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Modification of Sheep Plasma Kininogen by Free Radicals

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Riboflavin sensitized photodynamic modifications of high molecular weight Kininogen (HMWK) isolated from sheep (Avis-arias) plasma leads to inactivation of antiproteinase activity and formation of aggregated products. A continued disappearance of the inhibitory activity towards papain and formation of high molecular weight adducts was observed with increasing concentration of riboflavin and varying time periods of incubation reaching a maximum value of over 85% (loss in activity). Aggregates resisted dissociation upon heating at 100°C in 1% SDS. Aggregation and photoinactivation of HMWK was promoted by the substitution of H_2O for deuterium oxide (D_2O), which is known to prolong the life span of singlet oxygen, and suppressed by sodium azide a known singlet oxygen quencher. Mannitol and thiourea (hydroxyl radical scavenger) did not protect the antiproteinase activity of HMWK. Treatment with reducing agent resulted in decrease of the aggregated products suggesting the possible involvement of disulfide linkages in protein crosslinking. Tryptophan fluorescence was completely lost and significant production of dityrosine was detected in photoinactivated HMWK aggregates. Changes in the far Ultra violet circular dichroism (u.v.c.d.) spectrum of HMWK was indicative of loss of secondary structure. Analysis of modifications induced in HMWK by riboflavin reveals that the processes proceed *via* a singlet oxygen mediated pathway. It is concluded that the susceptibility of HMWK to oxidation may arise from oxidative modifications by reactive oxygen species generated in plasma.

Keywords: Kininogen; Cysteine proteinase inhibitor; Aggregation; Inactivation; Singlet oxygen

INTRODUCTION

Kininogens are multifunctional glycoproteins present in plasma and secretions of mammalian species.[1–3] In human and bovine, two forms of kininogens (*H-kininogen*, high molecular weight kininogen or HMWK and *L-kininogen*, low molecular weight kininogen or LMWK) and several other species are present.[2,3] HMWK is composed of a N terminal heavy chain. The heavy chain and kinin segment of HMWK can be subdivided into four domains, the cystatin like domains D1, D2 and D3, and the kinin segment D4. The light chain of HMWK can be subdivided into D5 and D6.[4–6] D1 does not inhibit proteases. D2 and D3 contain the conserved pentapeptide QVVAG, responsible for proteinase inhibition, which is also found in class II cystatin and stefins.[7–9] The procoagulent activity of HMWK resides exclusively in D5 and D6.[7–10]

Functional changes have frequently been suggested to occur in proteins with oxidation-sensitive amino acid residues like cysteine, methionine, histidine, and tryptophan located at or near the sites of specific interaction in proteins.[11] Oxidative modification of HMWK markedly reduces kinin production when exposed to plasma or tissue kallikreins.[12] Anastassios *et al.*[13] reported that chemical modification of the light chain of HMWK affects the procoagulent activity, surface binding and the binding of protein with zinc indicating that histidine imidazoles are involved in surface and zinc binding, free carboxyl and tryptophan aromatic side groups participate in protein binding. Chemical modification of Trp-104 markedly affects the binding of chicken cystatin to papain.[14] Chicken cystatin forms a tight equimolar complex (kd ~ 60 fm) with papain, blocking the active site of the enzyme.[15] From computer docking experiments three regions

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of the chicken cystatin are suggested to interact with the enzyme, namely the segment of the chain around Gln-Leu-Val-Ser-Gly sequence at residues 53–57, and the loop around Trp-104 of the inhibitor.[16]

Flavins are known to photooxidize amino acids and also effect the conformation of proteins.[17–20] Riboflavin is known to generate singlet oxygen, hydroxyl radical and flavin triplet state on photoillumination.[21] The present work was carried out to examine riboflavin mediated photodynamic modifications of HMWK to elucidate the location of the reactive site, types of ROS involved and various structural changes associated with them. Our studies indicate that the oxidation of tryptophan residues of HMWK inactivate this inhibitor towards papain. Spectroscopic characterization of photoinactivated HMWK aggregates have shown marked difference relative to native protein. Singlet oxygen and flavin triplet state were found to be involved in these modifications by the use of various scavengers and enhancer. The present findings indicate dityrosine formation in HMWK on exposure to riboflavin. We present a comprehensive characterization of these changes by various spectroscopic methods.

MATERIALS AND METHODS

Papain, Trypsin, Phenylmethylsulphonylflouride (PMSF), Ethylene diamine-tetraacetic acid (EDTA), Casein, Uric acid, Thiourea, Mannitol, Sodium azide, Ascorbic acid, Potassium iodide, Riboflavin, Casein, Sephacryl-S300HR, D_20 were from Sigma Chemical Company (St. Loius, MO). High molecular weight protein markers were from Genie India Limited. All the other reagents were of highest purity commercially available.

Purification of HMWK

HMWK was purified from sheep plasma. Sheep blood was routinely collected at slaughter (within few minutes after the animal were killed) in plastic bottles containing 1/10 volume of acid citrate dextrose containing 1 mM PMSF/STI (50 mg/l of blood). HMWK was purified in high yield by a simple two-step procedure. Protein precipitated between 40 and 60% saturation of ammonium sulphate was dialyzed against 50 mM sodium phosphate buffer pH 7.5. This was subsequently chromatographed on Sephacryl-300 (82 × 1.5) in the same buffer. The fraction containing inhibitory activity against papain were pooled and concentrated. The purified protein gave single band on 4.5% native polyacrylamide gel electrophoresis (PAGE) and 4.5% SDS-PAGE. Molecular weight of HMWK estimated by SDS-PAGE was 80 kd.

