3-HYDROXYKYNURENINE–MEDIATED MODIFICATION OF HUMAN LENS PROTEINS: STRUCTURE DETERMINATION OF A MAJOR MODIFICATION USING A MONOCLONAL ANTIBODY

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Running title: Protein modification by 3-hydroxykynurenine

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Tryptophan can be oxidized in the eye lens by both enzymatic and non-enzymatic mechanisms. Oxidation products, such as kynurenines, react with proteins to form yellow-brown pigments and cause covalent crosslinking. We generated a monoclonal antibody against 3-hydroxykynurenine (3OHKYN)-modified keyhole limpet hemocyanin and characterized it using 3OHKYN-modified amino acids and proteins. This monoclonal antibody reacted with 3OHKYN-modified N’-acetyl lysine, N’-acetyl histidine, N’-acetyl arginine and N’-acetyl cysteine. Among the several tryptophan oxidation products tested, 3OHKYN produced the highest concentration of antigen when reacted with human lens proteins. A major antigen from the reaction of 3OHKYN and N’-acetyl lysine was purified by reversed phase HPLC, which was characterized by spectroscopy and identified as 2-amino-3-hydroxy[(5S)-5-acetamino-5-carboxypentyl amino]-oxo-benzene butanoic acid. Enzyme digested cataractous lens proteins displayed 3OHKYN-derived modifications. Immunohistochemistry revealed 3OHKYN modifications in proteins associated with the lens fiber cell plasma membrane. The low molecular products (<10,000 Da) isolated from normal lenses after reaction with glucosidase followed by incubation with proteins generated 3OHKYN-derived products. Human lens epithelial cells incubated with 3OHKYN showed intense immunoreactivity. We also investigated the effect of glycation on tryptophan oxidation and kynurenine-mediated modification of lens proteins. The results showed that glycation products failed to oxidize tryptophan or generate kynurenine modifications in proteins. Our studies indicate that 3OHKYN modifies lens proteins independent of glycation to form products that may contribute to protein aggregation and browning during cataract formation.

INTRODUCTION

Crystallins are the major proteins of the lens and they constitute 90% of the soluble proteins. A number of physicochemical changes occur in lens proteins during aging, and during cataract formation similar changes occur at an exaggerated rate. The most striking changes in these proteins include yellowing and browning of proteins, intra- and inter-molecular crosslinking and crosslinking with fiber cell membrane proteins (1-4). Several mechanisms have been proposed for such changes, including oxidation (5,6) and glycation (7-9). Recent studies suggest that these two processes are interrelated (10,11) and may thus synergistically contribute to the observed lens protein modifications in aging and cataract formation.

Oxidative modifications within the lens may occur either on proteins or protein-free constituents. Protein-free tryptophan in the lens undergoes both enzymatic (12) and non-enzymatic oxidation (13) to produce reactive kynurenines (Fig. 1). Absorption of ultraviolet light A by the lens is attributed in part to these products. The enzymatic oxidation to N-formylkynurenine (NFK) is initiated by indoleamine 2, 3-dioxygenase (IDO), an enzyme that is up-regulated by interferon--. The next enzymatic steps produce kynurenine (KYN), 3-hydroxykynurenine (3OHKYN), 3-
hydroxyanthranilic acid, quinolinic acid and nicotinic acid (12). The kynurenines within the lens become enzymatically glycosylated to O- glycoses. Quantification of these products in the human lens shows that 3-hydroxykynurenine O-D-glucoside (3OHKYNG) is the most abundant form, followed by 4-(2-amino-3-hydroxyphenyl)-4-oxabutanoic acid O-D-glucoside, L-kynurenine and 3-hydroxykynurenine (14).

The kynurenine products produced from protein-free tryptophan readily pass through cell membranes and could thus diffuse through the cortex of the lens. Kynurenines are unstable at physiological pH; they undergo side-chain deamination to produce alpha-beta unsaturated ketoalkenes (15). Kynurenine levels decrease in the lens with age (16) and cataract formation (17), which may be due to their reaction with lens proteins. Kynurenines react with nucleophilic amino acids, such as cysteine, histidine and lysine in lens proteins (18-20), and cysteinyl residue in glutathione (GSH) through Michael addition to form covalent adducts. One of the products of the reaction of 3OHKYNG with lens GSH is glutathionyl-3-hydroxykynurenine glucoside (21), which accumulates during lens aging and accumulates to relatively high levels in cataractous lenses (19). Vazquez et al. demonstrated modification of histidine and lysine residues of lens proteins by KYN and their accumulation in aging lenses (22), but these authors also noted that the modified products decrease in senile cataracts (23). Another recent study confirmed specific histidine modification in β-crystallin that was incubated with KYN; modification of histidine 83 was thought to affect its chaperone function (24). Other crystallins appear to be modified by kynurenines as well (25), suggesting that kynurenines are responsible, in part, for protein crosslinking during aging and cataract formation.

Kynurenines also play a role in reactive oxygen species (ROS)-mediated crystallin modification. For example, kynurenines such as, 3OHKYN that contain a hydroxyl group can generate ROS through transition metal reduction reactions (12). Such reactions are implicated in crosslinking of crystallins. Because kynurenine-modified crystallins can generate ROS through photochemical reactions (26), weakened defenses against oxidative stress due to age or cataract formation could exacerbate lens protein modifications.

Glycation is a firmly established mechanism for protein modification during lens aging and cataract formation (7,9,27,28). In this reaction, sugars, ascorbate and dicarbonyl compounds react with amino groups of lysine and arginine residues on proteins through the formation of ketoamine adducts on proteins (10,29). These adducts can produce ROS during their modification to advanced glycation end products (11,30). We reasoned that glycation-derived ROS might induce oxidation of tryptophan and contribute to lens protein modification by kynurenines.

The present study was conducted to understand the role of 3OHKYN in lens protein modification and to determine the effect of glycation on tryptophan oxidation. Using a novel monoclonal antibody raised against 3OHKYN-modified keyhole limpet hemocyanin (KLH), we demonstrate that 3OHKYN-derived products are present in human cataractous lenses, provide structure of a major antigenic product and show that glycation products do not influence tryptophan oxidation and kynurenine-mediated modification of proteins.

**EXPERIMENTAL PROCEDURES**

*Incubation of proteins with 3OHKYN:* Bovine serum albumin (BSA) or ribonuclease A (RNase A) at 10 mg/ml in 0.1 M sodium phosphate buffer (pH 7.4) were incubated under sterile conditions in the dark with 25 mM 3OHKYN for 3 days at 37 0C. The incubation mixture was stirred after every 24 hrs, and the pH was adjusted to 7.4. The incubated material was then dialyzed against 4 L PBS for 48 hrs at 4 0C.

