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Biochemical Analysis of Peroxynitrite Modified Human Serum Albumin (PN-HSA) in Rheumatoid Arthritis and Type I Diabetes

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ABSTRACT

Human tissues are continually exposed to a number of damaging reactive oxygen species. Peroxynitrite is more reactive, harmful and toxic to cells as it is a potent inducer of apoptic cell and causes apoptis in many cell types. Exposure to large amounts of peroxynitrite leads to necrotic cell death due to disruption of cellular metabolism and membrane integrity. It is also shown to be involved in pathogenesis of many diseases like acute and chronic inflammatory processes, atherosclerosis, rheumatoid arthritis, inflammatory bowel disease, adult respiratory distress syndrome and neurodegenerative disorders. Human serum albumin (HSA) is most abundant serum protein in circulatory system and comprises the main targets of reactive oxygen species. Among all, peroxynitrite have been implicated in the etiology of many human diseases including diabetes and rheumatoid arthritis. To understand the mechanisms of peroxynitrite cytotoxicity and its role in different diseases, it is important to characterize its reactivity toward different biomolecules. Therefore, the objectives of the present study were to carry out various biochemical studies to find the possible role of peroxynitrite modified HSA (PN-HSA) in type-1 diabetes and rheumatoid arthritis. This study presents modification of commercially available human serum albumin (HSA) with peroxynitrite, generated by the reactions of sodium nitroprusside, pyrogallol and EDTA.

INTRODUCTION

Living cells maintain a delicate balance between oxidizing and reducing species, and many disorders such as rheumatoid arthritis, diabetes, cancer and neurodegenerative diseases (e.g. Alzheimer's disease) have been associated with a disturbed intracellular 'redox equilibrium' [1]. To function properly, the living cell therefore needs to monitor, control and maintain an adequate intracellular redox balance. Several human diseases such as rheumatoid arthritis, neurodegenerative diseases (e.g. Alzheimer's disease), diabetes and cancer have been linked to a disturbed intracellular redox balance, also known as 'oxidative stress' [2–3]. The latter is characterized by dramatically increased intracellular concentrations of highly reactive, oxidizing species, that are commonly known under names such as 'reactive oxygen species' (ROS) and 'reactive nitrogen species' (RNS) [4]. In addition, an increase in 'free', adventitious transition metal ions that have been oxidatively liberated from proteins is commonly associated with oxidative stress [5]. Excess generation of reactive oxygen and reactive nitrogen species have the ability to damage proteins, DNA and other biomolecules. Among these, Peroxynitrite is a reactive oxygen species generated by reaction of superoxide (O_2) with nitric oxide (NO) which is consequently involved in a number of pathological mechanisms including inflammation, cell damage (apoptosis and cytotoxicity), and may interfere with NO•-mediated signaling [6].

Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disease primarily affecting synovial membranes of joints. [7]. Pathogenesis of Rheumatoid Arthritis is a multistep process where cellular and humoral interactions are mediated. At the site of inflammation, activation of T cells and macrophages leads to a large increase in oxygen consumption, whose corollary is increased release of ROS [8]. The disease shows prevalence in middle aged females. It has a major impact on the physical as well as mental health status of patients [9]. Elevated ROS/RNS have been reported in RA patients [10]. Normal cellular metabolism generates ROS and RNS and each body cell is exposed to 10000-20000 hits of free radicals per day [11]. Proinflammatory cytokines such as IL-1B and TNF-alpha can induce peroxynitrite generation by increasing nitric oxide synthetase activity [12]. The peroxynitrite can cause irreversible modification in variety of macromolecules through nitration, nitrosation or oxidation. [13]. The peroxynitrite generated during inflammatory conditions may cause structural changes in albumin that has been trapped in synovial cells. The nitroxidized-albumin released from the cells may persist in circulation for a

longer period than its native counterpart because of its poor susceptibility to proteases. The immunoregulatory cells may consider nitroxidized-albumin as foreign and start producing antibodies. Presence of autoantibodies against nitroxidized-albumin may be an indicator of the initiation or progression of RA [14].

