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Tryptophan mediated denaturation of β-lactoglobulin A by UV irradiation.

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Abstract

β-Lactoglobulin A, a genetic variant of one of the main whey proteins, was irradiated at 295 nm for 24 hours. After irradiation 18% of the protein was denatured (determined by reverse-phase chromatography). The fluorescence spectrum of the irradiated protein was red shifted compared to that of the native protein, indicating a change in protein folding.

Sulfhydryl groups, which are buried in native β-lactoglobulin, were exposed following irradiation and became available for quantification using the Ellman assay. The quantity of exposed sulfhydryls increased, but the number of total sulfhydryl groups decreased. Gel permeation chromatography showed that some protein aggregation occurred during irradiation. Fourier transform infrared (FTIR) spectroscopy of irradiated β-lactoglobulin revealed changes in the secondary structure, comparable to that of early events during heat-induced denaturation. There was evidence for some photo-oxidation of tryptophan

Key words: β-lactoglobulin, UV irradiation, Tryptophan, Disulfide, Photo-oxidation

INTRODUCTION

β-Lactoglobulin is the main whey protein in bovine milk making up ~50% of the total whey protein; consequently it has been widely studied. The structure has been well characterized. It contains 163 amino acid residues and has a molecular weight of 18,362 Da (β-lactoglobulin A, primary accession number 02754). The protein contains five cysteines, which give rise to two intermolecular disulfide bonds, between Cys66 and Cys160, and between Cys106 and Cys119 (1). The cysteine in position 121 has a free sulfhydryl group, which remains inaccessible for chemical reaction around neutral pHs. β-Lactoglobulin also contains two tryptophan residues, located at positions 19 and 61. Spatially these are located close to the disulfide bridges, based on three-dimensional structures of β-lactoglobulin A (PDB code 1CJ5). Changes in the functionality of β-lactoglobulin functionality can be induced by unfolding of the protein, whereby reactive groups such as the free sulfhydryl is exposed on the protein surface. This enables aggregation of the molecules. Normally, unfolding is induced by heat, pH or high pressure.

As mentioned above, β-lactoglobulin contains two tryptophan residues. The fluorescence of Trp61 is partly exposed to the aqueous solvent and is quenched because of its proximity to the Cys66 – Cys160 disulfide bond. Trp19 is located in an apolar environment within the cavity of β-lactoglobulin, making it easily detectable by fluorescence measurements. This means that the intrinsic fluorescence of β-lactoglobulin is mainly due to Trp19, which is located in a hydrophobic area at the core of the protein. The non-polar location increases the intensity of the Trp19 fluorescence and the emission maximum of the protein is blue shifted compared to the light emitted by tryptophan in a polar environment.

Dose et al. (*2*) suggested that disulfide bonds could be photolysed in the presence of aromatic amino acids. Disulfide bonds can quench the fluorescence of tryptophan when they are located in close proximity (*3*). Cleavage of the disulfide bond can lead to an increase in the fluorescence intensity from tryptophan excitation. An example of this phenomenon has been described for *Fusarium solani pisi* cutinase (*4*). An increase in free sulfhydryl was observed along with an increase in fluorescence intensity, before a decrease due to photo bleaching. Energy (*5*)or electron transfer between the excited tryptophan and the disulphide bond (*6*) have been suggested as possible mechanism for the cleavage of disulphide bonds leading to free sulfhydryl in the protein.

A detailed study has been carried out on other globular whey proteins. Vanhooren et al. (*7*) studied the effect of UV light on goat α-lactalbumin. They found a number of interesting effects of UV radiation: a red-shift and a decrease in the intensity of the emitted light were observed as the protein was irradiated. Importantly they also noticed an increase in the amount of free sulfhydryl present as a function of irradiation time. Gel-permeation chromatography and electrophoresis showed that there was some aggregation of the irradiated protein. UV irradiation of Bovine Serum Albumin (BSA) in the presence of cysteine brought about structural changes in the BSA and induced gelation (*8*). More recently Dalsgaard et al. studied the photo-oxidation of milk proteins in the presence of riboflavin as photo-sensitizer (*9*). The results show that β-lactoglobulin underwent changes in both secondary and tertiary structure upon irradiation. The degree of polymerization of the globular whey proteins was lower than the caseins. The observed formation of *N*-formylkynurenine and kynurenine were attributed to exposure of tryptophan to singlet-oxygen.