Reaction of Photosensitized Riboflavin with HMWK

HMWK (500–600 nmoles) was photoilluminated with increasing concentration of riboflavin (5–40 μM) in a final volume of 1000 μl at room temperature (28–30°C). Riboflavin was freshly prepared at 2 mM concentration in 50 mM sodium phosphate buffer pH 7.5. HMWK was photoilluminated for different time intervals (0–60 min) with 40 μM riboflavin in a final reaction volume 1000 μl at room temperature. Further, HMWK was incubated with riboflavin for different time intervals in which H_2O was replaced by D_2O. The light incubations were done by setting tubes at a distance of 1 cm from 40 W cool fluorescent lamp. The light intensity was 0.768 milliwatt at a distance of 1 cm measured by a Model 351A-Powermeter.

Riboflavin-HMWK Interaction in the Presence of Scavengers and Antioxidants

HMWK (500–600 nmoles) was incubated with 40 μM riboflavin for 1 h along with sodium azide, potassium iodide, thiourea, mannitol, uric acid and ascorbic acid.

HMWK Antiproteolytic Activity

The inhibitory activity of HMWK against papain was monitored by the method described by Kunitz.[22]

SDS Polyacrylamide Gel Electrophoresis

HMWK (500 nmoles) treated with increasing concentration of riboflavin and different time intervals of incubation with riboflavin were examined by SDS-PAGE using 4.5% gel essentially as outlined by Laemmli[23] using tris-glycine buffer pH 8.3. Different scavengers and antioxidants were used in the incubation mixture of HMWK and riboflavin.

Fluorescence Measurements

Fluorescence measurements were performed with Hitachi F-2000 spectrofluorimeter with a 10 nm bandwidth for emission. The emission spectra of the inhibitor at 25°C in the region 300–400 nm were recorded (protein concentration 500–600 nmoles) after exciting at 280 nm.[24] Tryptophan oxidation of protein incubated with riboflavin was monitored by loss of fluorescence peaks. Fluorescence emission spectra of HMWK incubated with riboflavin in presence of sodium azide, potassium iodide, thiourea, mannitol, ascorbic acid and uric acid were taken to determine the protective effects on tryptophan residues.

Dityrosine Formation

Dityrosine was assessed with intrinsic fluorescence spectroscopy.[25] To investigate the dityrosine linkages involved in aggregation oxidized HMWK (500–600 nmoles) was concentrated and subjected to trypsin (10 μg/ml) digestion for 24 h at 37°C and precipitated by a one third dilution (v/v) with 1.6 M perchloric acid (PCA). Following centrifugation at 4000*g* and 61620*g* the supernatants were neutralized with 2 M NaOH. The neutralized PCA supernatants were next subjected to HPLC separation on a Bond pack C-18 column (5 μm : 4.6 × 25 cm). HPLC conditions were as follows: flow rate 0.8 ml/min, solvent A: methanol/water (25/75, v/v), solvent B: acetonitrile/water (80/20, v/v) and both solvents contained 0.1% (v/v) trifluoroacetic acid. The elution program consisted of solvent A (100%) from 0 to 10 min, solvent B at a gradient from 0 to 90% during 10–20 min; solvent B (100%) from 20 to 30 min. In order to detect dityrosine the fluorescence excitation wavelength was 284 nm and the emission wavelength was 410 nm characteristics for dityrosine. Retention time was detected on comparison with authentic dityrosine standards, prepared as described.[26]

CD Spectroscopy

Far-u.v.c.d. measurements were made with Jasco-810 spectropolarimeter, calibrated with 0.1%d-10 camphor sulphonic acid solution. The spectra were recorded with a scan speed of 50 nm min^{-1} with a response of 2 s. Each spectrum was recorded as an average of two scans. Changes in the secondary structure of HMWK were monitored between 200 and 250 nm with a protein concentration of 500–600 nmoles in a 1 mm path length cuvette.[27]

Samples for all spectroscopic measurements were photoilluminated with 40 μM riboflavin for 1 h and were extensively dialyzed against 50 mM sodium phosphate buffer pH 7.5 before taking the spectra.

UV Absorption Spectra

Absorption spectra were carried out on Cintra 5 spectrophotometer at 28°C. The protein concentration for absorbance measurement was between 1.30 and 1.50 μmoles. Protein estimation was carried out by the method of Lowry *et al.*[28]

RESULTS

Riboflavin Induced Inactivation and Aggregation of HMWK

To directly quantitate the effects of riboflavin exposure on HMWK functions, its antiproteolytic potential was assessed by the caseinolytic assay with papain. As shown in Table I exposure of HMWK (500 nmoles) with increasing concentration of riboflavin (0–40 μM) results in rapid decrease of inhibitory activity (85% loss) towards papain. Similarly increase in length of exposure (0–60 min) of HMWK (500 nmoles) with riboflavin (40 μM) results in loss of antiproteolytic activity towards papain (Fig. 1). Samples of both native and treated inhibitor were analyzed by non-reducing SDS-PAGE. The detrimental effects of photodynamic action of riboflavin on HMWK are shown in Figs. 2 and 3. The untreated inhibitor moves on SDS-PAGE 4.5% as a single band which still retains the original mobility on SDS-PAGE, but decrease in band intensity as well as increase of aggregated products was observed with increasing concentration of riboflavin and increasing time periods of incubation with riboflavin. The diverse distribution pattern of aggregated products after SDS-PAGE suggests that molecular weight of HMWK has been modified. Untreated HMWK on SDS PAGE has a molecular weight of 80 kd and aggregated products were 200 kd as determined in the presence of markers (Fig. 4).

