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Original Article

Proteolytic Sensitivity, *In Vitro* Glycation Efficiency of Diabetic and Non-diabetic Human Serum Albumin

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Abstract

Background: Glycation of human serum albumin (HSA) leads to disturbances in its stability, activity, and other properties which, in turn, affect the functional properties of HSA. Modification of albumin by glycation shows considerable potential as a significant biochemical biomarker for diagnosing diabetes. The characteristics of the glycation process in proteins have not been fully examined yet and, therefore, there is insufficient knowledge about them in the field.

Objectives: This study aimed to clarify the differences between diabetic and non-diabetic HSA as well as their structure-function relationship.

Methods: The physiological and laboratory characteristics of glycated albumin as well as HSA were explored. A total of 30 subjects were enrolled in this study in which 15 normal healthy individuals were assigned into the control group, and 15 type-2 diabetic patients were included in the diabetic group. Patients with type-1 diabetes, pregnant women, and individuals with other diseases were excluded from the study. Protein estimation, polyacrylamide gel electrophoresis, ammonium sulphate fractionation, dialysis, glycation of HSA followed by gel electrophoresis of glycated samples, digestion of BSA, as well as HSA by α-chymotrypsin and their documentation and stoichiometry were all performed.

Results: Various characteristic differences were observed between diabetic and non-diabetic HSA including proteolytic susceptibility and *in vitro* glycation efficiency. Hypoalbuminemia was, particularly, observed in diabetic patients, which was suggestive of a relationship between hyperglycemia and hypoalbuminemia.

Conclusion: Peculiar contrariety between diabetic and non-diabetic HSA, specific differences in their glycation efficiencies, as well as proteolytic susceptibility and their innuendos were precisely traced out. It was concluded that albumin may have been regarded as a significant clinical biomarker for diagnosing diabetes.

Keywords: Diabetes, Human serum albumin, Glycation, Hypoalbuminemia, Proteolytic susceptibility

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Background

Deficiency of insulin or resistance to it, which leads to disorder in the body, is known as diabetes. Insulin hormone is responsible for regulating the glucose in the circulation (1). One of the causes of long-term effects of diabetes is associated with protein glycation. Glycation is the binding of reducing sugars or their reactive degradation products, non-enzymatically to the primary or secondary amine groups on proteins (2). Glycation of human serum albumin (HSA) leads to disturbances in its stability, activity, and other properties that further affect the functional properties of HSA (3). The reaction proceeds in the presence of glucose by forming acid-labile Schiff bases that reshuffles the initial glycation Amadori adducts-fructosamine. These Amadori products are more stable and further react to cause oxidation and polymerization, producing intermediate reactive dicarbonyl compounds or α-oxoaldehydes (4-6). HSA goes through non-enzymatic glycation by reducing sugars in the body. The modification ranges from 1% to 10% in a healthy person, which may be increased 2- or 3-fold during diabetes mellitus condition. Conclusively, modification of albumin by glycation has the potential to be regarded as a significant biochemical biomarker for diagnosing diabetes (7,8). Out of three main binding sites on the protein, two sites (site I and site II) are located on sub-domains IIA and IIIA, respectively; and have been found to have affinity for binding, specifically, to aromatic and heterocyclic ligands (9,10). The native conformation and efficiency of these binding sites are expected following the protein modification which is induced by physiological and pathological changes (11). A novel class of uremic toxins, known as advanced glycation end products (AGEs) and capable of binding to plasma proteins, has emerged (12). AGEs are indicators of various diseases, such as arteriosclerosis, renal failure, Alzheimer disease or diabetes, and have been also found to be elevated during the process of normal aging (13). Human and bovine albumin both have been the subjects of *in vitro*

and *in vivo* studies performed on structural properties of glycated albumin. Homology of 80% has been revealed for human and bovine albumin in the process of sequence comparison (14). There has been an increase in total molecular weight of the protein due to glycation among other structural modifications induced by glycation of albumin (15). The main abundant protein in serum (i.e., HSA) also undergo the glycation process (16). Field of proteomics has yet to fully characterize the process of glycation in proteins. A great need exists to identify and quantify the Amadori and AGEs which are of great clinical relevance in age related chronic diseases and for the utilization of recombinant proteins in therapy (17). The unfolding, degradation, and activity of protein glycation have an important deduction line (18). Only a few studies have investigated the effects of these post-translational modifications on the secondary and tertiary structure of proteins (19). The structural surrounding around the open glycation sites is a big dependent factor involved in the glycation reactivity and its end product development (20). The glycation reactivity and its end products very much depend on the structure and orientation of the target protein in a biological state. The glycation patterns of HSA in normal healthy person and diabetic patient are excellent examples (21). Since the major oxidized form is reversibly the oxidized HSA, the proportion of reduced HSA [HSA reduced%] changes according to surrounding conditions:
HSA (red)% = [reduced HSA/(reduced HSA + reversibly oxidized HSA)] × 100