The aim of this study was to examine the possibility of using UV radiation to denature β-lactoglobulin A. The extent of denaturation was determined using chromatographic and spectroscopic techniques. UV denatured β-lactoglobulin was compared to heat-denatured β-lactoglobulin.

MATERIALS AND METHODS

All chemicals were from Sigma Aldrich (Dublin, Ireland) unless otherwise stated. Milli-Q water was used through-out.

β-Lactoglobulin purification

β-Lactoglobulin was purified from whey protein concentrate (WPC), which was prepared in-house from fresh milk (pilot plant of Moorepark Technology Ltd.). To produce a WPC with minimal heat treatment raw milk was creamed, microfiltrated to remove caseins, ultrafiltrated to reduce the lactose content and concentrate the whey proteins. 25 mL of 10 % (w/w) WPC reconstituted in mobile phase was injected on a Q-Sepharose column (Amersham Biosciences). A 10 mM Tris-HCl pH 7.0 buffer with a 0 to 400 mM NaCl gradient was used to elute the proteins. The β-lactoglobulin A fractions were collected, dialyzed extensively to remove salts and freeze-dried. The purity of β-lactoglobulin A was greater than 99% by reverse-phase chromatography (RP-HPLC).

UV treatment of β-lactoglobulin

Solutions of β-lactoglobulin A were prepared in 20 mM phosphate buffer pH 7.0 containing 50 mM NaCl. The concentration of β-lactoglobulin A was determined

from the absorbance of the solution at 280 nm, using the specific extinction coefficient of 0.96 $Lg^{-1}cm^{-1}$, and the final concentration was adjusted to 10 gL^{-1} (545 μM using 18,362 $gmol^{-1}$ for the molecular weight of β-lactoglobulin A) with 20 mM phosphate buffer pH 7.0 containing 50 mM NaCl. The irradiation was carried out at a wavelength of 295 nm for 24 hours using a Cary Eclipse fluorescence spectrofluorimeter (Varian, Inc., USA). A low volume cuvette (Hellma®, USA) was used; the cuvette had an excitation window of 8 × 5 mm, the emission window, located at a 90° angle to the excitation one, was 2 × 5 mm. The intensity of the excitation beam was ~20 μW, the size of the excitation beam was close to that of the excitation window. 170 μL of β-lactoglobulin solution was placed in the cuvette. The excitation slit was set to 5 nm for the duration of the irradiation. The irradiation was carried out at 4°C to minimize evaporation from the cuvette.

Emission spectra

The same instrument and cuvette outlined above was used to carry out all emission measurements (25°C). The measurements were made immediately prior to and following the 24 hours of irradiation. The protein concentration was the same as used for the UV treatment, 10 gL^{-1}. The instrument settings were as follows: emission slit 1.5 nm, excitation slit 10 nm, excitation wavelength 295 nm, emission scan 298 to 450 nm, scan rate 120 nm min^{-1}. A control sample, in a 1 cm×1 cm quartz cuvette, containing 2 gL^{-1} β-lactoglobulin was used to measure any changes in the intensity of the lamp over the 24 hours of irradiation. The emission spectrum of the control was read before and after the 24 hours of irradiation, the control was not irradiated during the 24 hour period.

Absorption spectra

The absorption measurements were performed on a one in ten dilution of β-lactoglobulin A before and after irradiation. The absorption spectrum was measured from 250 to 400 nm using a Cary Scan 1 instrument.