Type 1 diabetes mellitus is characterized by chronic insulin deficiency and hyperglycemia due to extensive destruction of insulin-producing beta cells. The autoimmune nature of this process is supported by the presence of a pool of autoantibodies against beta cell antigens [15]. Elevated blood glucose induces oxidative stress and changes in the cellular redox state. NADPH oxidase has been responsible for the formation of high levels of reactive oxygen species (ROS) in response to high glucose [16]. A second source of ROS production is the excessive production of non-enzymatic formation called advanced glycation end products (AGEs). Two AGEs, namely carboxymethyl-lysine and pentosidine, are related to the severity of diabetic nephropathy and are known biomarkers for 'carbonyl stress' [17]. The main toxic effect of both ROS and AGEs is the induction of abnormal posttranslational modifications of self-antigens and the generation of neo-antigens, thus bypassing immune tolerance and contributing to the development of autoimmune responses [13].

Human serum albumin (HSA) is the most abundant serum protein whose redox modifications modulate its physiological function, as well as serves as bio-marker for oxidative stress. It has been well documented that HSA is quite vulnerable to reactive oxygen species [18]. Therefore it is continuously exposed to oxidative stress so that alternation of conformation and function of HSA could occur resulting in modification of its biological properties. Many studies show the presence of elevated levels of oxidized albumin in patients with diabetes mellitus (DM), aging, patients with chronic hepatitis-C. [12]. Hyperglycemia increases the production of free radical and decreases their scavenging by numbers of mechanisms. It is well documented increased oxidative stress leads to protein oxidation [19].

During pre-clinical phase of RA there is a high uptake of albumin at the sites of inflammation which serves as the metabolic fuel for highly up-regulated synovial cells [20]. The nitroxidized-albumin released from the cells may persist in circulation for a longer period than its native counterpart because of its poor susceptibility to proteases. The immunoregulatory cells may consider nitroxidized-albumin as foreign object and start producing antibodies. Presence of

autoantibodies against nitroxidized-albumin may be an indicator of the initiation or progression of RA. Co-existence of type 1 diabetes and RA in the same individuals has been reported and a large study of 3,093 individuals showed that type 1 diabetic patients are two to five times more likely than healthy individuals to develop RA [21].

Therefore, in the present study, various biochemical studies were carried out to find the possible role of peroxynitrite modified HSA (PN-HSA) in type-1 diabetes and rheumatoid arthritis. In view of this, the present study was designed to evaluate the influence of oxidative byproducts such as peroxynitrite (ONOO-) damaged HSA and to explore its biochemical and structural characteristics. Evaluation of the effect of procynitrite (ONOO-) on the biological properties of isolated HSA from diabetes and rheumatoid arthritis patients were also attempted.

MATERIALS AND METHODS

Normal human sera were obtained from healthy subjects. Sera of patients with rheumatoid arthritis and type 1 diabetes mellitus were collected respectively visited in Janaki Medical College and Teaching Hospital (JMCTH) and were decomplemented by heating at 56^0C for 30 min, stored in aliquots at 0^0C with sodium azide as preservative [22]. All patients consented to participate in the study. The work protocol was approved by the institutional ethical committee.

Protein Estimation

Protein was estimated by the Lowry's Method with slight modification [23] and HSA concentration was measured spectrophotometrically using $E^{1\%}_{1cm}$ of 5.30 at 280 nm[24]. .

Polyacrylamide Gel Electrophoresis

PAGE was performed as described by Laemmli with slight modification [25].

Modification of Human Serum Albumin

Commercially available human serum albumin (500 µg/ml) in phosphate buffer, pH 7.4 was modified by peroxynitrite, generated by the synergistic action of sodium nitroprusside (0.5 mM), pyragallol (0.5mM) in presence of EDTA (0.5mM) by a published procedure [26]. The reaction was carried out for 3 hour at 37 ± 0.5^0C such that protein was damaged by generated peroxynitrite. After completion of reaction, protein samples were dialyzed against PBS, pH 7.4.

Spectroscopic Analysis

The ultraviolet spectra of native and peroxynitrite modified HSA samples were recorded in the wavelength range of 200-400 nm on a Shimadzu UV-1700 spectrophotometer.

