, K.J.A. Davies, Methods Enzymol. 233 (1994a) 363-371.

[31] R. Kanwar, D. Balasubramanian, Exp. Eye Res. 68 (1999) 773-784.

[32] A.J. Gross, I.W. Sizer, J. Biol. Chem. 234 (1959) 1611-1614.

[33] S.S. Lehrer, G.D. Fasman, Biochemistry 6 (1967) 757-767.

[34] D.A. Malencik, S.R. Anderson, Amino Acids 25 (2003) 233-247.

[35] K.J. Davies, M.E. Delsignore, S.W. Lin, J. Biol. Chem. 262 (1987) 9902-9907.

[36] A. Kochman, C. Kośka, D. Metodiewa, Amino Acids 23 (2002) 95-101.

[37] L. Galeazzi, P. Ronchi, C. Franceschi, S. Giunta, Amyloid 6 (1999) 7-13.

[38] S. Pennathur, W. Jackson-Lewis, S. Przedborski, J.W. Heinecke, J. Biol. Chem. 274 (1999) 34621-34628.

[39] M. Abdelrahim, E. Morris, J. Carver, S. Facchina, A. White, A. Verma, J. Chromatogr. B. Biomed. Sci. 696 (1997) 175-182.

[40] A. van der Vliet, M. Hristova, C.E. Cross, J.P. Eiserich, T. Goldkorn, J. Biol. Chem. 273 (1998) 31860-31866.

[41] F. LaBella, P. Waykole, G. Queen, Biochem. Biophys. Res. Commun. 30 (1968) 333-338.

[42] F. LaBella, F. Keeley, S. Vivian, D. Thornhill, Biochem. Biophys. Res. Commun. 26 (1967) 748-753.

[43] D.J. Raven, C. Earland, M. Little, Biochim. Biophys. Acta 251 (1971) 96-99.

[44] S.O. Anderson, Biochim. Biophys. Acta 93 (1964) 213-215.

[45] A. Mateja, T. Cierpicki, M. Paduch, Z.S. Derewenda, J. Otlewski, J. Mol. Biol. 357 (2006) 621-631.

[46] I.A. Slepneva, V.V. Glupov, S.V. Sergeeva, V.V. Khramtsov, Biochem. Biophys. Res. Commun. 14 (1999) 212-215.

[47] A.J. Nappi, E. Ottaviani, Bioessays 22 (2000) 469-480.

[48] S.M. Kim, S. Han, Biochem. Biophys. Res. Commun. 312 (2003) 642-649.

**Figure Legends**

**Figure 1.** **Fluorescence spectra of native JHBP.** **(A)** Excitation (dashed line) and emission (solid line) spectra of 2 µM JHBP were recorded in the presence of 0.1 M of sodium phosphate, pH 7.2. The excitation spectrum was monitored at fixed λem = 308 nm, and the emission spectrum was monitored at fixed λex = 280 nm. **(B)** pH-dependent fluorescence spectra of JHBP. Excitation spectra (monitored at a fixed λem = 420 nm) and emission spectra (obtained with a fixed λex = 315 nm) are shown. 2 µM of JHBP in: 0.1 M of carbonate, pH 9.9 (solid lines), 0.1 M of phosphate, pH 7.2 (dashed lines), or 0.1 M of an acetate buffer, pH 3.0 (dotted lines). **(C)** The effect of borate on JHBP fluorescence. Excitation and emission spectra of 2 µM of JHBP were recorded in the presence of 0.5 M of borate, pH 8.6 (solid lines) and 0.1 M of carbonate, pH 9.9 (dashed lines). Excitation spectrum was monitored at a fixed λem = 420 nm and emission spectra was monitored at a fixed λex = 304 nm. Slit widths: 5 nm.

**Figure 2.** **Fluorescence spectra of oxidized JHBP.** Fluorescence emission spectra of native (solid lines) and riboflavin-photosensitized (dashed lines) 2 μM JHBP samples in 0.1 M of sodium phosphate, pH 7.2. Photosensitization was performed as described in Materials and Methods. Spectra were recorded at the excitation wavelength of 280 nm **(A)** and 315 nm **(B)**. Additional emission maximum was observed at ~415 nm. Slit widths: 5 nm.

**Figure 3.** **Detection of DT cross-links using Western blotting.** Protein samples were subjected to SDS-PAGE (15%) performed using aliquots of native JHBP (10 µg; line 2), oxidized bovine serum albumin (5 µg; line 3) as a positive control and native bovine serum albumin (10 µg; line 4) as a negative control. Anti-dityrosnie monoclonal antibody was used for Western blot analysis. Numbers on the left (line 1) refer to the size of the molecular weight markers. Arrows indicate the positions of the monomeric JHBP and dimeric BSA that contained DT.

**Figure 4.** **Far-UV CD spectra of native and oxidized JHBP.** Mean residue ellipticities ([]) of 4 μM JHBP samples in 0.1 M of sodium phosphate, pH 7.2, were calculated for native (solid line) and photooxidized (dashed line) protein as described in Materials and Methods.

**Figure 5.** **Location of DT in** **the primary and three-dimensional structure of JHBP.** **(A)** The primary structure of JHBP. The ten tyrosyl residues are underlined – Y128 and Y130 are in red, whereas the remaining Tyr residues are in blue, disulfide bridges are in yellow. The shaded region represents the G124-E140 peptide containing Y128 and Y130 residues, obtained after cleavage of JHBP with a V8 protease. **(B)** The PyMOL-generated cartoon of the JHBP structure (Protein Data Bank identifier 2rck). Distances (in Å) between the two *ortho* carbon atoms of the phenyl rings of pairs of the adjacent Tyr residues (black dashed lines) are indicated and labeled.

**Figure 6. Changes in the G124-E140 peptide mass resulting from JHBP oxidation.** Deconvoluted ESI-MS of native **(A)** and oxidized **(B)** JHBP peptide at  873.93 (+2). **(C)** MS/MS spectrum acquired from the parent ion of  873.94 (+2), which corresponds in mass to the peptide GGAAYSYSVKTDDKGVE obtained after V8 protease cleavage of JHBP molecule. Only fragments containing +1 ions are shown.