Determination of the quantity of native protein

For the purpose of this study denatured protein was defined as protein which precipitated at pH 4.6 or had had an altered retention time in the reverse-phase HPLC method described below. The quantity of remaining native protein after irradiation was quantified using a previously described method (*10*). A Source^{TM} 5RPC column (Amersham Biosciences, UK limited) and a Waters 2695 separation module with a Waters 2487 absorbance detector were used. The proteins were detected at 214 nm.

Ellman's assay

To determine the amount of exposed and total sulfhydryl groups (SH groups) in the irradiated β-lactoglobulin solutions, a variation of the assay developed by Ellman (*11*) was used. The protein solutions were diluted to a concentration of 0.5 gL^{-1} protein in a 50 mM Tris-HCl buffer pH 7.0 (for the determination of exposed SH) or in a 50 mM Tris-HCl pH 7.0 with 8 M urea (total SH groups). 2.75 mL of the diluted solution was placed in a cuvette and 0.25 mL of a 1 gL^{-1} 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) solution in 50 mM Tris-HCl buffer was added. The solutions were incubated at room temperature for 30 min prior to reading the absorbance at 412 nm. The molar extinction coefficient of free thio-bis(2-nitrobenzoic acid) TNB, 14,150 $M^{-1}cm^{-1}$ (*12*) was used to calculate the concentration of the sulfhydryl groups.

High-performance gel permeation chromatography

The samples were analyzed on a TSK G3000 SWXL column (Toshibas, Montgomeryville, PA. USA). The same HPLC system as above was used. The separation was carried out using a mobile phase of 20 mM sodium phosphate, 50 mM NaCl, pH 7.0, buffer at a flow rate of 0.8 mL min^{-1}. The absorbance was measured at 214 and 321 nm.

Blocking of sulfhydryl groups with IAEDANS

The sulfhydryl blocking agent 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (IAEDANS) (Invitrogen, Dublin, Ireland) was used to block the exposed sulfhydryl groups as described previously (*13*). Briefly, 20 μL of IAEDANS (2.26 gL^{-1} in 20 mM phosphate 50 mM NaCl) was added to 100μL of protein solution (10 gL^{-1}) and incubated overnight in the dark at 37°C. The samples were dialyzed against phosphate buffer to remove excess IAEDANS.

Reduction and alkylation of proteins

100 μL of the β-lactoglobulin solution, which was treated with IAEDANS and subsequently dialyzed, was diluted with 400 μL of 50 mM Tris-HCl buffer containing 6 M urea (pH 8.5) and 5 μL of 100 mM dithiothreitol (DTT in 50 mM Tris-HCl buffer containing 6 M urea at pH 8.5). The reduction was carried out at 56ºC for 1 hour. After the reduction 20 μL of 2 M iodoacetamide (IAA) was added to the solution and incubated at room temperature in the dark. The solutions were dialyzed against a 10 mM carbonate buffer pH 8.5 overnight to remove excess reagents.

Protein hydrolysis

Dialyzed samples were adjusted to pH 8.0 by the addition of HCl. A stock solution of 1 gL^{-1} trypsin was added to the protein solution to bring it to an enzyme to protein ratio of 1:100 (w/w). The solutions were hydrolyzed overnight at 37ºC. 200 μL of hydrolyzed solution was removed and the pH was reduced below 3 with TFA in order to inactivate the enzyme.

Reverse phase chromatography of digested samples

The trypsin digested samples were separated on a reverse-phase Symmetry C18 column (2.1 × 125 mm; Waters, Milford, MA). The column was equilibrated with solvent A (0.106 % (v/v) trifluoroacetic acid in Milli-Q water) and eluted with a linear gradient of 3 to 60 % solvent B (0.1 % (v/v) trifluoroacetic acid in 4:1 (v/v) acetonitrile:Milli-Q water) over 60 min. RP-HPLC separations were achieved at 40°C at a flow rate of 250 $\mu Lmin^{-1}$. Peptides were detected simultaneously at 214 nm and 336 nm using a Waters 2487 dual wavelength absorbance detector.