*Production of a monoclonal antibody against 3OHKYN-derived modification:* Keyhole limpet hemocyanin (KLH) at 10 mg/ml in 0.1 M sodium phosphate buffer (pH 7.4) was incubated with 25 mM 3OHKYN for 3 days, followed by dialysis against 4 L PBS for 48 hrs at 4 0C. The antibody was prepared according to the method of Oya et al.
Briefly, mice were initially immunized by intraperitoneal (i.p.) injection with 30 \( \mu \)g of 3OHKYN-modified KLH followed by three booster i.p. injections with 20 \( \mu \)g of the modified protein. After the final booster injection, spleen cells were collected and fused with P3/U1 murine myeloma cells using polyethylene glycol. The hybridomas were cultured in hypoxantine/aminopterin/thymidine selection medium.

An ELISA was used to screen culture supernatants of hybridomas. Microplate wells were coated by incubation for 16 hrs at 4 °C with one of the following (1 \( \mu \)g/well) in 0.05 M sodium carbonate buffer (pH 9.7): unmodified BSA, unmodified RNase A, 3OHKYN-modified BSA or 3OHKYN-modified RNase A. The wells were washed three times with PBS containing 0.05% Tween-20 (PBST) and blocked with 300 \( \mu \)l of 5% nonfat dry milk (NFDM) in PBST for 2 hrs at room temperature. The wells were then washed three times with PBST and incubated with 50 \( \mu \)l of hybridoma supernatant for 2 hrs at room temperature in a humid chamber. Following incubation, the wells were washed three times with PBST and incubated with 50 \( \mu \)l of either goat anti-mouse IgG (1:15,000 dilution in PBST) or goat anti-mouse IgM (1:2,500 dilution in PBST) antibody for 1 hr as described above. The wells were finally washed three times with PBST and incubated with 100 \( \mu \)l of 3,3',5',5'-tetramethylbenzidine substrate (Sigma). The enzyme reaction was stopped by addition of 50 \( \mu \)l of 2N H 2SO4 and absorption of the chromophore was measured at 450 nm in a Dynex MRX 5000 Microplate Reader.

An IgG antibody producing hybridoma with high specificity against 3OHKYN-modified proteins was expanded. We then purified the antibody from the culture medium on a protein G-Sepharose column (Amersham Biosciences) and stored aliquots at -20°C. The monoclonal antibody was determined to be of IgG1k subclass.

Modification of human lens proteins and N'-acetyl amino acids by tryptophan oxidation products:

Water soluble proteins (WS-HLP) were extracted from human lenses by homogenizing each lens in 2.0 ml of PBS followed by centrifugation at 20,000 Xg for 30 min, at 4 °C. The supernatant was dialyzed against 4 L of PBS for 24 hrs at 4°C. Samples of this material (5 mg/ml) were then incubated at 37 °C for 7 days in 0.1 M sodium phosphate buffer (pH 7.4) with one of the following: tryptophan (TRP), 3OHKYN, anthranilic acid (AA) (all from Sigma), KYN (Fluka) or NFK. NFK was synthesized according to Simat and Steinhart (32). We also used benzoic acid (BA) and 4-hydroxybenzoic acid (HBA) (Sigma) for incubations to test specificity of the antibody. TRP and its oxidation products were used at 10 times the molar concentration of lysine in lens proteins. After incubation, the mixtures were dialyzed extensively against PBS for 48 hr at 4 °C with a change of dialysis medium after 24 hrs.

N'-acetyl lysine, N'-acetyl arginine, N'-acetyl histidine and N'-acetyl cysteine (all from Sigma) were incubated independently with either tryptophan or tryptophan oxidation products at a 5:1 molar ratio for 7 days at 37 °C as described above.

Purification of antigen from the reaction mixture of N'-acetyl lysine and 3OHKYN: N'-acetyl lysine (59 mM) was incubated with 3OHKYN (9.9 mM) in 0.1 M sodium phosphate buffer (pH 7.4) at 37 °C for 7 days under sterile conditions. A sample (250 \( \mu \)l) of the reaction mixture was loaded on semi-preparative reversed phase HPLC on a C18 reversed-phase column (VYDAC, 218TP1010, 10 \( \mu \)m, 10 x 25 cm, Separations Group, Hesperia, CA). A linear gradient was established with solvent A [0.1% trifluoroacetic acid (TFA) in water] and solvent B (50% acetonitrile and 0.1% TFA in water). The following program was applied: 0-5 min – 0 % B, 5-50 min – 40% B, 50-60 min –100% B, 60-68 min –100% B and 68-78 min-0% B with a flow rate of 2.0 ml/min. We used an online UV detector (Jasco Corporation, Japan, Model UV-970) to monitor the column effluent for absorbance at 365 nm. Four major fractions were collected and dried in a Savant Speed Vac Concentrator (Savant Instruments, Hicksville, NY) and re-suspended in 200 \( \mu \)l of water. Each fraction (17 \( \mu \)l/well) was tested in a competitive ELISA for immunoreactivity against the antibody (ELISA...
procedure described below). The material eluted at ~30 min had the strongest reaction with the antibody in this assay.

To obtain a higher amount of pure antigen, we modified the HPLC method (described above) so that the reaction mixture of N\(^\text{\textsuperscript{\text{\textdegree}}}\)-acetyl lysine and 3OHKYN was first passed through a Sep-Pak Light C\(_{18}\) Cartridge (Waters, Milford, MA). After application of the sample, the cartridge was sequentially eluted with stepwise gradient of acetonitrile in 0.1% TFA. The eluate from 0.1% TFA in water was collected, dried in a Savant Speed Vac Concentrator and finally suspended in water. This material was then purified with the same semi-preparative reversed phase HPLC that we used previously for the crude mixture. A linear gradient of solvent A (0.1% TFA in water) and solvent B (50% acetonitrile and 0.1% TFA in water) was used. The program was slightly altered to improve separation of the compound: 0-5 min ~0% B, 5-50 min ~25% B, 50-80 min ~100% B, 80-88 min ~0% B. The flow rate was 2.0 ml/min. The antigen peak now eluted at around 36 min. We collected this peak from 10 injections of 500 µl each and pooled. This pooled sample was dried, suspended in water and re-injected to the same column under same HPLC conditions. A single homogeneous peak at R\(_t\)~36 min was obtained by this procedure. This material was lyophilized, and the product was characterized by spectroscopy.