Fluorescence Measurements

Fluorescence measurements were performed on spectrofluorimeter (Hitachi RF-1501, Japan). The fluorescence spectra were measured at 25 ± 0.1˚C with a cell of 1 cm path length. The excitation and emission slits were set at 5 and 10 nm, respectively. Intrinsic fluorescence was measured by exciting the protein solution at 280 or 295 nm and emission spectra were recorded in the range of 300-400 nm. Loss of fluorescence intensity (F.I.) was calculated using the following equation :

$$\% \text{ Loss of F.I.} = (\text{F.I.}_{\text{native HSA}} - \text{F.I.}_{\text{modified HSA}}) / \text{F.I.}_{\text{native HSA}}) \times 100$$

SDS-PAGE under Non-Reducing Conditions

Native and peroxynitrite modified HSA were characterized by SDS-PAGE under non-reducing conditions, using 7.5% (w/v) acrylamide gels, respectively. For non-reducing SDS-PAGE, samples were not heated and samples buffer contained all above chemicals except mercaptoethanol. Gels were electrophoresed at 50 V for 3-4 h and proteins were visualized using coomassie brilliant blues R-250 or silver nitrate staining.

Absorption-Temperature Scan

Thermal denaturation profile of protein was performed on a Shimadzu UV-1700 spectrophotometer equipped with a temperature programmer and controller assembly [27]. Protein samples were in PBS, pH 7.4 and absorbance was recorded at a fixed wavelength of 280 nm.

Assay of Carbonyl Formation

Carbonyls contents of native and peroxynitrite-modified HSA were analyzed according to Levine method [25] with slight modifications. The reaction mixture containing 15 µM native HSA or peroxynitrite -HSA, (0.5 ml) 10 mM 2, 4- dinitrophenylhydrazine (DNPH) / 2.5 M HCl was added and thoroughly mixed. After addition of 1 ml 250 µM TCA (20%) and centrifugation, the pellet was collected and washed three times with 1 ml ethanol : ethylacetate (1:1) mixture.

The pellet was then dissolved in (1 ml) 6 M guanidine solution and incubated at 30˚C for 15 min. After centrifugation, the supernatant was collected and carbonyl contents were estimated from the absorbance at 370 nm using a molar absorption coefficient of 22,000 $M^{-1}cm^{1}$. Samples were spectrophotometrically analyzed against a blank of 1 ml of guanidine solution (6 M). Protein concentration was determined in the samples by Lowry method [23]. Carbonyl contents were expressed as μmol/ mg protein. Similar procedure was used for the estimation of protein carbonyl contents in normal human, type 1 diabetes Mellitus and Rheumatoid arthritis serum protein

Isolation of Human Serum Albumin

Human serum albumin (HSA) was prepared according to the method described by Tayyab S, Qasim MA with slight modification [28].

Gel Chromatography

Column of Sephadex G-100 (30x5cm) was packed according to the published procedure [28] and procedure for chromatography was followed. Protein in each fraction was monitored by Lowry method [23].

RESULTS

Peroxynitrite Modification of HSA

A solution of human serum albumin (HSA) in PBS, pH 7.4 was modified with peroxynitrite (PN) generated by a mixture of sodium nitroprusside (SNP), pyragallol and EDTA. The native and modified samples were dialyzed against PBS extensively. The native and modified samples were then analyzed by UV absorption spectroscopy on a Shimadzu spectrophotometer in wave length range of 200 nm to 400 nm shows UV absorption spectra of PN- modified HSA with characteristics hyperchromicity of 45.5% at 280 nm.The characterization of native and PN-modified HSA has been summarized in Table 1.

Absorbance Temperature Scan

During temperature-induced denaturation the protein absorbance changes with temperature until the process of unfolding is completed. Thermal melting profile of native and peroxynitrite modified HSA showed melting temperature (Tm) of native and modified HSA as 58^{0}C and

60.5^{0}C, respectively (Fig. 1). The complete unfolding of native and damaged HSA was occurred at 70^{0}C and 85^{0}C, respectively. After these temperatures both native and damaged HSA samples showed precipitation or aggregation as abnormal increase in their absorbance was noticed.