Fourier transform infrared spectroscopy (FTIR)

FTIR measurements were carried out using a Bruker Tensor 27 instrument in transmission mode with a thermally controlled AquaSpec™ cell. Spectra were obtained for native and irradiated β-lactoglobulin at 25°C in the 20 mM phosphate buffer pH 7.0, 50 mM NaCl buffer. The spectra used were an average of 250 scans at a resolution of 2 cm^{-1}. The quantity of β-sheets and α-helix present were determined using the CONFOCHECK™ system, part of the Opus version 5.5 software, which is based on a multivariate calibration using 35 known proteins. After atmospheric compensation the spectra were vector normalized and the spectrum of the native

sample (1%) was subtracted from that of the irradiated one. The resulting curves were compared to those obtained from heated β-lactoglobulin (1%, 78ºC) in the same buffer to assign changes in the secondary structure.

RESULTS

The intensity of light emitted by β-lactoglobulin decreased with the length of irradiation at 295 nm. Initially there was a large decrease in the intensity emitted at 340 nm, but as the irradiation continued the decrease in emission intensity slowed (data not shown). The controls demonstrated that there was no change in lamp intensity over the period of the measurement, Figure 1. Integration of the emission spectra showed a decrease of almost 50%. A red shift of 9 nm in the emission maximum was also observed (Table 1.)

The irradiation of β-lactoglobulin caused an increase in the absorbance between 300 and 350 nm (Figure 1). A shoulder centered at 290 nm was also more pronounced in the case of the irradiated sample.

The gel permeation profile of β-lactoglobulin before irradiation showed a peak eluting at 12 minutes, Figure 2A. The intensity of this peak decreased after irradiation. There was also material eluting before the peak at 12 minutes in the irradiated sample, which can be associated to aggregated β-lactoglobulin. The chromatograms (214 nm) for the irradiated samples were integrated between 9 and 15 minutes. It was found that 93% of the protein was contained in the monomer peak (11.4 – 15 min). The chromatogram was also recorded at 321nm. The results show that there was an increase in absorbance at 321nm for the monomer peak after irradiation, Figure 2B. The newly formed aggregates also absorbed at 321 nm.

The reverse phase HPLC results show that there was a decrease in the concentration of β-lactoglobulin in the samples after irradiation (Table 1.). It was previously observed that changes in the profile of RP-HPLC are a good indicator for irreversible structural changes. Non-native monomers such as those described by Croguennec et al. (*14*) do not elute like native proteins in RP-HPLC, making it a better method than either SDS-PAGE or HP-GPC for studying minor changes in the protein structure.

Given that 93% of the protein was monomeric after irradiation, 11% of the protein must have been non-native monomer, (93% - 82%). Non-native monomers are brought about by disulfide rearrangements in the β-lactoglobulin molecule and may contain exposed sulfhydryl groups.

After the irradiation, the protein which eluted as native in the RP-HPLC method had an increased absorbance at 321 nm. This indicates that even though the protein has not unfolded some changes have occurred in the primary structure of the protein.

Ellman assay results showed there was 0.96 ± 0.02 mol total sulfhydryl per mol of β-lactoglobulin present in the sample prior to heating. This is close to the expected value of one mol sulfhydryl per mol of β-lactoglobulin. Only very small quantities of these sulfhydryls were exposed (Table 1.). After irradiation there was a decrease in the concentration of total sulfhydryl present but there was a greater quantity of exposed sulfhydryls (Table 1.).