Involvement of Singlet Oxygen in Inactivation and Aggregation

Riboflavin on photoillumination generates singlet oxygen, hydroxyl radicals and flavin triplet state.[21] Free radical scavengers and enhancer were used to determine the type of ROS involved. In order to identify whether singlet $^1O_2$ is involved in the aggregation and inactivation, photooxidation reaction was carried out in H_2O and D_2O media to compare the extent of cross linking and inactivation in these media. Since the life time of singlet $^1O_2$ is approximately 10 times shorter in H_2O than in D_2O.[29] Therefore reactions involving singlet $^1O_2$ are enhanced in D_2O. As shown in Fig. 1 the rate of HMWK aggregation was enhanced in D_2O. Aggregated products were formed within 5 min of incubation and concomitant loss of native bands were also enhanced. HMWK lost its caseinolytic activity towards papain within 10 min of incubation

TABLE I Loss of HMWK antiproteolytic activity after treatment with increasing concentration of riboflavin

Conditions	HMWK activity "units"
Native HMWK	80 ± 7.8
HMWK+5 μM riboflavin	45 ± 2.6
HMWK+10 μM riboflavin	30 ± 2.6
HMWK+20 μM riboflavin	21 ± 1.5
HMWK+30 μM riboflavin	19 ± 1.6
HMWK+40 μM riboflavin	10 ± 1

Concentration of riboflavin shown is as in the final reaction mixture. All incubations were in light for 1 h (28°C) in sodium phosphate buffer pH 7.5. HMWK activity was assayed by the method of Kunitz.[22] Results represent the mean ± SEM calculated from four separate experiments.

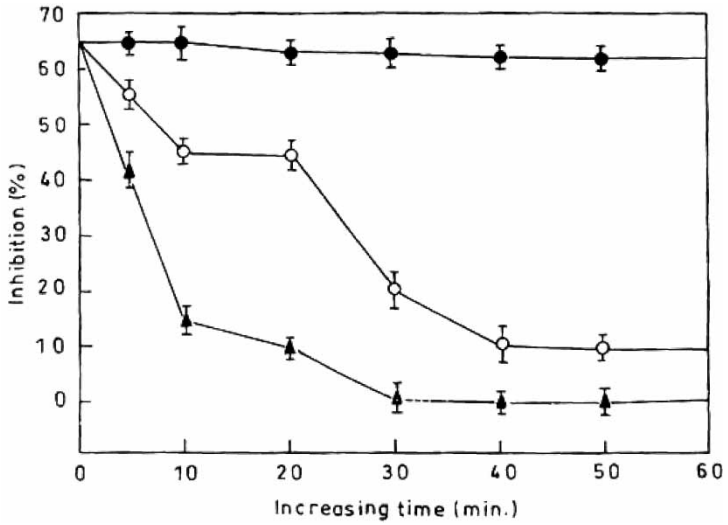

FIGURE 1 Native HMWK (500 nmoles) was incubated with riboflavin (40 μM) for increasing time periods (0–60 min) in a final volume of 1 ml at room temperature under fluorescent light. (○) HMWK was assayed for loss in anti-proteinase activity by caseinolytic assay. (▲) Another sample of HMWK was incubated in the presence of riboflavin for increasing time periods in D_2O. (●) Untreated HMWK is shown for comparison purposes. Values are means of four independent determinations.

(75% loss) by the substitution of D_2O for H_2O (Fig. 1). Further support for the role of singlet $^1O_2$ in photodynamic modification of HMWK is based on the fact that photoinactivation was greatly inhibited by sodium azide (25 mM), which is supposed to be a good quencher of singlet $^1O_2$[30] whereas the presence of mannitol and thiourea, hydroxyl radical scavengers[30] on inactivation has less effect. Protection of antiproteinase activity by KI, a triplet state quencher[30] (Table II) is taken as an evidence for the involvement of triplet flavin state.[30] Uric acid and ascorbic acid which are known as biological antioxidants[31–34] were also used to determine their protective effects against photodynamic modifications. Among biological antioxidants only ascorbic acid was able to protect antiproteinase activity effectively whereas uric acid was not able to fully protect the antiproteinase activity (Table II). No aggregated products were observed in treated HMWK in the presence of sodium azide, potassium iodide, thiourea, mannitol and ascorbic acid. However in the presence of uric acid, inhibition of aggregation was less effective (Fig. 5a). These results collectively suggest that HMWK photoinactivation and aggregation proceeds mainly *via* a singlet $^1O_2$ pathway.

Tryptophan Oxidation of HMWK by Singlet Oxygen

The fluorescence emission spectrum of riboflavin treated HMWK was completely quenched in the emission range 300–400 nm when excited at 280 nm, compared with untreated protein which had emission maximum at 347 nm (Fig. 6). This was consistent with tryptophan oxidation. Sodium azide, potassium iodide and ascorbic acid, resulted in maximum retention of fluorescence emission with a slight decrease in energy. However the protective effects of mannitol, thiourea and uric acid were less pronounced (Table III). The protective effects verify the involvement of singlet oxygen and flavin triplet state in tryptophan oxidation.

Dityrosine Formation

The stability of HMWK aggregates was characterized by the reduction with 2-mercaptoethanol (reducing agent). There was a considerable decrease in aggregated products on reduction confirming

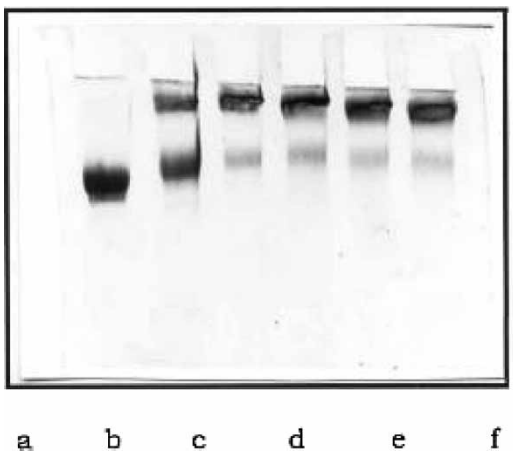

FIGURE 2 SDS polyacrylamide gel electrophoresis of HMWK (500 nmoles) incubated with different concentrations of riboflavin (0–40 μM) for 1 h. Lane a contains untreated HMWK, Lanes b, c, d, e, f, g contain HMWK incubated with 5, 10, 20, 30, 40 riboflavin for 1 h.