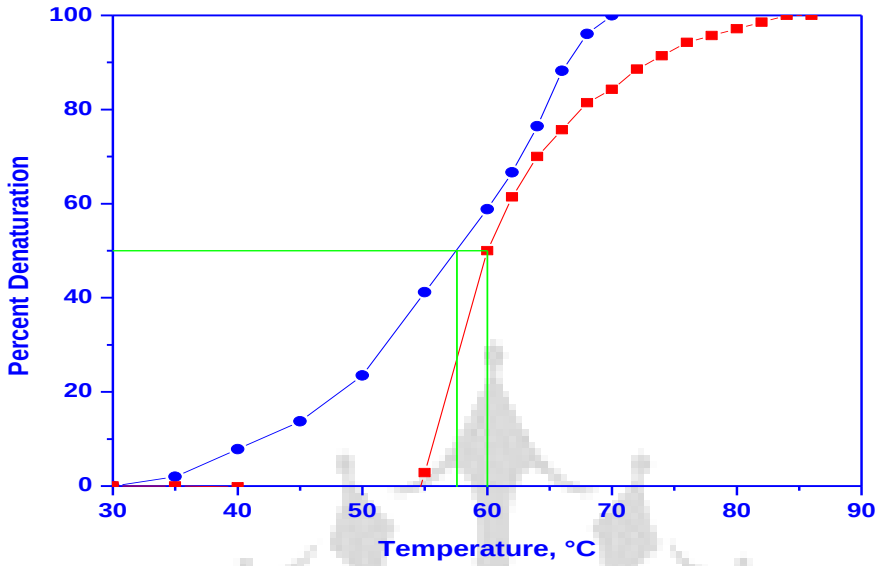

Figure 1. Thermal denaturation profile of native (●) and peroxynitite modified (■) HSA. The samples were in PBS, pH 7.4 at a concentration of 3µM.

Fluorescence Spectroscopy

HSA has single tryptophan residue (trp-214) located in domain –II. To examine the conformational variations around this residue in HSA and HSA- domain-II, protein was excited at 295 nm. The oxidation of tryptophan residue on peroxynitrite damaged HSA was evident by loss of 7.18% fluorescence intensity at 330 nm using an excitation wave length of 295 nm. The damage of the tryptophan residue was confirmed by the loss of 6.20% fluorescence intensity at 320 nm using an excitation wave length of 280 nm summarized in Table 1.

Polyacrylamide Gel Electrophoresis

SDS-PAGE of native and peroxynitrite modified HSA under non reducing condition are shown in Fig. 2. Intensity of peroxynitrite modified HSA band becomes dark as compared to native HSA on damaged by peroxynitrite. The PN- HSA band also shows slight shift towards low

molecular weight side as compared to band of native HSA. Both native and modified samples were run on 7.5% gel.

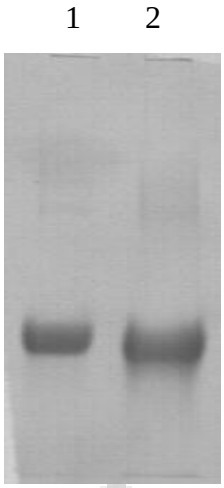

Figure 2. SDS - Polyacrylamide gel electrophoresis of native (lane 1) and peroxynitrite modified (lane 2) HSA, under non reducing conditions both protein samples were 35 µg each. Electrophoresis was carried out on 7.5% of gel for 3 hours at 50 volts.

Estimation of Carbonyl Contents in Native and Peroxynitrite Modified HSA

Oxidation- induced carbonyl formation in native and peroxynitrite modified HSA samples were 25 and 45 µM /mg of HSA, respectively. There is an increase of 55.5% of carbonyl contents in PN-HSA as compared to its native analogue. The characterization of native and peroxynitrite modified HSA has been summarized in Table 1.

Table 1: Determination of Serum Protein and Purified Albumin Oxidation

Parameter	**Native has**	**PN- HSA**	**Modification**
UV absorbance at 280 nm	0.6	1.1	45.5% (hyperchromicity)
Fluorescence intensity (At ext.295 nm and emm.330 nm	104.55	97.03	7.18% loss
Fluorescence intensity (At ext. 280 nm and emm. 320 nm)	237.75	223.0	6.20% loss
Carbonyl contents (µmol/mg of HSA)	25	45	55.5% increase
Melting temperature, ^{0}C	58^{0}C	60.5^{0}C	——