The sulfhydryl groups which had been exposed by irradiation were blocked with IAEDANS. The peptides containing cysteines blocked with IAEDANS were easily identified, as IAEDANS absorbs at 336 nm. The chromatograms obtained for the separation of peptides from irradiated β-lactoglobulin treated with IAEDANS are shown in Figure 3A and B. Figure 3D shows that in the absence of IAEDANS there were some peptides absorbing at 336 nm. This intrinsic absorbance was considered

when analyzing the significance of the results for the irradiated sample. Comparing the data from the current study with that obtained previously (*13*), allowed several peptides containing cysteines blocked with IAEDANS to be identified. The results showed cysteines 121 and 119 were blocked with IAEDANS. Cysteine 121 has a free sulfhydryl in native β-lactoglobulin; however, this is normally unavailable for reaction with IAEDANS. Peptides containing Cysteine 119 blocked with IAEDANS were also identified. Cysteine 119 forms a disulfide linkage with cysteine 106 in native β-lactoglobulin but cysteine 119 has previously been shown to readily interchange with cysteine 121 during heat-denaturation (*14*). Cysteine 66 was also found blocked with IAEDANS.

The amide I band in FTIR spectra is very sensitive to changes in the secondary structure of proteins. The denaturation, aggregation and gelation of globular proteins (including β-lactoglobulin) have been studied previously by looking at changes in the amide I region of the FTIR spectrum. The spectra of native, irradiated and heat-treated β-lactoglobulin are shown in Figure 4A. The spectra show that there are structural differences between the β-lactoglobulin treatments. Using a multivariate analysis it was determined that in native β-lactoglobulin, the β-sheet content was 35.4% and the α-helix content was 17.6%. Irradiation for 24 hours did not significantly alter these values. The irradiated samples contained 33.9% β-sheet and 14.9% α-helix. The subtraction of the native spectrum from that of the irradiated sample showed that there was a significant change in intensity at 1627 cm^{-1}. A similar change was observed for that of heat-denatured β-lactoglobulin, see Figure 4. The band around 1627 cm^{-1} was previously assigned to strongly bonded β-sheets (15,16). The formation of anti-parallel intermolecular β-sheets caused by aggregation caused the appearance of a band around 1617 cm^{-1} (17,18), which increased with heating time. UV irradiation of

β-lactoglobulin did not cause the appearance of a band around 1617 cm^{-1}. An increase in intensity at 1645 cm^{-1} was also observed in both the heated and irradiated samples. This band has been assigned to both unordered structure and α-helix (19,20). In the case of the irradiated sample the calculated quantity of α-helix did not change significantly. Therefore, this change in the intensity at 1645 cm^{-1} can be assigned to an increase in unordered structure. Similarly, the broadening of the band between 1656 to 1580 cm^{-1} could be due to an increase in turns or β-sheets. However, the calculated β-sheet content did not change upon irradiation; hence this band could be associated to an increase in turns. β-Lactoglobulin that had been heat-denatured for 10 minutes at 78ºC showed the greatest degree of homology to the irradiated sample in terms of the FTIR spectrum. However, when the heated sample was analyzed using the HPLC method above, 42% of the protein had been denatured. This compares to only 18% denatured in the irradiated sample (determined by RP-HPLC).

DISCUSSION

The irradiation of β-lactoglobulin brought about some structural changes, which manifested themselves in a number of ways. There was a red-shift in the intrinsic tryptophan fluorescence of β-lactoglobulin. The red-shift is indicative a change in the tertiary structure of the protein. The red-shift observed here was similar to that seen in other globular proteins, such as goat α-lactalbumin (*7*). As well as the red-shift there was a decrease in the intensity of the emitted light. Disulphide rearrangements which occur in this study could give rise to an increase in the intensity of emitted light as they would act to move disulphide bonds further from tryptophan residues.