One-dimensional and two-dimensional \(^1\text{H}\) and \(^13\text{C}\) NMR spectra were recorded on a Varian Inova 600 MHz spectrometer. Samples were dissolved in D\(_2\)O for \(^1\text{H}^-\text{\textsuperscript{\text{\textdegree}}}\text{C}\) correlation experiments (HSQC, HMBC). FAB-MS analyses were done in the Mass Spectrometry Facility at Michigan State University, East Lansing, MI in a JEOL HX-110 double focusing mass spectrometer. UV-visible spectra were recorded using Molecular Devices Spectra Max 190 spectrometer (Sunnyvale, CA 94089).

Treatment of 3OHKYN with ninhydrin: The procedure was as described by Miyata and Monnier (33). 40 mM 3OHKYN was incubated with 10 mM ninhydrin in 300 µl of ethanol/acetic acid, pH 5.0 for 10 min at 65 °C. 25 mM L-lysine was then added and incubated at 65 °C for 10 min. The mixture was dried on Savant Speed Vac Concentrator, dissolved in 200 µl of water and analyzed by an ELISA outlined below. A control experiment in which L-lysine was used in the place of 3OHKYN, was run simultaneously.

Preparation of protein-free filtrate from human lenses: Four normal lenses from donors of 30-50 years of age were each homogenized in 2 ml of water and centrifuged at 20,000Xg for 30 min, at 4°C. The supernatant fraction was filtered through a Centricon YM-10 filter (Millipore, Bedford, MA) and the filtrate was lyophilized and reconstituted in 100 µl of water.

Incubation of proteins with human lens protein-free filtrate: One half of the protein-free filtrate was digested with α-glucosidase (Sigma) to obtain 3OHKYN from its glucoside form. For this step, we used a 1% enzyme solution in 0.04 M sodium phosphate buffer (pH 5.6) and incubated the material for 2 hr, at 37 °C. The sample was then filtered through a Centricon YM-10 filter to remove the enzyme. The digested and non-digested protein-free filtrates were incubated for 5 days at 37 °C with 1.0 mg of either RNase A or WS-HLP (see above) in 1.0 ml of 0.1 M sodium phosphate buffer (pH 7.4). The incubated samples were then dialyzed against 2 L PBS at 4 °C. Finally, 30 µg of modified protein in 50 µl PBS per well was tested for antigen using a competitive ELISA (see below).

Enzyme-linked immunosorbent assay (ELISA): Microplate wells were coated with 3OHKYN-modified RNase A in 0.05 M carbonate buffer (pH 9.7) at a concentration of 1µg/50 µl, incubated at 4°C over night, then washed three times with PBST. Before use in the assay, the wells were blocked for 2 hr at room temperature with 300 µl of 5% NFDM in PBST and washed three times with PBST. The monoclonal antibody (1:200 diluted in 1% NFDM/PBS for proteins modified TRP oxidation products and 1:60 diluted for amino acids modified by 3OHKYN) was pre-incubated with the competitor for 2 hr at 37 °C, then dispensed into specified wells and incubated for 1.5 hr at room temperature. The washed plates were then incubated with HRP-conjugated goat anti-mouse IgG (Promega Corp., Madison, WI, USA).
diluted 1:15,000) for 1 hr at 37 °C as described earlier. The enzyme reaction was assessed by addition of 100 μl of 3,3’,5’,5’-tetramethylbenzidine (Sigma) followed by the addition of 50 μl of 2N H₂SO₄, and measurement of chromophore absorbance at 450 nm. Results were expressed as the ratio: B/B₀, where B is the absorbance in presence of competitor, and B₀ is absorbance in the absence of competitor.

Western blotting: WS-HLP from cataractous and normal lenses were digested with proteinase K (2% w/w in PBS) for 30 min at 37 °C, aliquots of the digest corresponding to 20 μg of protein were separated on 18% reducing gels, and the proteins were transferred electrophoretically to Immobion P membranes (Millipore). Comparable gels were stained with BioSafe Coomassie (BioRad, Hercules, CA). The membranes were then blocked with 5% NFDM in PBS for 2 hrs and incubated overnight at 4°C with a 1:20 dilution of the anti-KLH-3OHKYN (10.3 μg/ml) monoclonal antibody. After washing 5 times for 10 min with PBST, the membranes were incubated with goat anti-mouse IgG conjugated with HRP (Promega Corp.) diluted 1: 15,000. After repeated washing (5 times for 10 min with PBST), the membranes were treated with SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL) for 5 min and exposed to x-ray film (Pierce, CL-XPosure Film). Proteins modified by TRP oxidation products were similarly subjected to Western blotting, except that 12% gels were used and 2 μg protein/ lane was loaded.

Immunostaining: HLE-B3 cells (from Dr. Usha Andley, Washington University, St. Louis, MO) (between passages 13 and 19) were cultured in chamber slides with minimal essential medium (MEM) containing 20% fetal bovine serum (FBS), 2 mM L-glutamine and 50 μg/ml gentamycin. The cells were washed twice with PBS and treated with MEM containing 0 (control), 20, 50, 100 or 200 μM of 3OHKYN in the absence of FBS for 24 hours. The treated cells were washed twice with PBS and fixed with 4% paraformaldehyde at –20 °C for 15 min. After washing twice with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS at –20 °C for 5 min, then washed 5 times with PBS to remove the detergent. Next, the slides were blocked with 3% NFDM/1% BSA for 30 min at room temperature and washed twice for 5 min each time with PBS. The slides were incubated with the anti-KLH-3OHKYN antibody (10.3 μg/ml, diluted in 0.1% BSA) or non-immune mouse IgG diluted to the same concentration for 1 hr at room temperature and washed twice for 5 min each time. In some experiments, we pre-incubated the antibody for 1 hr at 37°C with 3OHKYN-modified RNase A (0.2 mg/ml). All slides were incubated with secondary antibody (anti-mouse IgG) conjugated with Oregon Green (Molecular Probes, Eugene, OR). The secondary antibody was diluted in 0.1% BSA/PBS and applied to the slides for 1 hr at room temperature. After this incubation, all slides were washed twice for 5 min with PBS and then developed by incubation for 20 min at room temperature with Texas red-Phalloidin (Molecular Probes) diluted in 0.1% BSA/PBS and washed as described above. Finally, all slides were incubated with DAPI (Molecular Probes, diluted in PBS) for 1 min and washed twice for 5 min with PBS. After mounting, the slides were observed with an Olympus System (Model BX60) fluorescence microscope, and images were acquired with an attached digital camera (Diagnostic Instruments, Inc. Spot RT Slider) connected to a Macintosh computer using Spot RT Slider Software v3.5.5.