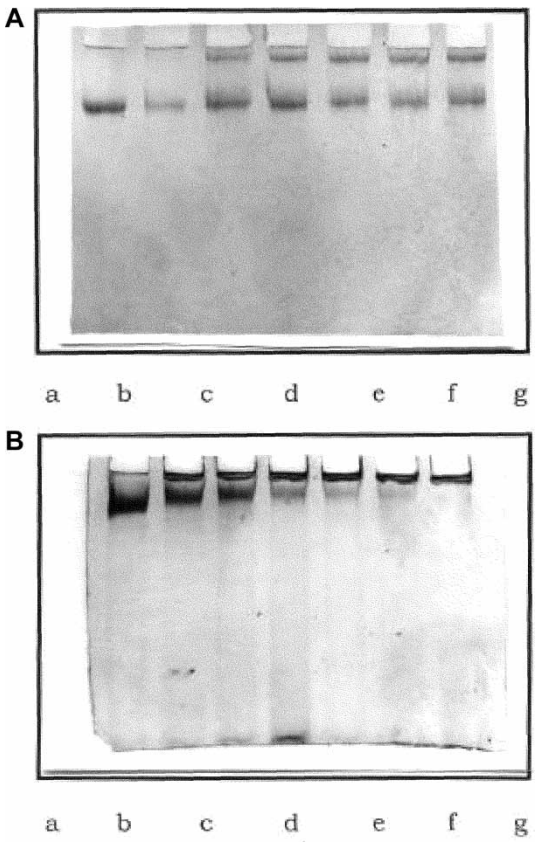

FIGURE 3 (A) HMWK (500 nmoles) was incubated with riboflavin (40 μM) for different time intervals. SDS polacrylamide gel electrophoresis (4.5%) was conducted. Lane a contains untreated HMWK, lanes b, c, d, e, f, g contain HMWK incubated with riboflavin for 5, 10, 20, 30, 40, 60 min. (B) HMWK (500 nmoles) was incubated with riboflavin (40 μM) in D_20 for different time intervals. Aggregates were formed within 5 min of incubation. SDS polyacrylamide gel electrophoresis was conducted. Lane a contains untreated HMWK, lanes b, c, d, e, f, g contain HMWK incubated with riboflavin for 5, 10, 20, 30, 40, 60 min.

the involvement of disulphide linkages (data not shown). To further characterize the nature of covalent linkages in aggregates, dityrosine cross linkages were assessed by intrinsic fluorescence spectroscopy and HPLC analysis. There was an increase in emission of oxidized protein in the range 410–420 nm when excited at 325 nm (Fig. 7), which is an indication of dityrosine formation. HPLC analysis of aggregated HMWK was done to confirm the involvement of dityrosine linkages in aggregation. Oxidized HMWK was incubated with trypsin and subjected to PCA precipitation. Trypsin and chymotrypsin are known to produce 3–4 fold increases in dityrosine release.[36] PCA soluble peptides after a series of HPLC steps provide a peptide with a fluorescence spectrum characteristic of dityrosine (retention time after 10 min) (Fig. 8a and b). Therefore cross-linking through dityrosine appears also to be responsible for the aggregation of HMWK after exposure to the oxidizing agent.

Loss of Secondary Structure and Unfolding of the Protein

Changes in the secondary structure were followed by measuring the c.d. spectrum in far-u.v. region. Photodynamic modifications significantly altered the far-u.v.c.d. spectra of native HMWK. Judging from the ellipticties at 222 nm, which reflects the α helical content of protein,[35] increasing time periods of incubation of HMWK with oxidizing agent leads to a significant loss of α helicity in the protein (Fig. 9). The changes of ellipticity at 222 nm is usually supposed to be associated with the change of the secondary structure of the polypeptide chain[35] i.e. they reflect the change of the conformation of the entire molecule. Far-u.v.c.d. spectrum of treated HMWK is changed completely to random coil by one week of storage at 4°C (results not shown). UV absorption spectra of treated HMWK relative to intact protein suffered a blue shift to 275 nm with an increase in energy (Fig. 10).

DISCUSSION

Proteins are critical targets of reactive oxygen species because they are present inside and outside the cell in high concentration and since many are catalytic in nature, modifications by free radicals can have an amplified affect on their activity i.e. susceptibility to proteolysis, inactivation and aggregation. Davies[37] reported that proteins, which have been exposed to certain radicals, exhibit altered primary, secondary and tertiary structure, and can

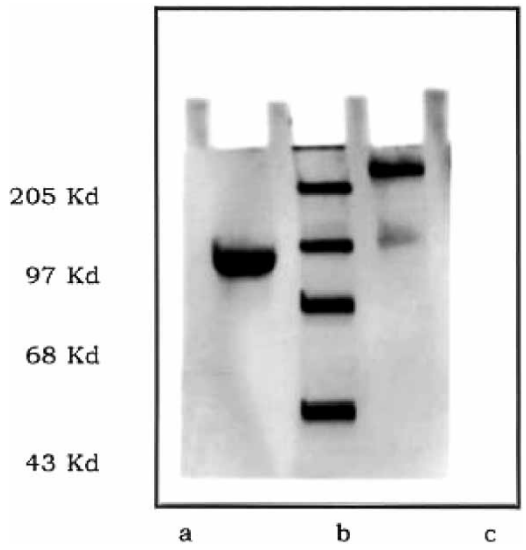

FIGURE 4 HMWK (500 nmoles) was incubated with riboflavin (40 μM) for 1 h. Molecular weight of aggregates was determined in the presence of markers. Lane a corresponds to untreated HMWK. Lane b consists of markers; Lane c, consists of HMWK treated with riboflavin.