337 The increase in the absorbance spectrum around 320 nm is indicative of the
338 formation of an oxidation product of tryptophan, *N*-Formylkynurenine. Numerous
339 studies have shown that the irradiation of tryptophan can lead to the formation of
340 *N*-formylkynurenine (21,22). *N*-formylkynurenine is formed by the oxidative cleavage
341 of the 2, 3 – bond of the indole ring in tryptophan. The structure of tryptophan and
342 *N*-formylkynurenine are shown in Figure 1. Assuming the increase of absorbance at
343 320 nm is caused by the formation of *N*-formylkynurenine, it was estimated that 30%
344 of the tryptophan present had been converted to *N*-formylkynurenine after 24 hours of
345 irradiation (using the extinction coefficient of 3200 L $mol^{-1}cm^{-1}$ at 315 nm (23)) The
346 formation of this oxidation product also explains the decrease in the intensity of the
347 emitted light observed in the fluorescence experiments.
348 The gel permeation chromatography results showed that aggregate formation is
349 induced by the irradiation. The formation of these aggregates may be responsible for
350 the observed decrease in the quantity of sulfhydryl groups present in the
351 β-lactoglobulin, assuming the aggregates are covalently linked. Another consideration
352 is that the aggregates are linked by other means such as hydrophobic or electrostatic
353 interactions and the observed decrease in sulfhydryls is due to some other cause. It
354 had been shown previously that goat α-lactalbumin formed aggregates when it was
355 irradiated (*7*). Even though the majority of the irradiated protein remained
356 monomeric, RP-HPLC analysis revealed that not all of these monomers were native
357 β-lactoglobulin. The formation of non-native monomers is considered to be the first
358 step in the aggregation process during heat-denaturation at pH 7.0 (24). Combining
359 the results of reverse-phase and gel permeation chromatography allowed the quantity
360 of non-native monomer to be determined, 11%. These non-native monomers could

explain the increase in the concentration of exposed sulfhydryl groups in the irradiated protein.

The non-native monomers formed during this study exhibit different characteristics to a non-native monomer formed by heat-denaturation (25). The monomers studied contained a free cysteine 119 and exhibited an increase in intrinsic fluorescence. However, photo bleaching would not occur in heat-denatured samples. More importantly no shift in the emission maximum was observed in the heat-denatured monomer, showing the environment surrounding the tryptophans had not altered. When β-lactoglobulin was heated for longer periods of time then a red-shift in intrinsic fluorescence occurred (26). When β-lactoglobulin was denatured using hydrostatic pressure the same red-shift and increase in intrinsic fluorescence was observed (27).

The FTIR results showed that the denaturation by irradiation brought about different structural changes to those that occurred during heat-denaturation. The largest difference was that light-denatured β-lactoglobulin did not form anti-parallel β-sheets. The formation of these sheets has been attributed to the formation of non-covalent linked dimers in heated samples.

This study demonstrated that UV radiation can denature β-lactoglobulin. There was evidence of two different processes occurring in irradiated β-lactoglobulin: cleavage of disulfide bonds leading to protein unfolding and aggregation and, photo-oxidation of tryptophan into *N*-formylkynurenine. Previous studies have shown that the amount of denaturation was related to the intensity and duration of the radiation (*5*), so it could be possible to denature and aggregate β-lactoglobulin to a greater extent by altering the conditions of irradiation. However, the depletion of tryptophan and the

formation of oxidation products would have to be taken into consideration before irradiated β-lactoglobulin were used as a food ingredient.

Legends for tables and figures

Table 1. Summary of results for native β-lactoglobulin A and β-lactoglobulin A irradiated for 24 hours at 295 nm

Figure 1. Absorbance and emission profiles of native and irradiated β-lactoglobulin. Excitation was at 295 nm for the emission measurements. The control measurements were made before and after irradiation to verify the stability of the lamp intensity over the measurement period. The structures show tryptophan (left) and *N*-formylkynurenine (right).

Figure 2. Gel permeation chromatography profiles for native β-lactoglobulin (black line) and irradiated β-lactoglobulin (grey line). Detection at 214 nm (A) and 321 nm (B) for the detection of *N*-formylkynurenine.

Figure 3. Reverse-phase chromatograms for the separation of tryptic digests of β-lactoglobulin. Irradiated sample (A & B) and irradiated sample not treated with IAEDANS (C & D). Chromatograms A & C were detected at 214 nm. Chromatograms B & D were detected at 336 nm.