The oxidation of a protein typically results in an increase in carbonyl contents this increase is due to the oxidation of Lysine, Arginine or other amino acids residues. The data showed serum protein carbonyl contents were increased in type 1 diabetes patient, rheumatoid arthritis patients compared with normal subjects. The carbonyl contents of diabetes serum protein, rheumatoid serum protein and normal human serum protein were 11.7, 11.8 and 10.3 µM/mg of protein respectively (Figure 4). To investigate the extent of alteration in the biological properties of HSA in type 1 diabetes and rheumatoid arthritis patient, HSA was isolated from type 1 diabetes patient (DM-HSA), rheumatoid arthritis patient (RA-HSA) and also from the normal subject (NH-HSA), and their carbonyl contents were compared. The purity and homogeneity of patient purified HSA is given in Figure 3. The carbonyl contents of purified DM-HSA, RA-HSA and NH-HSA were 4.9, 5.1, 4.1 µM/mg of protein respectively (Figure 4). The results were similar to those obtained from sera (Figure 4).

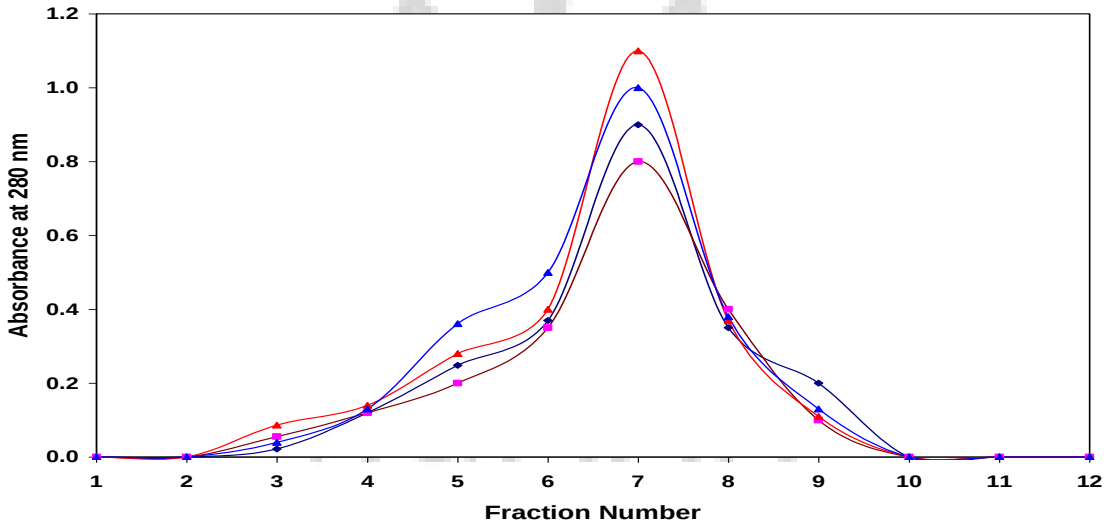

Figure 3. Elution profiles of purified HSA from the blood of type 1 diabetes patient (▪), Rheumatoid arthritis (▪), normal human subject (▪) , and commercial BSA (▪) on Sephadex G- 100 column. Elution was performed with 10 mM sodium phosphate buffer, pH 7.4, at flow rate of 15 ml per hour in 3 ml fractions.

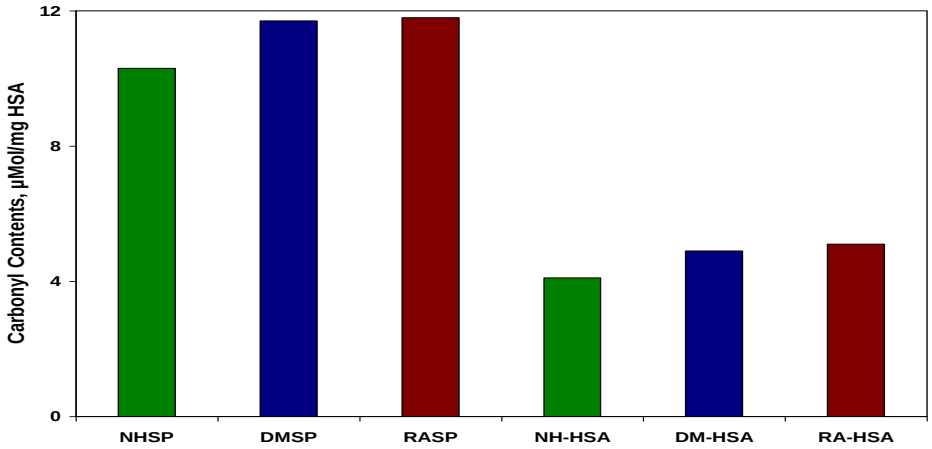

Figure 4. Comparison of carbonyl contents in normal human serum protein (NHSP), type 1 diabetes serum protein (DMSP), rheumatoid arthritis serum protein (RASP), purified HSA from normal human (NH-HSA), diabetes patient (DM-HSA) and rheumatoid arthritis patient (RA-HSA).