Immunohistochemistry: Cataractous and age-matched normal lenses from donors between ages of 65-70 years were fixed in 10% neutral buffered formalin, embedded in paraffin and cut into 5 μm sections. Following dehydration, heat-induced epitope retrieval was done in 10 mM citrate buffer (pH 6.0), and the sections were treated with 3.0% hydrogen peroxide to block endogenous peroxidases. The sections were blocked with 1.5% normal horse serum and incubated for 2 hrs with either the anti-KLH-3OHKYN antibody (10.3 μg/ml) diluted in PBS or with non-immune mouse IgG, diluted to the same protein concentration as the primary antibody. For adsorption experiments, the antibody was pre-incubated overnight at 4°C with 3OHKYN-modified RNase A in PBS (0.4 mg/ml). After washing, the slides were incubated with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) followed by ABC reagent (Vector Laboratories, Burlington, CA). They were stained initially with 3,3’-
diaminobenzidine, counterstained with hematoxylin and then permanently mounted.

**Incubation of N\(^\alpha\)-acetyl tryptophan with ribated lysine-Sepharose-4B:** To determine whether glycation could catalyze oxidation of tryptophan, we incubated N\(^\alpha\)-acetyl-tryptophan with ribated lysine-Sepharose (Amersham Biosciences). One gram of lysine-Sepharose was washed thoroughly with 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 1 mM DTPA, then incubated at 37 \(^\circ\)C for 2 days with 500 mM ribose in the same buffer. The tubes were bubbled with argon for 15 min and sealed before incubation. The ribose-treated gel was extensively washed with PBS to remove unbound ribose. Finally, 250 mg samples of either modified or unmodified (control) gel were incubated at 37 \(^\circ\)C with 5.0 mM N\(^\alpha\)-acetyl tryptophan in 0.1 M sodium phosphate buffer (pH 7.4) for 3, 7 and 10 days. Aliquots were subjected to C\(_{18}\) reversed phase HPLC using a gradient program consisting of solvents A [water with 0.01 M heptafluorobutyric acid (HFBA)] and B (70% acetonitrile in water and 0.01 M HFBA). The column was eluted with a linear gradient of B from 5-50 min at a flow rate of 1.0 ml/min and the column effluent was monitored for fluorescence at excitation/emission wavelengths of 290/320 nm. N\(^\alpha\)-acetyl tryptophan eluted at \(R_t = \sim 22\) min.

We also incubated N\(^\alpha\)-acetyl tryptophan during glycation; ribose (6.7 mM), lysine (0.6 mM), arginine (0.6 mM) and N\(^\alpha\)-acetyl tryptophan (13.2 mM) were incubated for 5 days at 37 \(^\circ\)C in 0.1 M sodium phosphate buffer (pH 7.4). Other incubations included N\(^\alpha\)-acetyl tryptophan + ribose, N\(^\alpha\)-acetyl tryptophan + arginine + ribose, N\(^\alpha\)-acetyl tryptophan + lysine + ribose, all at concentrations as above. All samples were analyzed by HPLC for N\(^\alpha\)-acetyl tryptophan oxidation as described above.

In another experiment, we incubated lysine-Sepharose (500 mg) with 500 mM ribose in the presence or absence of 6.7 mM N\(^\alpha\)-acetyl arginine in 0.1 M sodium phosphate buffer, pH 7.4 under sterile conditions at 37 \(^\circ\)C for 6 days. After the incubation, the gel was extensively washed with PBS. The washed gel (18 mg, glycated or non-glycated) was incubated with 16.7 mM L-tryptophan for 20 hrs in PBS at room temperature. After the incubation, the supernatant was incubated with 0.3 mg of RNase A in PBS for 3 days at 37 \(^\circ\)C. The protein was then dialyzed against PBS for 16 hrs at 4 \(^\circ\)C and tested in competitive ELISA for reaction with KLH-3OHKYN antibody.

To identify formation of 3OHKYN-derived products in proteins during glycation, human lens water soluble proteins (4.3 mg/ml) were incubated under sterile conditions for 7 days at 37 \(^\circ\)C with one of the following: 15.5 mM glucose, 15.5 mM ribose, 15.5 mM ascorbate, 7.5 mM methylglyoxal or 7.5 mM glyoxal in 0.1 M sodium phosphate buffer (pH 7.4). We also incubated protein in the absence of glycatig agents, which served as control in this experiment. After incubation, the material was dialyzed extensively against PBS at 4 \(^\circ\)C and tested for reaction with antibody by competitive ELISA and Western blotting.

**RESULTS**

**Characterization of monoclonal antibody for 3OHKYN modification:** The monoclonal antibody was characterized by a competitive ELISA using TRP oxidation products-modified human lens proteins. We found the strongest reaction with proteins modified by 3OHKYN (Fig. 2A), and 2 \(\mu\)g of 3OHKYN-modified protein blocked the antibody completely. None of the other oxidation products blocked the antibody completely at concentrations used in the assay. We did observe some reaction with proteins modified by KYN with the antibody. However, it was much less than proteins modified by 3OHKYN when tested between 1-10 \(\mu\)g of protein. At high concentrations (>10 \(\mu\)g), proteins incubated with NFK also displayed slight reaction. Proteins incubated with just PBS or incubated with TRP also showed reaction comparable to that of protein incubated with NFK. These observations suggest that some amount of antigen was already present in the protein isolated from young human lenses, and incubation with either NFK or TRP produced no additional antigens. Surprisingly, protein treated with the three acids, HBA, AA and BA all had no reactivity with the antibody, which might
be due to masking of antigen as a result of conformational changes or destruction of antigen by the acid. The latter is unlikely as our incubations were done in 0.1 M sodium phosphate buffer, pH 7.4 and the pH remained at 7.4 after addition of the reactants.

Protein staining of the SDS-PAGE gel revealed that 3OHKYN-modification resulted in high molecular aggregates of lens proteins (Fig. 2B). Western blotting experiments confirmed the ELISA results. Lens proteins modified by 3OHKYN reacted with the antibody (Fig. 2C, lane 8). Among the many high molecular weight proteins, we assume that some that display immunoreactivity could be crosslinked aggregates.

To establish which amino acids are involved in kynurenine modifications, we incubated N'-acetyl amino acids with kynurenines. In these incubations, we used 5 times higher concentrations of amino acids relative to kynurenines to complete reaction of kynurenines. In addition, since 3OHKYN is unstable in aqueous neutral solutions, we do not expect free 3OHKYN to remain after 7 days of incubations. We used the reaction products as competitors in ELISA. Our results show that 3OHKYN-modified N'-acetyl lysine, N'-acetyl cysteine and N'-acetyl arginine reacted similarly with the antibody (Fig. 3). The reaction with 3OHKYN-modified N'-acetyl histidine was slightly stronger than other amino acids tested. Unmodified amino acids did not react with the antibody. These results indicate that the antibody recognized a structure common to all modifications.