undergo spontaneous fragmentation or increased proteolytic susceptibility.[38]

In vivo conditions large amounts of oxidants are released from phagocytes at the inflammatory sites in the host-defense response against invading microorganisms. Most of this activity is attributed to macrophages, neutrophils[39] mast cells[40] and release of reactive oxygen species.[41] All of these inflammatory oxidative processes are primarily used to kill foreign organisms. However host proteins that are sensitive to oxidation e.g. α_{1-} PI and plasminogen activator inhibitor-1 may also be affected so that they are no longer functional.[42,43] HMWK like other proteins could be inactivated by reactive oxygen species. HMWK are multipurpose proteins that are involved in kinin generating pathway, blood coagulation cascade, inhibitor defense system and acute phase response.[1,2] Previous studies show that chemical modifications of the histidine residues in HMWK resulted in over 90% loss in procoagulent activity.[12] Oxidation of methionine residues in HMWK markedly reduces kinin production when exposed to plasma or tissue kallikrein.[13] For the study of HMWK properties, HMWK from sheep plasma was isolated by simple two-step purification procedure coupled with its resemblance to human HMWK in quaternary structure, proteinase inhibition and other properties[2] it was selected for further characterization.

In the present investigation it has been shown that sheep plasma HMWK is susceptible to reactive oxygen species generated by photosensitized riboflavin,[21] which mimics biological exposure to oxygen radicals.[44] HMWK susceptibility was measured by its ability to be used as proteinase inhibitor after photodynamic modification. Photodynamic actions of various sensitizers have long been applied to delineate structural and functional properties of large number of enzymes and other biologically active proteins.[45,46] Incubation of HMWK with increasing concentration of riboflavin (Table I) and varying time periods of incubation with riboflavin inactivates its antiproteinase activity towards papain (Fig. 1) owing to destruction of active site tryptophan residues. Tryptophan fluorescence was completely lost and a decrease in fluorescence intensity was observed in HMWK on incubation with riboflavin (Fig. 6). Loss of tryptophan fluorescence results from both tryptophan oxidation and conformational change. There was clear protection of tryptophan residues from oxidation, observed by retention of native tryptophan fluorescence in the presence of various scavengers and antioxidants (Table III). The utilization of scavengers, quenchers and enhancers is the method

TABLE II Percent residual HMWK activity after treatment with riboflavin (40 μM) in the presence of scavengers and antioxidants

Conditions	Percent HMWK activity
Native HMWK	100 ± 2.8
HMWK+40 μM riboflavin	20 ± 1
HMWK+Sodium azide (25 mM)	116 ± 7.8
HMWK+Potassium Iodide (25 mM)	80 ± 3.1
HMWK+Thiourea (25 mM)	54 ± 36
HMWK+Mannitol (25 mM)	60 ± 1.2
HMWK+Uric acid (25 mM)	48 ± 2.4
HMWK+Ascorbic acid (100 mM)	98.1 ± 2.0

Concentration of scavengers shown is as in the final reaction mixture. All incubation were in light for 1 h (28°C) in 50 mM sodium phosphate buffer pH 7.5. HMWK activity was assayed as described by Kunitz.[22] Results represent the mean ± SEM calculated from four independent experiments and are expressed as a percentage of the HMWK activity.

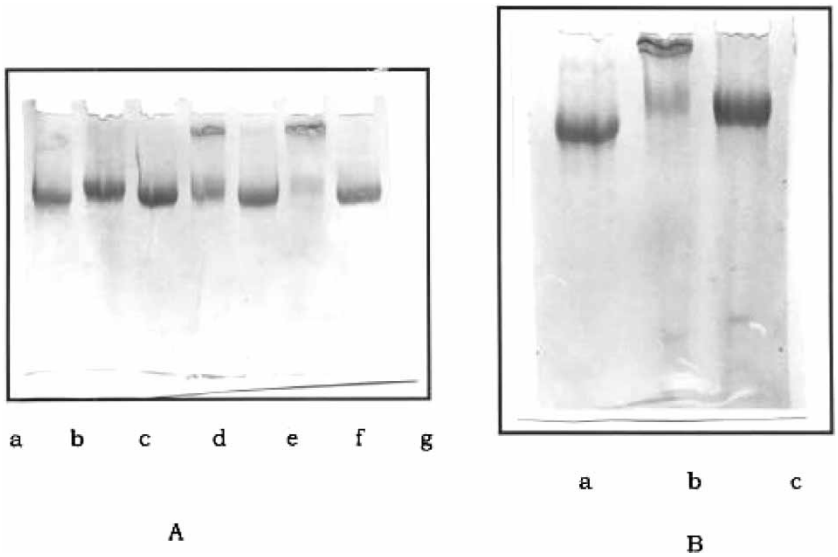

FIGURE 5 (A) HMWK (500 nmoles) incubated with 40 μM riboflavin along with antioxidants and scavengers (25 mm) in a final volume of 1 ml for 1 h. Lane a contains untreated HMWK, lanes b, c, d, e, f, g, contain HMWK treated with riboflavin along with thiourea, mannitol, uric acid, sodium azide and potassium iodide, respectively. Lane f contains riboflavin treated HMWK. (B) Lane a contains untreated HMWK. Lane b contains HMWK incubated with riboflavin; lane c consists treated HMWK along with ascorbic acid (100 mM).