Figure 4. (A) FTIR Spectra; vector normalized amide I bands, spectra of native, irradiated and heat-treated (10 gL^{-1}, 78°C) β-lactoglobulin are shown. (B) FTIR spectra with the spectrum of native β-lactoglobulin subtracted.

	Native	Irradiated
Exposed SH (mol SH per mol β-lg)	0.03 ± 0	0.08 ± 0.01
Total SH (mol SH per mol β-lg)	0.96 ± 0.02	0.76 ± 0.02
Native (RP-HPLC) % of total β-lg	100	82
Monomer (GPC) % of total β-lg	100	93
Non-native monomer % of total β-lg	0	11
Emission max (nm)	335 ± 1	344 ± 4
Emission area (Integration of spectrum)	100%	50%
% α-helix (by FTIR)	17.6	14.9
% β-sheet (by FTIR)	35.4	33.9

Table 1.

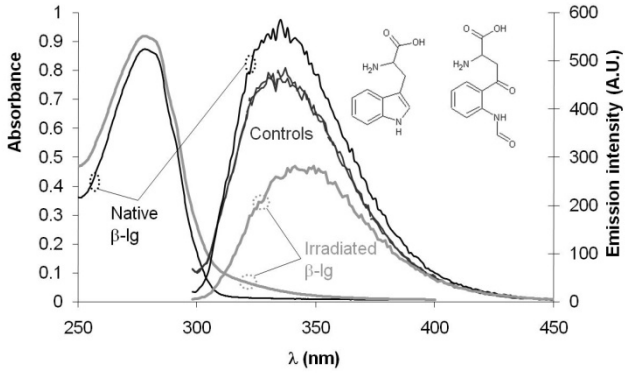

Absorbance
Emission intensity (A.U.)
λ (nm)
Native
β-lg
Controls
Irradiated
β-lg
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
600
500
400
300
200
100
0
250
300
350
400
450

Figure 1.

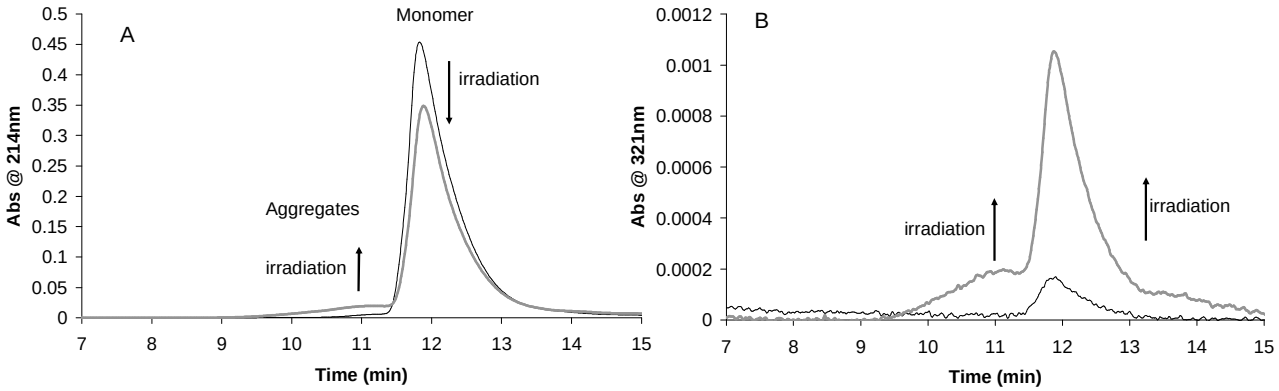
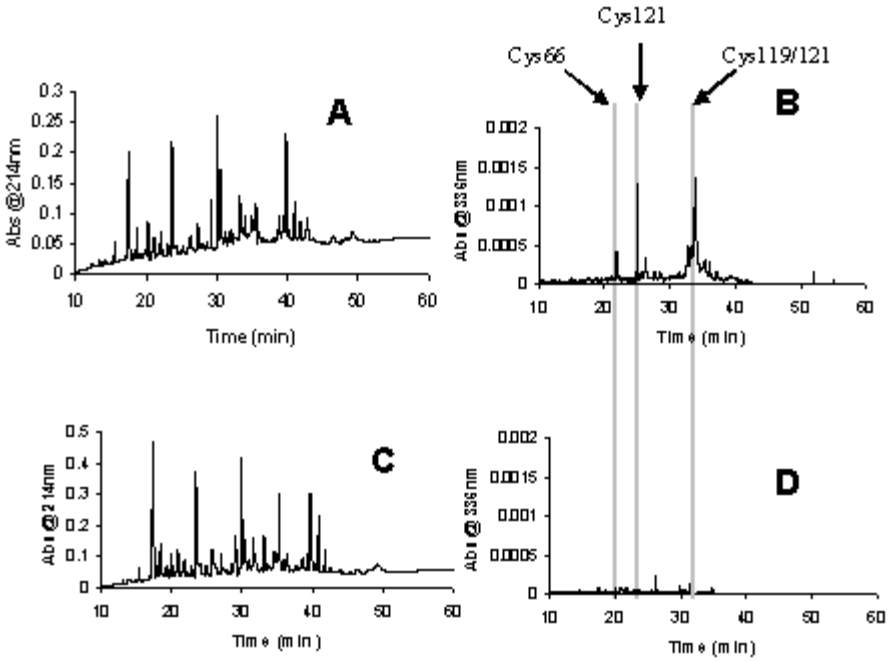