The reduced HSA percentage has been generally found to be lower in patients with various disease conditions like liver disease (22,23), kidney disease (24), temporomandibular joint disorders (25), aging (26), and fatigue (27). The HSA fulfills various functions including: (*a*) performing free radical scavenging activity; (*b*) functioning as a source of amino acid in malnutrition conditions; (*c*) regulating osmotic balance; and (*d*) transporting various metabolites like hemin, bilirubin, steroids etc. (28-34). This study aimed to clarify the differences between diabetic and non-diabetic HSA as well as their structure-function relationship.

Materials and Methods

Chemicals

The chemicals used in this study included bovine serum albumin (SRL, India), alkaline copper reagent (Sigma, USa), Folin's reagent (Loba Chemie, India), sodium carbonate (Qualigens, India), sodium hydroxide (Fisher Scientific, USA), Tris glycine (Fisher Scientific, USA), acrylamide (Sigma, USA), glycerol (Fisher Scientific, USA), TEMED (Sigma, USA), ammonium persulphate (Sigma, USA), sodium dodecyl sulphate (SDS, SRL, India), ammonium sulphate (SRL, India), dextrose glucose (SRL, India), copper sulphate (Merck, Germany), potassium sodium tartrate (SRL, India), coomassie brilliant blue (CBB, HiMedia, India), glacial acetic acid (Thomas Baker, India), β-mercaptoethanol (Sigma, USA), α-chymotrypsin (Sigma, USA), and phenyl methyl sulfonate (Sigma, USA).

Study Design and Blood Samples

Diabetic patients approaching the JNMC Hospital of Aligarh Muslim University, Aligarh were selected for the present study. After obtaining the consent of the subjects, their blood samples (~5 mL from each) were collected. Individuals representing control group were also well-informed about the sampling, and their non-dependency on any kind of medication during the previous three months was ensured. The selection of the subjects was performed randomly, and it was not age-related or gender-based. A total of 30 subjects were enrolled in this study in which 15 normal healthy individuals were assigned to control group, and 15 type-2 diabetic patients were included in diabetic group. Exclusion criteria were patients with type-1 diabetes, pregnant women, and individuals with other diseases like thyroid and arthritis. Blood samples collected from 30 subjects were transferred to EDTA vials for analysis. Using sterilized syringe and venipuncture procedure, about 5 mL of blood was obtained from each subject and, then, the samples were transported to the lab in ice jar. The blood samples were stored at room temperature for about 4-5 hours to ooze out the sera. Low speed centrifugation was performed at 2000 rpm for 10 minutes at 0°C, and the resulting pale yellow colored serum of each sample was collected using micropipette. Sera samples were either analyzed afresh or stored at -20°C for further analysis. The comprehensive history of laboratory and clinical evidence of the subjects were reviewed. All aspects regarding age, sex, etc. of the subjects were recorded during the study period.

Protein Estimation of Sera Samples

Protein concentration was estimated in sera samples based on the method adopted by Lowry et al and by using bovine serum albumin (BSA) as standard (35). Optical density (absorbance) was read at 660 nm on a UV-1700Pharma-spec UV-visible spectrophotometer.

Polyacrylamide Gel Electrophoresis (PAGE) of Sera Samples

Polypeptide Profiling by Native PAGE and Localization by CBB Staining

Polyacrylamide gel electrophoresis (PAGE) was performed following the procedure of Laemmli (1970) with the change that gels were lacking SDS (36). Equal amounts (2 µg) of sera were loaded according to protein concentration on gel which was run at 75 V, 10 mA for 4 hours at room temperature. When run time was over, the gel was stained with CBB for 20 minutes and destained in 5% acetic acid.