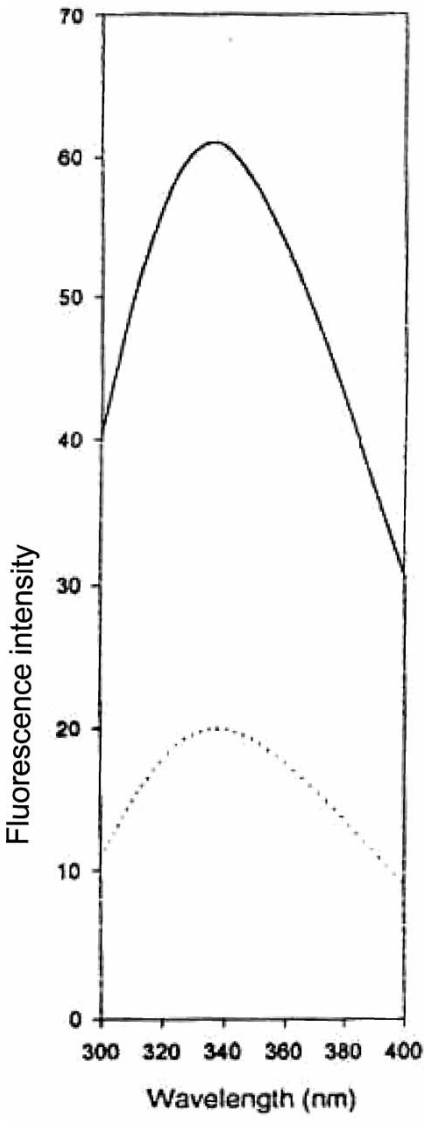

FIGURE 6 The fluorescence spectra of untreated and riboflavin treated HMWK excited at 280 nm in the emission range (300–400 nm) with a slit bandwidth of 10 nm. Fluorescence studies were performed at pH 7.5 with 50 mM sodium phosphate buffer. (—) The emission spectrum of untreated HMWK. (. . .) The emission spectra HMWK incubated with riboflavin.

most often used to investigate the role of ROS in photosensitization reaction.[47] In order to determine the type of ROS involved in inactivation and aggregation of HMWK the mechanism was investigated by the use of enhancer D_20 that increase the life span of singlet $^1O_2$.[29] Sodium azide and potassium iodide that are known to quench singlet $^1O_2$ and flavin triplet state, respectively[30] were also used. The rate of HMWK photoinactivation and aggregation was enhanced in D_2O (Figs. 1 and 3b) and decreased in the presence of sodium azide and potassium iodide (Fig. 5, Table II). Singlet $^1O_2$ and flavin triplet state are known to be involved in polymerization of proteins.[48–50] Effects of mannitol and thiourea (known to eliminate hydroxyl radicals)

TABLE III Retention of Tryptophan fluorescence in the presence of different scavengers and antioxidants

Conditions	% Retention of tryptophan fluorescence
HMWK+Sodium azide (25 mM)	83
HMWK+Potassium iodide (25 mM)	71
HMWK+Thiourea (25 mM)	45
HMWK+Mannitol (25 mM)	50
HMWK+Uric acid (25 mM)	40
HMWK+Ascorbic acid (100 mM)	79

HMWK (500–600 nmoles) incubated with 40 μM riboflavin for 1 h. Results are percentage retention of tryptophan fluorescence emission intensity (which is dependent upon, but not synonymous with the number of tryptophan residues) measured at 280 nm excitation and 300–400 nm emission with a slit width of 10 nm. Fluorescence studies were performed at pH 7.5 with 50 mM sodium phosphate buffer. Values are means of four independent determinations for which standard errors were less than 10%.

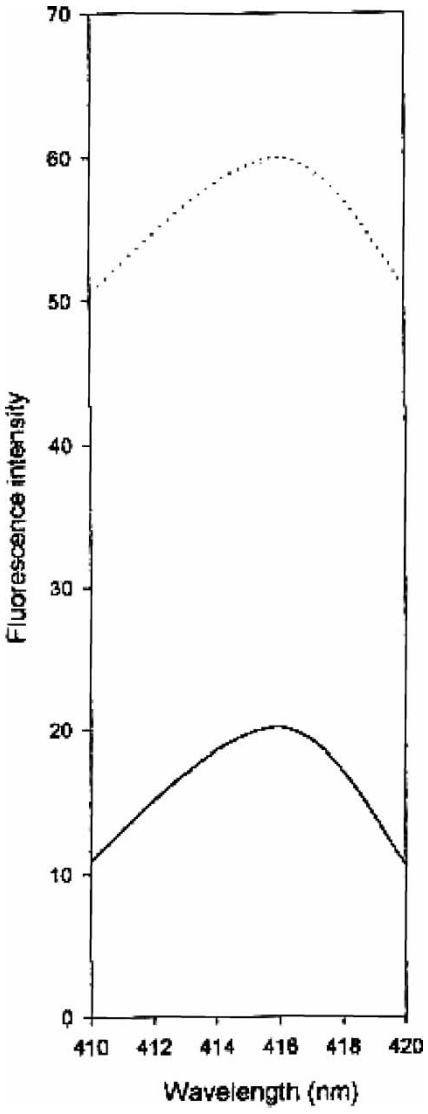

FIGURE 7 Fluorescence emission spectra of untreated HMWK (500 nmoles) and riboflavin treated HMWK were excited at 325 nm and emission in the range of 410–420 nm for dityrosine formation. (—) Corresponds to fluorescence emission spectra of native HMWK. (. . .) Emission spectra of HMWK treated with riboflavin.