A
Monomer
irradiation
Aggregates
irradiation
Abs @ 214nm
Time (min)
B
irradiation
irradiation
Abs @ 321nm
Time (min)

Figure 2.



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465 Figure 3.

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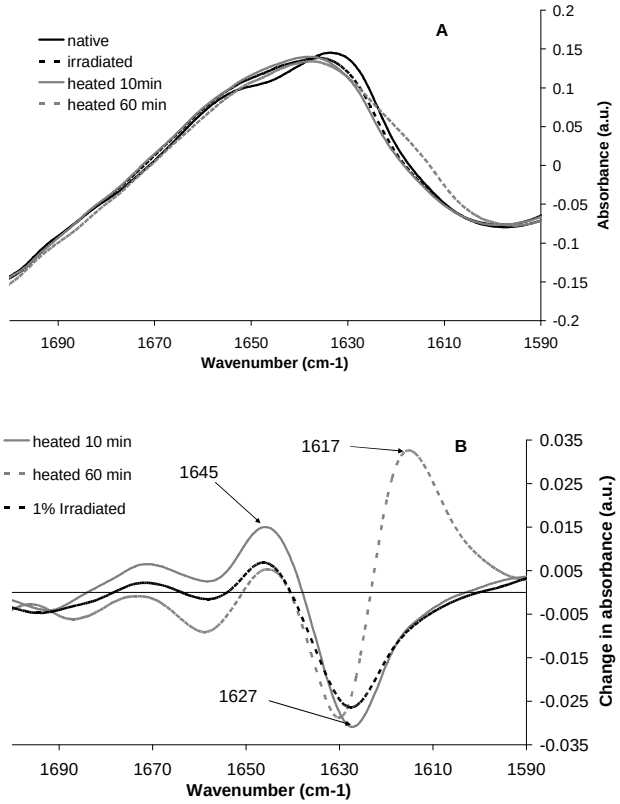
A
native
irradiated
heated 10min
heated 60 min
Absorbance (a.u.)
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.2
1690
1670
1650
1630
1610
1590
Wavenumber (cm-1)
B
heated 10 min
heated 60 min
1% Irradiated
1645
1617
1627
Change in absorbance (a.u.)
0.035
0.025
0.015
0.005
-0.005
-0.015
-0.025
-0.035
1690
1670
1650
1630
1610
1590
Wavenumber (cm-1)

Figure 4.

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