Denaturing PAGE

PAGE was essentially carried out in the presence of SDS according to the protocol of Laemmli (36). Equal amounts of proteins were loaded in the gel, and the gel was run at 75 V for 3-4 hours at room temperature. After the completion of run, the gel was washed overnight to remove

SDS and stained with CBB. Silver staining of the SDS-Polyacrylamide gels from digested samples was performed according to the protocol of Nesterenko et al (37) after the runs were over.

Ammonium Sulphate Fractionation

Sera samples of diabetic and non-diabetic subjects were pooled. Standard protocol of ammonium sulphate fractionation was followed to obtain albumin fractions of sera proteins (38). Two cuts at 60% and 70% (w/v) of ammonium sulphate were selected. The precipitates obtained following the low-speed centrifugation for 10 minutes at 4°C were collected and saved for further study. Pooled precipitates obtained after ammonium sulphate fractionation were extensively dialyzed against chilled phosphate buffer (100mM, 7.2 pH) for 3 days in order to remove salt from them.

Glycation of HSA

Glycation treatments were conducted according to the protocol of Kańska and Boratyński (39). First, 150 µL of non-diabetic HSA (5 mg/mL) was mixed with 150 µL of sugar solution (10 mg/mL glucose) and, then, 150 µL of 0.1 M phosphate buffer (pH 8.0) was added. Samples were frozen at -40°C, freeze dried and heated at 25°C, 37°C, and at 55°C for 30, 60, 120 and 180 minutes. The same procedure was followed with diabetic samples. Now, 150 µL of diabetic HSA (4 mg/mL) was mixed with 150 µL of sugar solution (8 mg/mL glucose) and, then, 150 µL of 0.1 M phosphate buffer (pH 8.0) was added. Samples were frozen at -40°C, freeze dried and heated at 25°C, 37°C, and at 55°C for 30, 60, 120 and 180 minutes. The difference in concentrations of non-diabetic and diabetic HSA was neutralized by the difference in concentrations of the sugar solutions so as to keep the ratio of serum albumin concentration to sugar equal (i.e. 1:2).

Gel Electrophoresis of Glycated Samples

Glycoconjugates from each treatment were resolved in 10% SDS-PAGE. Protein load in each slot was 2 µg, and gels were stained with CBB.

Digestion of BSA and HSA by α-Chymotrypsin

Limited proteolysis of diabetic and control HSA samples along with BSA was carried out by α-chymotrypsin (40). One mg of glycated HSA was dissolved in 1.3 mL of 50 mM NH_4HCO_3 buffer solution (pH 8.3). After adding 15 µL of a solution 45 mM of β-mercaptoethanol, the mixture was heated at 50°C for 15 minutes, then incubated with α-chymotrypsin (200 µL of a solution 100 ng/µL, substrate to enzyme ratio = 25:1 w/w) overnight at 37°C. The reaction was stopped with 80 µL of 10% PMSF.

Documentation and Stoichiometry

Stained PA-gels were photographed using SONY-CYBERSHOT digital camera (zoom-4X, 14.1 megapixels). The photographs were transferred on the LCD and converted into compatible formats for software analysis. Gels were also processed through Adobe Photoshop (version 7.0; Windows XP) to obtain the best contrast for densitometric analysis through Scion Image (Scion Corporation: 4.0). Molecular weight was estimated using GelPro (Media Cybernetics, USA) software.

Results

Polyacrylamide Gel Electrophoresis of Sera Samples

Figure 1 shows the sera collected from non-diabetic and diabetic subjects along with the densitogram of the sera samples of some lanes from both categories. Generally, quantitative differences in various sera fractions were observed; in case of diabetics, however, the peak area of albumin was lower compared to that in the control. This was indicative of the decreased level of albumin (glycated) in diabetics. The densitometric analysis of the polyacrylamide gels obtained under denatured conditions demonstrated that the quantity of polypeptide and albumin levels was elevated in non-diabetic in comparison to diabetic subjects.