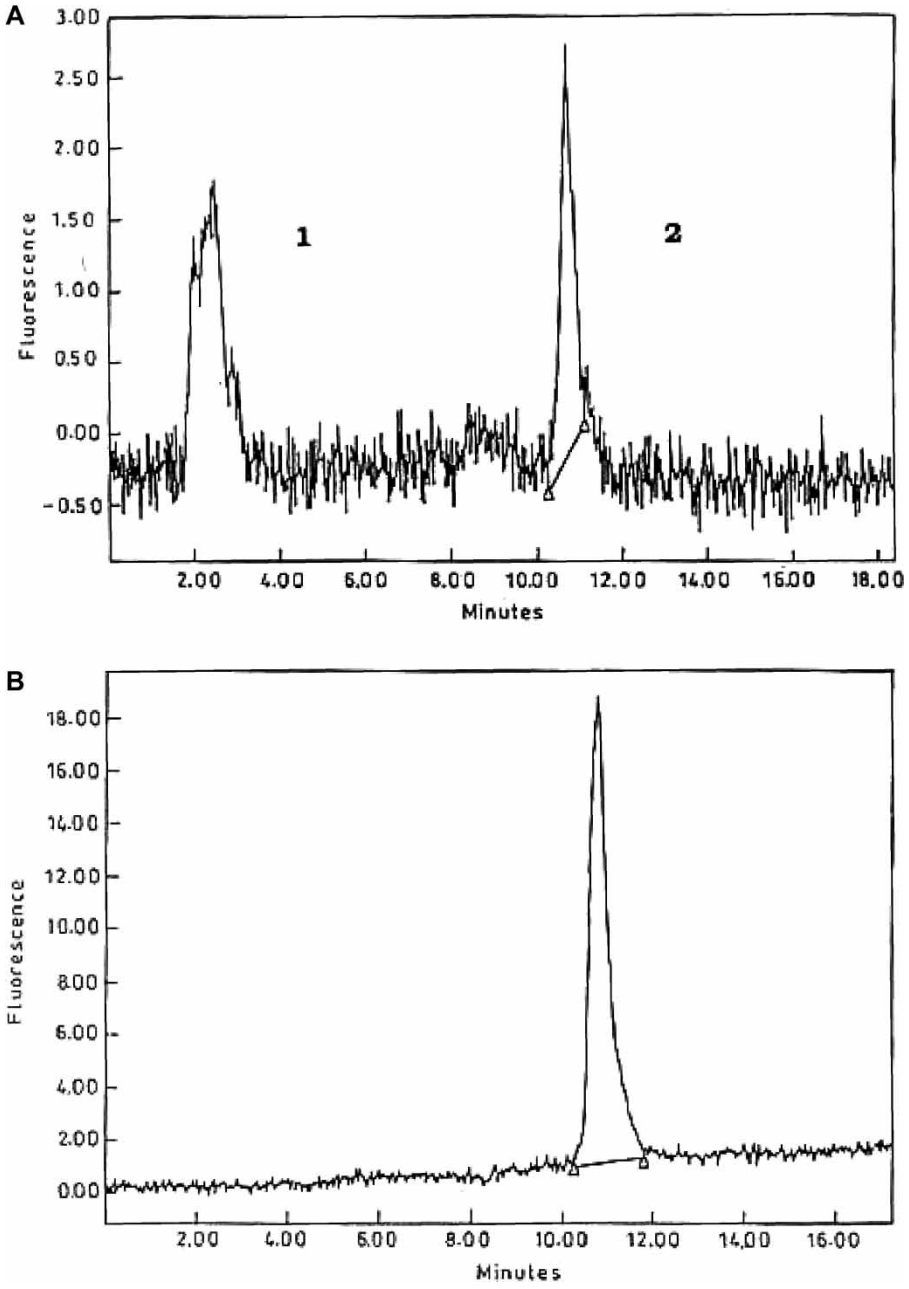

FIGURE 8 (A, B) HPLC of riboflavin treated with HMWK. HMWK (500 nmoles) treated with riboflavin were incubated with proteolytic enzyme Trypsin (10 μg/ml) for 24 h before undergoing PCA precipitation and NaOH neutralization as described under "Materials and Methods Section". The results shown in both trace A and trace B are representative chromatograms from multiple experimental incubations and HPLC separations. Trace A shows a chromatogram obtained by trypsin digestion and PCA precipitation of oxidized HMWK. Peak 2 corresponds to dityrosine which was confirmed by elution of dityrosine standard and fluorescence spectrum $\lambda_{ex} = 284$ nm; $\lambda_{em} = 410$ nm (Trace B Dityrosine standard).

on photoinactivation was less pronounced as compared to sodium azide and potassium iodide (Table II). Retention of inhibitory activity in treated HMWK was pronounced in the presence of ascorbic acid (Table II), which is a known biological antioxidant and is important in lungs and at sites of inflammation.[51,52] Uric acid also a known biological antioxidant however was not able to effectively inhibit photoinactivation (Table II). This is attributed to the fact that uric acid can generate uric acid radicals with certain oxidizing species, that are capable of doing biological damage[53] e.g. by inactivating certain enzymes. These findings indicate HMWK inactivation and aggregation proceed *via* singlet oxygen pathway.

The formation of protein aggregates during incubation with riboflavin could be due to the formation of intermolecular disulfide bridges (HMWK are known to consist of cysteine residues), exposure of hydrophobic regions on the protein following the perturbation of tryptophan residues, or the dityrosine cross linkages. Exposure to