In another experiment, we wanted to know if the antibody could react directly with TRP and its oxidation products. 3OHKYN alone strongly reacted with our antibody, and we achieved a complete blockade of the antibody binding at 12.5 nmoles of 3OHKYN (Fig. 4). Among the other products tested, we found some degree of immunoreactivity with KYN and 3OHKA, although these were at least 50-60% lower than 3OHKYN-modified protein at 12.5 nM concentration. The antibody was unreactive against HBA, AA or AP. Our results indicate that the monoclonal antibody requires 2-amino and 3-hydroxyl groups on the benzene ring in order to recognize an antigen. The low reactivity with KYN compared to 3OHKYN suggests that the –OH group at position 3 makes 3OHKYN the better antigen.

To determine the structure of antigenic epitope in amino acid modifications, we purified the major immunoreactive product of the reaction of N'-acetyl lysine and 3OHKYN. The mixture was separated on C18 reversed phase HPLC. Four peaks were collected by repeated injections (Fig. 5A). The product in peak at R =30 min (peak labeled 1) was the most effective inhibitor of the antibody (Fig. 5B).

Next, we used a C18 reversed phase cartridge for the first stage of a preparative scale purification. The purification protocol on C18 reversed phase HPLC was altered as described in Experimental Procedures to achieve clear separation of the peak of interest and scaled-up purification. Peak 1 in Fig. 5A eluted at ~36 min (Fig. 5C). The yield was calculated at 2.1 % based on the amount of 3OHKYN used for synthesis.

**Spectroscopy:** The purified compound had UV absorption maxima at 270 nm and 370 nm (Fig. 5C inset), which was very similar to the product isolated from the reaction of KYN and N'-t-BOC-lysine. Figure 6 shows the $^1$H-NMR spectrum in D$_2$O. –H 7.44 (d, 1H, J=8.0 Hz, H-4), 7.10 (d, 1H, J=7.9 Hz, H-6), 7.04 (t, 1H, J=8.0 Hz, H-5), 4.2 (dd, 1H, J=5.0, 9.0 Hz, H-15), 4.15 (t, 1H, J=5.0 Hz, H-9), 3.73 (m, 2H, H-8), 3.03 (m, 2H, H-11), 1.88 (s, 3H, CH3-18), 1.76 (m, 1H, H-14), 1.64 (m, H-12,12',14', H-12), 1.34 (m, 2H, J=7.3, 14.1 Hz, H-13,13', H-13); C 199.9 (C-7), 176.0 (CO), 172.1 (CO), 147.9 (C-2), 147.9 (C-18), 128.3 (C-3), 123.7 (C-5), 123.4 (C-4), 122.8 (C-6), 121.1 (C-1), 56.7 (C-9), 52.7 (C-15), 47.2 (C-11), 38.7 (C-8), 28.5 (C-12), 28.0 (CH3), 25.5 (C-14), 22.6 (C-13). FAB-MS spectrum showed M+H of 396.1, which is comparable to the calculated 396.2 empirical formula of C$_{18}$H$_{25}$N$_3$O$_7$. Based on these results, we determined the structure of the product shown in Fig. 6 inset.

**Reaction of the purified product with the monoclonal antibody:** We tested the purified product for reaction with the antibody using a
competitive ELISA. The purified compound completely blocked antibody binding at 12.5 nmoles (Fig. 7). The reaction of the purified product with antibody was very similar to that of 3OHKYN. Based on observations following incubation with various amino acids and the purified product, we were able to identify a core structure that is recognized by the antibody. This structure is (2-amino-3-hydroxy)-4-oxo-benzene butanoic acid (Fig. 8). Our results suggest that attachment of lysine at C-9 does not influence the antibody recognition. To confirm that the antibody recognizes the core structure, we treated 3OHKYN with ninhydrin. We expected ninhydrin to deaminate 3OHKYN to a keto acid or an aldehyde. We found that the resulting product had nearly 4-fold enhanced reactivity with the antibody (Fig. 9). Lysine treated with ninhydrin in the same way failed to react with the antibody. Taken together, these observations confirm that the antibody reacts with a core structure in modified N\textsuperscript{2}-acetyl lysine, N\textsuperscript{2}-acetyl histidine, N\textsuperscript{2}-acetyl arginine and N\textsuperscript{2}-acetyl cysteine.

Modification of proteins by protein free filtrates from human lens: Protein-free filtrates of material obtained from normal human lenses were treated with \(\alpha\)-glucosidase to remove glucose from kynurenine glucosides and then incubated at 37 °C for 5 days with either RNase A or WS-HLP. The modified proteins were then analyzed in a competitive ELISA. Controls were from incubations of proteins with protein-free filtrates that were not treated with \(\alpha\)-glucosidase. Fig. 10 shows that 30 µg of the filtrate-modified proteins (RNase A as well as WS-HLP) inhibited binding of the antibody nearly 100% if pretreated with \(\alpha\)-glucosidase. These results support findings that human lenses contain kynurenine glucosides, and further indicate that kynurenine glucosides by themselves may not produce antibody-recognizable products, but after removal of glucose react with proteins to produce immunoreactive products.

3OHKYN-modifications in the human lens: To detect kynurenines modifications that had occurred in vivo, we extracted water-soluble proteins from normal and cataractous lenses and digested them mildly with proteinase K. This treatment was necessary to expose buried antigenic epitopes. The samples were then subjected to SDS-PAGE (Fig. 11A) followed by immunoblotting. We noted immunoreactivity in cataractous lenses (lanes 2-4) but not in normal (non-cataractous) age-matched lenses (lane 1). A representative Western blot is shown in Fig. 11B.

We used immunohistochemistry to localize the kynurenine modifications in paraffin sections of normal and cataractous human lenses, and while we noted 3OHKYN modifications in the cataractous lens (Fig. 11C, panel 2), we found none in the normal lens (panel 1). The positive reactivity from a cataractous lens preparation was markedly reduced when the antibody was pre-incubated with 3OHKYN-modified RNase A (panel 3).

Since it appeared that the antibody reaction occurred primarily in the fiber cell plasma membrane (Fig. 11C), we used an antibody against aquaporin-0 to confirm that the 3OHKYN-modifications were indeed localized in the lens fiber cell plasma membrane. Aquaporin-0, which is abundant in lens fiber cell plasma membrane, is also known as MP26. The staining pattern indicates that 3OHKYN modifications occurred primarily in proteins within the plasma membrane or proteins associated with plasma membrane (Fig. 11, panel 5).