Ammonium Sulphate Fractionation

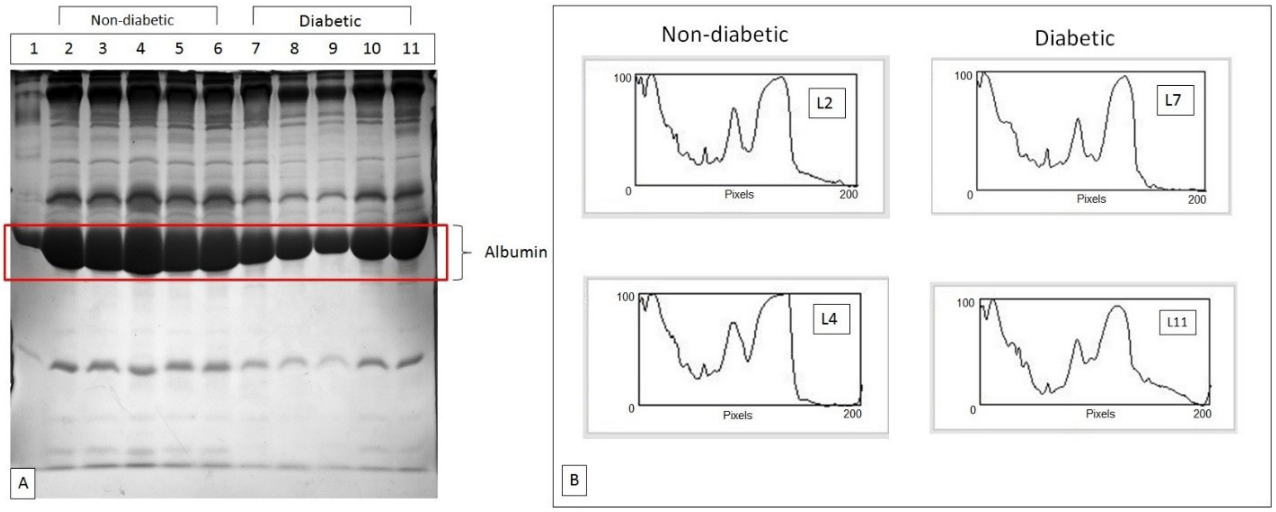

Figure 1. (A) SDA-PAGE Profiles of non-diabetic and diabetic samples showing depleted albumin levels in diabetic patient samples; (B) Densitograms of protein profiles of control and diabetic patients showing the differences in the quantity of various sera fractions and evidently decreased level of albumin in diabetic patients.

The ammonium sulphate fractionation successfully purified the albumin protein from the sera samples of both diabetic and non-diabetic samples. There was no specific difference between the polypeptide profiles of fractionated albumin of both diabetic and non-diabetic samples.

Glycation of HSA and Gel Electrophoresis of Glycated Samples

After fractionation and dialysis, pooled non-diabetic and diabetic albumin samples were glycated *in vitro* using glucose (Figure 2A and 2B). Pooled non-diabetic and diabetic albumin samples were glycated for different time durations (30, 60, 120, and 180 minutes) at 37°C and run on gel under denatured conditions (Figure 2A and 2B). The albumin purified from non-diabetic samples showed more glycated efficiency compared to that from diabetic samples. Moreover, both non-diabetic and diabetic albumin samples glycated for different time durations were also monitored, and the samples were run on gel under denatured condition at different temperatures (25°C, 37°C, and 55°C) (Figure 3A and 3B). As was evident from the gels, the purified human albumin under physiological conditions with respect to pH and ion composition incorporated glucose in time dependent manner.The rate of glucose uptake was also dependent on temperature. Marked peaks in Figures (3A and 3B) are significant. Figure 3B demonstrates the PAGE profiles of different varieties of diabetic samples (i.e., control serum, fractionated sample, glycated samples treated for different temperature and time duration). This SDS-PAGE profile confirmed the presence of polypeptides in the range of 14.3-203 kD. PAGE profile demonstrated that albumin was a band of ~66 kD.

Digestion of BSA and HSA by α-Chymotrypsin

Major differences were observed in the polypeptide profiles of both BSA and fractionated purified HSA after proteolysis by α-chymotrypsin (Figure 4A and 4B). Polyacrylamide gel electrophoresis answered some of the queries as whether the disease diabetes affected the susceptibility for proteolysis, and which one of the HSA samples had a higher proteolytic sensitivity. The results of time dependent proteolytic digestion manifested the presence of lesser number of bands in diabetics compared to non-diabetic HSA. This implied that the onset of diabetes led to the decreased susceptibility towards proteolysis. As the proteolysis proceeded, new peptide bands appeared with smaller molecular weights below HSA in non-diabetic samples. This suggested that HSA was specifically degraded into certain large fragments. Hence, the new peptide fragments exhibited in the non-diabetic HSA depicted its increased proteolytic sensitivity.