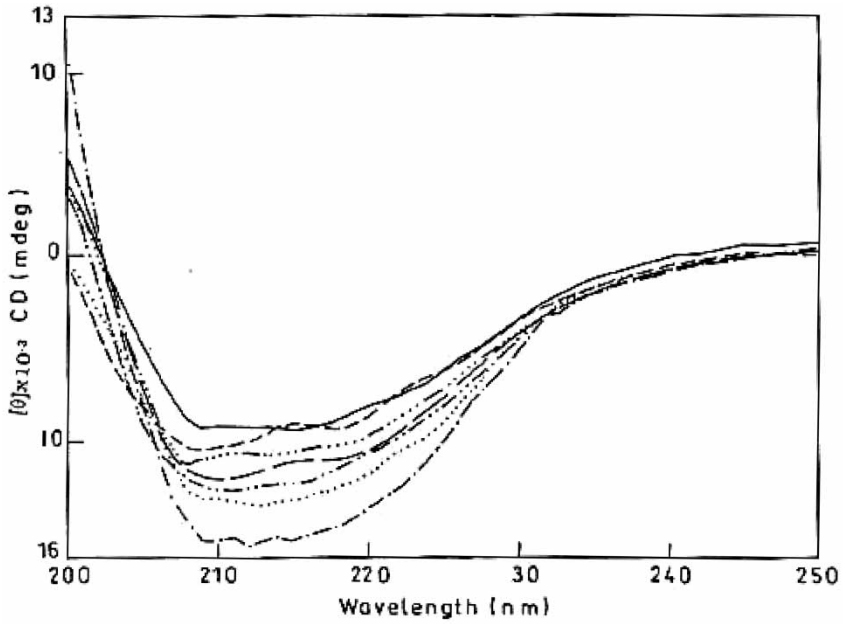

FIGURE 9 Far u.v.c.d. spectrum of HMWK exposed to timed intervals of riboflavin incubation. The time intervals for curves were 0, 10, 20, 30, 40, 50, 60 min for the lines from bottom to top, respectively.

2-mercaptoethanol results in considerable decrease of protein aggregates on SDS polyacrylamide gel electrophoresis indicating that inter-molecular disulfide bonds formed the aggregates. Our data indicate that dityrosine is formed during exposure of HMWK to oxidizing agent, observed by increase in emission at 410–420 nm when excited at 325 nm (Fig. 7). Dityrosine, which is known to be resistant to proteolysis since the bond formed, is 3′–3′ carbon–carbon bonds between the heterocyclic rings of two tyrosyl species. The 3′–3′ carbon bond of dityrosine is stable to hydrolysis by all other lytic enzymes.[36] Peptides with characteristic fluorescence of dityrosine were identified by HPLC analysis in oxidized HMWK (Fig. 8a). No aggregated products were observed when various scavengers and antioxidants were used. However uric acid was not able to prevent aggregation effectively in treated HMWK (Fig. 5a, Lane d).

Photoincubation of HMWK profoundly alters the far-u.v.c.d. spectrum. The c.d analysis of treated protein indicated that with increasing time periods of incubation there is decrease in the amount of α helix (Fig. 9). Simultaneously there is concomitant

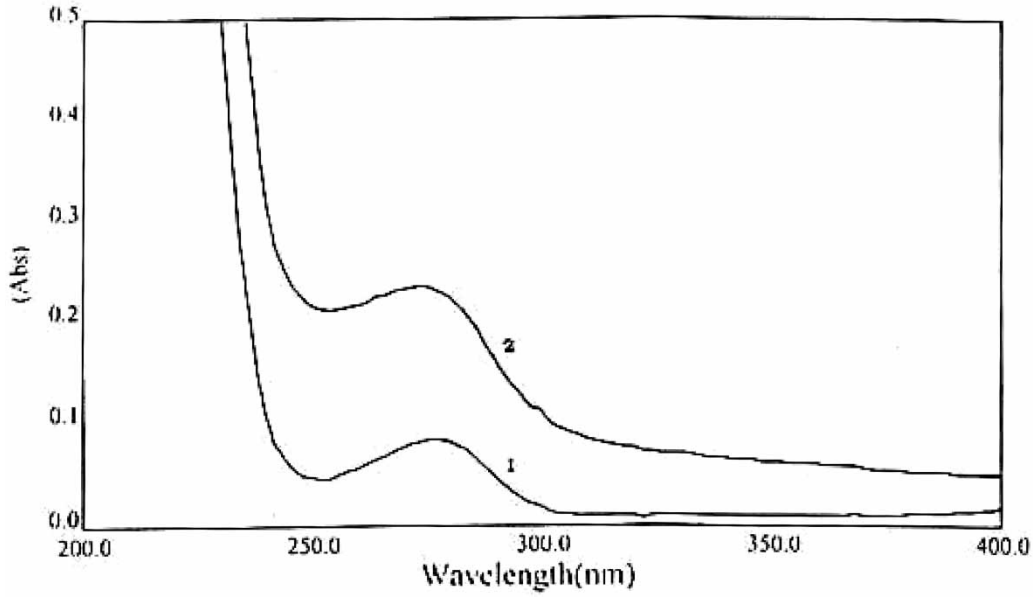

FIGURE 10 The UV spectra of native and treated HMWK were taken as described in the "Materials and Methods Section". Trace 1 is the absorption spectrum of riboflavin, trace 2 corresponds to absorption spectrum of riboflavin treated HMWK.

decrease in the inhibitory activity of the protein (Fig. 1). Furthermore the blue shift observed in the UV absorption spectra is generally considered to be a modification near the tryptophan environment, which lies to be in the interior of the protein.[54] (Fig. 10).

From these studies it appears that HMWK could be damaged by reactive oxygen species *in vivo*, which is evident, by the fact *in vitro*. HMWK is susceptible to oxidation and subsequently looses its ability to be used as a proteinase inhibitor. HMWK has been found in oxidized form in both human rheumatoid synovial fluid[54] and in lung washings from cigarette smokers.[55] Loss of antiproteinase activity by reactive oxygen species further suggests that there can be an imbalance of proteinase-antiproteinase activity during certain inflammatory disease, such as gout and arthritis, which may unleash a whole cascade of tissue destruction. The result of these experiments also allows certain conclusion concerning the reactive site of HMWK. It clearly shows that tryptophan residues are located in or near the proteinase-binding site. Earlier it had been reported that in chicken cysatin Trp-104 is directly or indirectly involved in the interaction with proteinase.[14] The position of tyrosine residues forming dityrosine crosslinkages, and tryptophan residue in HMWK involved in interaction with the proteinase inhibitor needs to be investigated.

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