Effect of 3OHKYN treatment on human lens epithelial cells: HLE-B3 cells on chamber slides were treated for 24 hrs with either 100 or 200 µM 3OHKYN in MEM without calf serum. The cells were fixed with 4% paraformaldehyde and treated with the monoclonal antibody followed by Oregon Green-conjugated secondary antibody. Cells treated with 100 µM 3OHKYN showed an antibody reaction within the cytoplasm that was even more intense with 200 µM 3OHKYN (Fig. 12, panels 1-3). The staining was markedly diminished when the antibody was pre-incubated with 3OHKYN-modified RNase A (data not shown). Non-immune mouse IgG caused no reaction (Fig. 12, panels 4-6). Further, HLE-B3 cells that were incubated without 3OHKYN showed no immunoreactivity (Fig. 12B, panels 7-9). These results indicate that immunoreactivity is
related specifically to intracellular 3OHKYN-derived products.

_Tryptophan oxidation by glycation:_ We examined the effect of ribose-mediated glycation on the oxidation of protein-free N'-acetyl tryptophan. HPLC results indicated no degradation of N'-acetyl tryptophan either from incubation with ribated lysine-Sepharose (Fig. 13A) or during glycation of lysine and arginine with ribose (Fig. 13B). We also studied tryptophan oxidation and modification of proteins by glycation products. This was achieved by incubating tryptophan with glycated lysine-Sepharose (by ribose + arginine) followed by incubation with RNase A. RNase A was then tested in a competitive ELISA for reaction with the KLH-3OHKYN antibody. The results showed absence of antibody recognizable products in the incubated protein (Fig. 13C).

We then studied glycation of human lens proteins with sugars, ascorbate and methylglyoxal as a possible mechanism for tryptophan oxidation and protein modification. WS-HLP was glycated with glucose, ribose and ascorbate and tested for reaction with 3OHKYN antibody in a competitive ELISA. None of the modified proteins were immunoreactive, even when the wells were coated with 1.4 μg of modified protein in a direct ELISA. Similarly, there was no immunoreactivity in a competitive ELISA (Fig. 13D). A slight reactivity was seen in ascorbate-modified proteins, but this could not be confirmed in Western blotting. All other glycated proteins including those reacted with methylglyoxal and glyoxal were negative in Western blotting as well (data not shown). These results suggest that tryptophan oxidation and formation of antibody reactive products are not produced during glycation with sugars, ascorbate and -dicarbonyls.

**DISCUSSION**

Our monoclonal antibody recognizes kynurenine-derived structures containing a hydroxyl group on benzene ring. Michael addition at C-9 on oxidized tryptophan has been well documented, and several additive products of lysine, histidine and cysteine have been isolated and detected in the human lens (12,19,22). Our antibody reacted nearly as well with several 3OHKYN-modifications, including N'-acetyl lysine, N'-acetyl histidine, N'-acetyl arginine and N'-acetyl cysteine, although the N'-acetyl histidine modifications reacted more strongly than products of the other amino acids. The fact that unmodified acetyl amino acids react only weakly or not at all with the antibody suggests a common epitope among the modified amino acids. The most likely epitope is (2-amino-3-hydroxy)-4-oxo-benzene butanoic acid. In fact, the structures identified by the reaction of KYN with acetyl amino acids all have a structure in common very similar to the one recognized by our antibody, but lacking the 3-hydroxyl group (22).

We used sequential HPLC methods and immunoreactivity to establish the structure of the antigen formed from the reaction of N'-acetyl lysine and 3OHKYN. We isolated and purified a major product recognized by our monoclonal antibody and found that it was very similar to N'-tert-Butoxycarbonyl (t-BOC)-L-lysyl-D,L-kynurenine, a product of the reaction of t-BOC-lysine and KYN (22). Vazquez et al. suggested that kynurenines under slightly basic conditions undergo deamination, and then nucleophilic amino acids can react with C-9 through Michael addition (22). We assume that similar reactions with 3OHKYN occurred under our experimental conditions, and to support this assumption, spectroscopy data indicate that N'-acetyl lysine is linked to the aliphatic chain of 3OHKYN through the -amino group. Mass spectrometry m/z of 396.1 (M+1) suggests that the structure is 2-amino-3-hydroxy-[(5S)-5-acetamino-5-carboxypentyl amino]-oxo-benzene butanoic acid. The absorption spectrum of the purified compound has maxima at 270 nm and 370 nm, similar to the lysylkynurenine described by Vazquez et al. (22).

Our antibody reacted equally well with 3OHKYN and the purified product, and the removal of an amino group at C-9 further enhanced the reaction of 3OHKYN. These findings suggest that the antibody requires 3-OH and 2-NH₂ groups in the benzene ring to recognize the antigen and the removal of the positive charge on the C-9 amino group by Michael addition of nucleophilic amino acids enhances reaction with
the antibody. Our detection of antigen(s) in the modified proteins suggests that it is the 3OHKYN-derived modifications and not the free 3OHKYN that are detected in lens proteins and in vitro modified proteins. 3OHKYN is unstable at physiological conditions; it becomes converted to xanthommatin type of compounds (34). Whether the compounds generated during protein and amino acid incubations are derived from these products is not known, but the purified antigen is a Michael adduct of N-acetyl lysine and 3OHKYN. Thus, we believe that Michael adducts are probably the major antigenic epitopes for the antibody.

Because we found 3OHKYN-modification in cataractous, but not in normal lenses, we suspect that these modifications contribute to cataract formation. Our immunohistochemical study showed that the products are primarily associated with plasma membrane of fiber cells, which further suggests that 3OHKYN modifies either plasma membrane proteins and/or proteins associated with plasma membrane. Binding of crystallins to the plasma membrane of fiber cells during cataract formation is a well recognized phenomenon (3). We cannot rule out modification of aquaporin-0, another component which occurs in relatively high amounts in the plasma membrane of lens fiber cells (35). In fact, experiments with appropriate antibodies and cataractous lenses showed co-localization of immunoreaction due to aquaporin-0 and 3OHKYN modifications. Because our attempts to immunoprecipitate aquaporin-0 from cataractous lenses were unsuccessful, we were unable to provide further proof for its modification by 3OHKYN.

Vazquez et al. (22) proposed that 3OHKYN might react with proteins similarly to KYN. Our own observations support this idea. We find that the major immunogenic product from the reaction of N′-acetyl lysine and 3OHKYN is similar to the product isolated by Vazquez et al. from the reaction of N′-t-BOC-lysine and KYN (22). We agree with Vazquez et al. that reactions of KYN and 3OHKYN likely proceed in similar fashion. The same authors also thought that o-aminophenol moiety in 3OHKYN might undergo oxidation to produce reactive oxygen species. If this happens within the lens, it could exacerbate protein modification by 3OHKYN. Reduced glutathione within the lens could inhibit kynurenine modification by competing for reaction with kynurenines. Since glutathione concentration is usually reduced in the inner nucleus of aging and cataractous lenses, KYN reactions can proceed unhindered in this part of the lens. Whether this also occurs with 3OHKYN is yet to be determined.