Discussion

Protein modifications by undergoing the process of glycation mainly occurs in diabetes which, in turn, leads to various other aggravations like atherosclerosis, renal failure, etc (41). The structural and functional properties of albumin are modified by glycation (42), including the protein affinity for therapeutic drugs (43). In the current scenario, diabetes poses a challenge to the healthcare systems. Disease is characterized by chronic

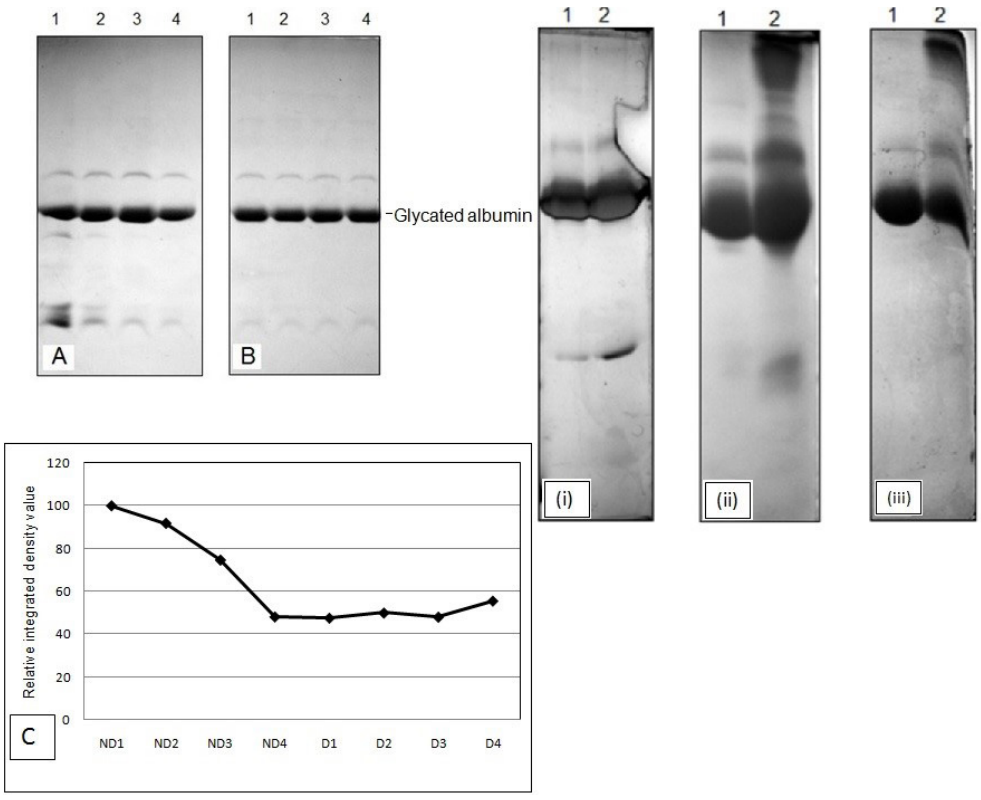

Figure 2. (A) SDS-PAGE profiles of fractionated non-diabetic sera samples glycated (B) SDS PAGE profiles of fractionated diabetic sera samples glycated; (C) relative integrated density value of non-diabetic and diabetic sera samples (i) 25°C (ii) 37°C (iii) 55°C