DISCUSSION

Continuously generating Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are regarded to be responsible for a variety of pathological conditions including cardiovascular diseases, SLE, diabetes, rheumatoid arthritis (RA) and aging. [29,30,31,32]. Peroxynitrite ($ONOO^-$) is a potent oxidant generated by various metabolic reactions under pathological conditions such as ischemia, reperfusion, injury, inflammation and sepsis [33].

Serum albumin is a most abundant serum protein whose redox modification modulates its physiological function as well as serves as biomarker of oxidative stress [34]. Many reported studies show the presence of elevated levels of oxidized albumin in patients with DM-type-1 and type-2 [35,36], patients with chronic hepatitis-C [37], patients with hepatocellular carcinoma. [38] Oxidized albumin is a reliable marker of oxidative stress in a hemodialysis patients [39,40] and alteration of redox state of HSA is also seen in patient under anesthesia and many other diseases. It has been well documented that HSA is quiet vulnerable to ROS [41]. In the present study, commercially available HSA was modified by peroxynitrite (PN), generated by the reaction of sodium nitroprusside, EDTA and pyrogallol. The PN caused extensive damage to HSA resulting in hyperchromicity at 280 nm of the spectral curve. This could be attributed to the

structural alterations in aromatic amino acids contributing to UV-light absorption or this could be the modification of chromophoric groups, modification of aromatic amino acids residues of HSA. The HSA contains only one tryptophan (trp 214), which is present in domain-2, of its structure, oxidation or nitration of trp 214 residue upon PN modification was confirmed by the decrease in fluorescence intensity after exciting the protein at 295 nm, clearly indicating conformational changes must have occurred in domain-2. These changes were further confirmed by exciting the protein at 280 nm. Thermal denaturation profile showed unfolding of peroxynitrite modified HSA required high temperature as compared to native HSA .The results indicates that stability of HSA increases upon peroxynitrite modification as PN-HSA required high temperature to melt. The data of SDS –PAGE under non-reducing condition reiterated the above results that substantial modification has been found on exposure of HSA by PN radical. Protein carbonyl contents are actually the most general indicator and by far the most commonly used biomarker of protein oxidation [42, 43, 44] and accumulation of protein carbonyl has been observed in many human disease [45]. Carbonyl contents in PN-HSA were found to be much higher as compared to its native form. This indicates that oxidation of HSA might have occurred upon peroxynitrite modification.

CONCLUSION

Commercially available human serum albumin was modified extensively with peroxynitrite, generated by a reaction of sodium nitroprusside, pyrogallol and EDTA. Peroxynitrite modified albumin resulted in UV hyperchromicity as compared to it native analogue. Oxidation of tryptophan residue (Trp-214) and conformational changes in domain-II of albumin was observed by a decrease of tryptophan fluorescence intensity. There conformational changes were further confirmed by exciting the protein samples at 280 nm. SDS-polyacrylamide gel electrophoresis further reiterates peroxynitrite modification of albumins. Thermal denaturation profile showed peroxynitrite damaged albumin was more stable than its native form. Total serum protein and purified albumin from type 1 diabetes and rheumatoid arthritis patients were found to be oxidatively modified as evident by the significant increase in the carbonyl contents. In human plasma all amino acids in the protein are susceptible to oxidative modification by oxidant such as peroxynitrite radical, hydroxyl radical (.OH) and hypochlorous acid [46]. Present data showed total serum protein carbonyl contents were increased in patients with diabetes type-1 and RA,

compared with normal subjects. Further, to investigate the extent of alteration in biological properties of HSA in diabetes and arthritis patients HSA was isolated from diabetes patient (DM-HSA), from RA-patient (RA-HSA) and also from normal human subjects (n-HSA) by ammonium sulphate fractionation followed by gel exclusion chromatography on sephadex G-100, and their carbonyl contents were compared. The present data suggests that NH-HSA offered significant protection against free radicals [47] compared to HSA.

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