The occurrence of glucosides of kynurenines in human lenses is fairly well established (21,36). Although both 3OHKYN and 3OHKYNG in the human lens decrease with age (possibly because of reaction with proteins), lenses from aged donors still contain significant amounts of these products. Combined cortical and nuclear concentrations up to ~7 nmoles/g lens 3OHKYN were reported for the aged human lens (16); concentrations of 3OHKYNG are much higher, with as much as 800 nmoles/g lens (16). Further evidence was provided by immunoreaction of proteins after incubation with protein-free filtrates of human lenses. We observed an enormous increase in the immunoreactivity of proteins after they were exposed to protein-free filtrates of -glucosidase-treated filtrates of human lens. Our results indicate that 3OHKYNG concentrations are much higher than 3OHKYN. This is in accord with the data of Bova et al. (16). The fact that human lens contains --glucosidase suggests that 3OHKYNG is converted to 3OHKYN and products that are detected by our antibody. The activity of --glucosidase increases in cataractous lenses (37), so the higher immunoreactivity with our antibody in cataractous lenses may be due, in part, to enhanced formation of 3OHKYN. However, there are conflicting reports on 3OHKYNG levels in cataractous lenses. One study found no difference in 3OHKYNG concentrations between brunescent and non-brunescent human lens nuclei (38), but the investigators did not measure the total glucoside concentration in the lens. Another study reported decreased levels of 3OHKYNG in cataractous lenses (17) which could be due to reaction of this compound with lens proteins during cataractogenesis.
The intense immunopositive reaction observed when we incubated human lens epithelial cells with 3OHKYN shows that 3OHKYN can pass through plasma membrane of lens epithelial cells. If fiber cells in the lens are permeable as well, we would expect 3OHKYN modifications to occur in both cortical and nuclear regions of the lens. However, decrease in glutathione with aging and cataract formation (39,40), especially in the nucleus may promote 3OHKYN-modifications in this region.

We anticipated that ROS generated during glycation might oxidize tryptophan and that the oxidation products of TRP would then react with proteins to chemically modify them. We were surprised to find that neither preformed glycation products nor glycation in the presence of tryptophan contributed to the oxidation of protein-free tryptophan. The monoclonal antibody against 3OHKYN-induced protein modifications failed to recognize either glycated proteins or proteins that were glycated in the presence of external tryptophan. The absence of immunoreactivity among various glycation products that we examined indicates that glycation does not lead to formation of kynurenines, although we cannot rule out an effect of glycation on the enzymatic oxidation of tryptophan. However, our antibody recognized proteins modified by 3OHKYN in the human lens. These findings imply that reactions of tryptophan oxidation products along with glycation occur in the human lens and that 3OHKYN-mediated reactions occur independently of glycation. Together these reactions are likely to contribute to cataractogenesis.

In summary, we showed that 3OHKYN-derived modifications can occur in the human lens, and we believe that these modifications may contribute to cataract formation. Our studies indicate that kynurenines are not produced during glycation of proteins. Since production of kynurenines is increased in the brains of patients with Huntington’s disease (41) and in plasma of end stage renal disease patients (42), our antibody might be useful for assessing the role of 3OHKYN modifications in these diseases. It is also noteworthy that the activity of IDO, the first enzyme in tryptophan metabolism to kynurenines, is up-regulated in viral and bacterial infection and inflammation (43-45). These conditions may favor kynurenine modification of proteins where the reactions may occur at an accelerated rate.

REFERENCES

214, 86-104
FOOTNOTES

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1 The abbreviations used are: IDO, indoleamine 2,3-dioxygenase; MEM, minimal essential medium; FBS, fetal bovine serum; AA, anthranilic acid; AP, 2-aminophenol; BA, benzoic acid; BSA, bovine serum albumin; DAPI, 4', 6-diamidino-2-phenylindole; DTPA, diethylene triamine pentaacetic acid; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FAB-MS, fast atom bombardment-mass spectroscopy; GSH, glutathione; HBA, 4-hydroxybenzoic acid; 3OHKYN, 3-hydroxykynurenine; HLE-B3, human lens epithelial cells B3; 3OHKYNG, 3-hydroxykynurenine O-D-glucoside; HFBA, heptafluorobutyric acid; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; KYN, D,L-kynurenine; NFDM, non-fat dry milk; NFK, N-formylkynurenine; NMR, nuclear magnetic resonance; 3OHA, 3-hydroxyanthranilic acid; PBST, PBS + 0.05% Tween-20; RNase A, ribonuclease A; ROS, reactive oxygen species; TFA, trifluoroacetic acid; TRP, L-tryptophan; WS-HLP, water soluble human lens proteins.

FIGURE LEGENDS

Fig. 1. Proposed pathway of kynurenine formation from tryptophan. Kynurenines react with lens proteins form brown pigmented, covalently crosslinked proteins.

Fig. 2. Characterization of monoclonal antibody to KLH-3OHKYN. A. Microplate wells were coated with RNase-3OHKYNG mAb was pre-incubated with water soluble human lens proteins (WS-HLP) modified by N-formylkynurenine (NFK), tryptophan (TRP), 3-hydroxykynurenine (3OHKYN), kynurenine (KYN), benzoic acid (BA), 4-hydroxybenzoic acid (HBA), anthranilic acid (AA) or incubated with PBS alone. B. Electrophoresis was performed on 12% polyacrylamide gel under reducing conditions, stained with Biosafe Coomassie reagent or blotted using the monoclonal antibody for KLH-3OHKYN. WS-HLP modified with 1, AA; 2, BA; 3, HBA; 4, PBS; 5, TRP; 6, NFK; 7, KYN; 8, 3OHKYN and 9, 3OHKYN-modified RNase A (positive control). C. Western blotting of HLP modified by tryptophan oxidation products. Sample details are same as in B.

Fig. 3. Characterization of KLH-3OHKYN monoclonal antibody. The monoclonal antibody for KLH-3OHKYN was pre-incubated with 3OHKYN + N-acetyl amino acid reaction mixture (described in Experimental Procedures) and tested by a competitive ELISA as described in Fig. 2. AcArg, N-acetyl arginine; AcCys, N-acetyl cysteine; AcHis, N-acetyl histidine; AcLys, N-acetyl lysine.