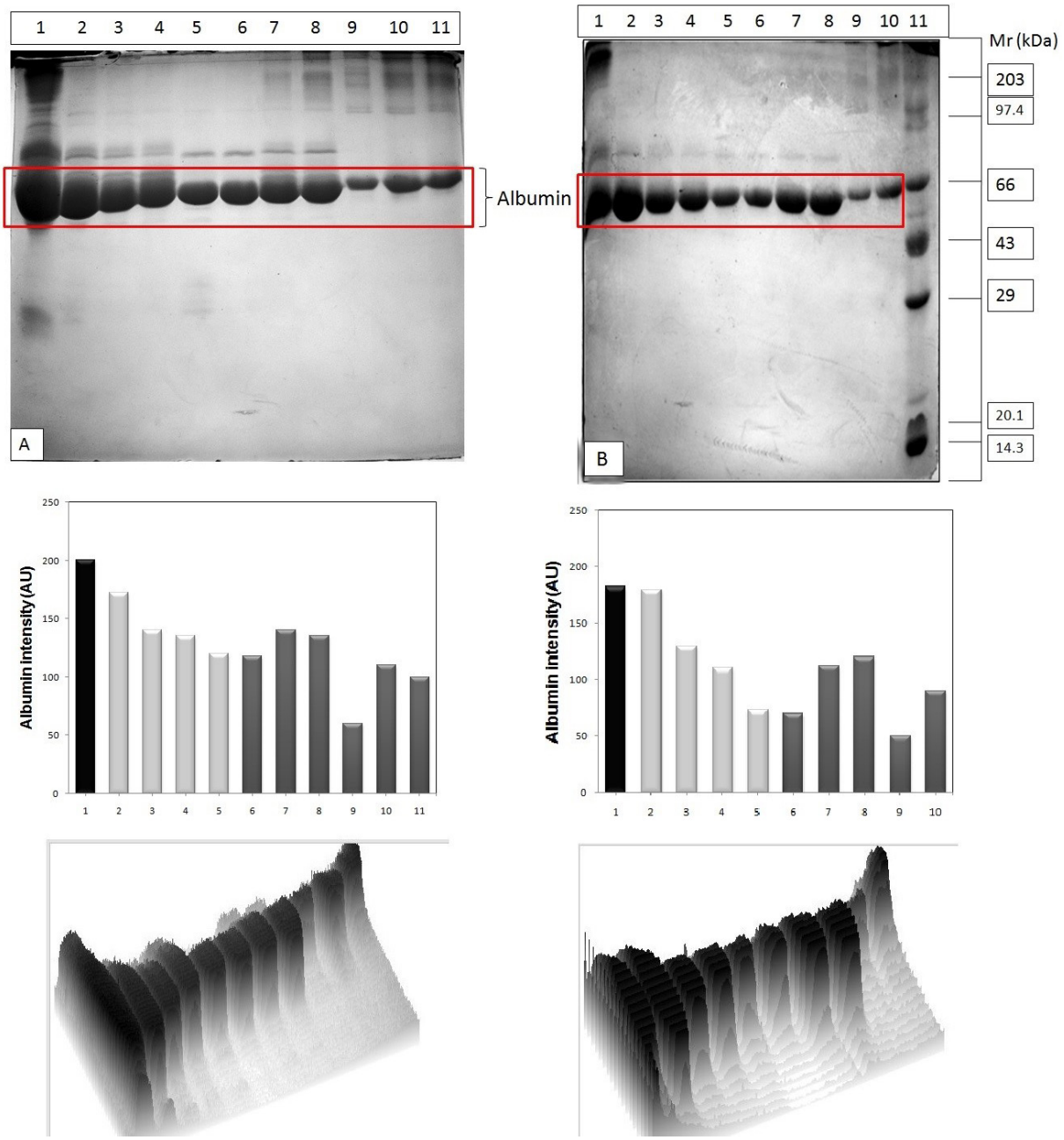

Figure 3. (A) (A) SDS PAGE profiles of non-diabetic sera samples sequentially glycated; Lane 1- Serum control, L2- Fractionated control, L3- Gly 30 min @25°C, L4-Gly 180 min @25°C, L5- Gly 30 min @37°C, L6-Gly 180 min @37°C, L7- Gly 30 min @55°C, L8-Gly 180 min@55°C, L9-BSA control (B) SDS PAGE profiles of diabetic sera samples sequentially glycated. GelPro and ScionImaging software analysis of the albumin intensity in the gel images is given in each corresponding column.

hyperglycemia, and its long-term effects cause more morbidity and mortality. If the disease remains untreated, it may cause further organ dysfunctions such as kidney failure, amputation of limbs, and blindness (44). These dysfunctions have proven to be highly costly for the healthcare systems, and reduces the life expectancy of the diabetic patients (44,45). Diabetes mellitus is diagnosed by using glycated hemoglobin (HbA1c), fasting plasma glucose and 2-hour post-prandial blood sugar, and an oral glucose tolerance test (44,46).