Fig. 4. Characterization of KLH-3OHKYN monoclonal antibody. Competitive ELISA was performed with unmodified tryptophan and its oxidation products. The products were incubated with KLH-3OHKYN monoclonal antibody at indicated concentrations, the rest of the ELISA procedure as in Fig. 2. 3-hydroxykynurenine (3OHKYN); kynurenine (KYN); 3-hydroxyanthranilic acid (3OHA); Anthranilic acid (AA); hydroxybenzoic acid (HBA); aminophenol (AP).
Fig. 5. Purification of a major antigen from the reaction of N\textsuperscript{-}acetyl lysine and 3OHKYN. Products were separated by reversed phase semi-preparative HPLC. The column effluent was monitored for absorption at 365 nm (trace A). Four major peaks were collected, dried, suspended in buffer and tested by ELISA for reaction with the antibody. Peak 1 with Rt~30 min showed the highest immunoreactivity against the antibody. B, reactivity of the collected fractions with mAb. The reaction mixture was passed through a C\textsubscript{18} cartridge and re-purified on the same column with a different gradient program (described in Experimental Procedures). The product eluted at Rt~36 min was collected by repeated injections (trace C). The inset shows the UV absorption of the purified compound.

Fig. 6. Characterization of the purified antigen. \textsuperscript{1}H-NMR in D\textsubscript{2}O. The structure of the purified product based on \textsuperscript{1}H-NMR, \textsuperscript{13}C-NMR and FAB-mass spectra is shown in the inset.

Fig. 7. Immunoreactivity of the purified antigen against KLH-3OHKYN monoclonal antibody. Antibody was preincubated with the purified product at indicated concentrations for 2 hrs before testing in the ELISA. Inhibition of the antibody binding by 3OHKYN was also tested.

Fig. 8. Proposed core structure of the antigen for KLH-3OHKYN antibody.

Fig. 9. Effect of ninhydrin treatment of 3OHKYN on immunoreactivity. 3OHKYN was treated with ninhydrin at 65°C for 10 min and lysine was added to trap unreacted ninhydrin. The mixture was dried, dissolved in 200 µl of water and tested against the monoclonal antibody in competitive ELISA. Reaction of 3OHKYN without ninhydrin treatment and L-lysine treated with ninhydrin are also shown for comparison.

Fig. 10. Formation of 3OHKYN-derived products in human lens proteins. Low molecular weight products were isolated from the water-soluble fraction of the human lens by filtration in a Mr. 10,000 cut off filter. The filtrate was either treated with \textgreek{\textalpha}-glucosidase or untreated and incubated with WS-HLP or RNase A. The resulting modified protein was dialyzed and tested in competitive ELISA.

Fig. 11. Immunochemical detection of 3OHKYN-modification in human lens proteins. A. SDS-PAGE of water-soluble proteins from a normal (lane 1) and three cataractous human lenses (lanes 2-4): 20 µg of protein were digested with proteinase K and loaded on 18% polyacrylamide gel. Lane 5 is 3OHKYN-modified RNase A (positive control) B. Immunoblotting using the anti-KLH-3OHKYN antibody. C. immunohistochemistry of normal lens (panels 1 and 4) and cataractous (panels 2, 3 and 5) probed with: mAb anti-AQP0 (panel 4, 5), mAb anti-KLH-3OHKYN (panel 1, 2), mAb anti-KLH-3OHKYN pre-incubated with RNase A modified by 3OHKYN (panel 3). Bar=50 microns.

Fig. 12. Detection of 3OHKYN-modification in HLE-B3 cells. Cells cultured on chamber slides were treated with A. 200 µM 3OHKYN in MEM B. MEM alone for 24 hours. Cells were fixed and incubated with the anti-KLH-3OHKYN monoclonal antibody (panels 1 and 7) or with non-immune mouse IgG (panel 4). The slides were probed with Texas red-Phalloidin and DAPI (panels 2, 5 and 8), developed with Oregon green-conjugated rabbit anti-mouse IgG. The slides were observed and photographed through a fluorescence microscope. Merged images are shown in panels 3, 6 and 9. Bar=50 microns.

Fig. 13. Effect of glycation on tryptophan oxidation and formation of KLH-3OHKYN antibody reactive products. Lysine-Sepharose (Lys-Sep) was glycated with 500 mM ribose for 2 days
and then thoroughly washed with PBS. The gel was then incubated with 5 mM N\(^{\text{\textsuperscript{-}}}\)-acetyl tryptophan (AcTrp) for 3, 7 and 10 days. Tryptophan content was analyzed by reversed phase HPLC by monitoring fluorescence at 320 nm (excitation at 290 nm) (A). N\(^{\text{\textsuperscript{-}}}\)-acetyl tryptophan was incubated with ribose and +/- lysine and +/- arginine for up to 5 days. Aliquots were withdrawn at specified time-intervals and subjected to HPLC to determine tryptophan content (B). Lys-Seph was glycated with ribose and in the presence of N\(^{\text{\textsuperscript{-}}}\)-acetyl arginine. The gel was incubated with tryptophan for 20 hrs. The supernatant from the incubation was then incubated with RNase A for 3 days at 37 \(^{\circ}\)C, dialyzed and tested in a competitive ELISA. Control = unmodified RNase A (C). WS-HLP was incubated with glycating agents as described in Experimental Procedures and tested in a competitive ELISA. Control = non-glycated WS-HLP (D).
Fig. 1

L-Tryptophan

\[ \text{O}_2 \xrightarrow{\text{INF} \gamma} \text{IDO} \]

\[ \text{N-Formylkynurenine} \xrightarrow{\text{Kynurenine Formamidase}} \text{L-Kynurenine} \xrightarrow{\text{Kynurenine hydroxylase}} \text{3-Hydroxykynurenine} \]

Lens proteins

Brown pigmented/cross-linked proteins
Fig. 2
Fig. 3

![Graph showing competitor concentration vs. B/B0 for various Ac peptides: AcArg-3OHKYN, AcCys-3OHKYN, AcHis-3OHKYN, AcLys-3OHKYN, AcLys, AcArg, AcCys, AcHis, AcLys.](image-url)
Fig. 4
Fig. 5B
Fig. 7

- Purified antigen
- 3OHKYN

B/B0 vs Competitor [µM]
Fig. 13

A

Peak area [AU]

AcTrp+Lys-Seph
AcTrp+Lys-Seph+Rib

Incubation time [days]

B

Peak area [AU]

AcTrp+Rib
AcTrp+Arg+Rib
AcTrp+Lys+Rib
AcTrp+Arg+Lys+Rib

Incubation time [hr]
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