Glycemic monitoring is carried out by the reference test HbA1c as it also reflects mean glycemia (47) and is associated with the long-term effects of diabetes mellitus (47,48). The clinical conditions involving hemoglobin metabolism are not suitable for using HbA1c (reference test for glycated hemoglobin) and, therefore, it is not recommended as well (46,49,50). This may be attributable to the interference in the results which cause misinterpretation. The monitoring of glycemic index in diabetes mellitus in the last decades has been tested by glycated albumin and some importance has been attached to as a laboratory test for diagnosing diabetes mellitus (51). Glycated albumin as a marker has some advantages; for instance, it reflects short term glycemia due to the half-life of the albumin, which is 3 weeks, and its measurement does not require the fasting levels. In some clinical conditions such as anemia, postprandial hyperglycemia, pregnancy, and diabetes mellitus, the glycated albumin

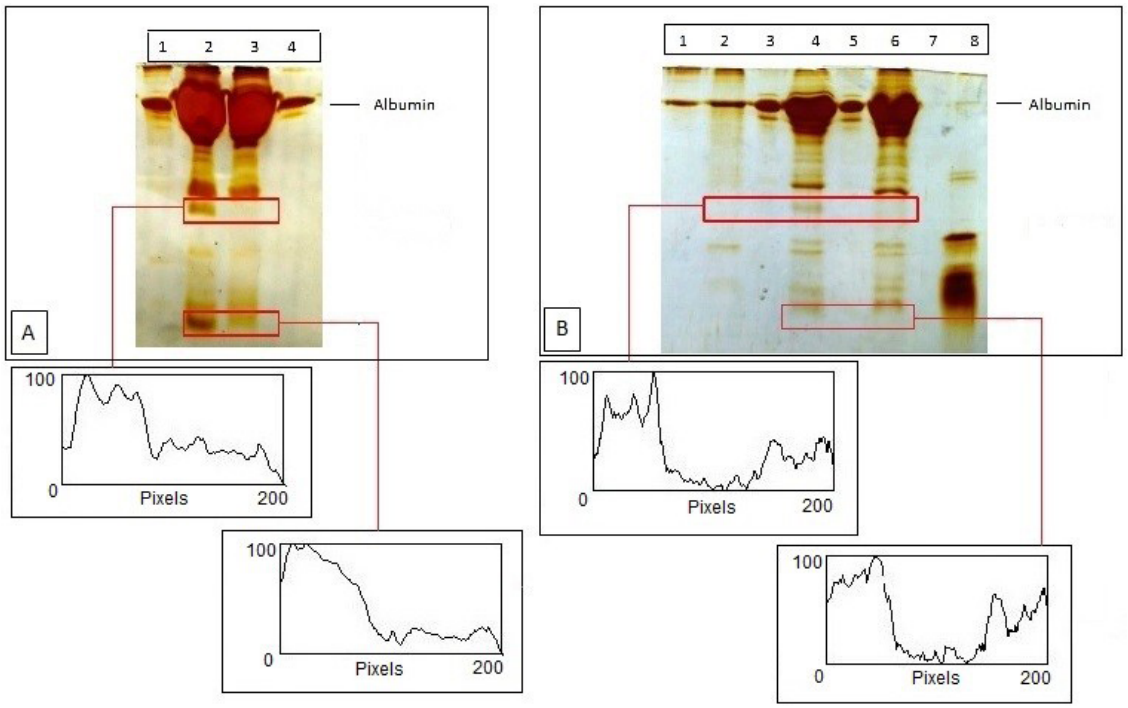

Figure 4. (A) Chymotryptic digestion of HSA of non-diabetic and diabetic samples (B) Lane 1- Undigested BSA, L2- Chymotryptic digestion of BSA Control Sample, L3- Undigested HSA of non-diabetic sample, L4- Chymotryptic digestion of HSA of non-diabetic sample, L5-Undigested HSA of diabetic sample and L6- Chymotryptic digestion of HSA of diabetic sample. Densitograms of gel lanes in each Figure is also given based on Scion Imaging software analysis.

has been detected to be more effective glycemic marker than HbA1c (52). Glycated albumin is used as a marker, especially, for patients on hemodialysis (53,54). In this regard, the present study explored the physiological and laboratory characteristics of glycated albumin as well as HSA.

Despite an established biomarker of glycemic control, HbA1c does not correlate well with the severity of the disease in conditions of hyperglycemia (55). It is noteworthy that the synthesis of albumin depends on an adequate amount of insulin reserve in the context of diabetes (56-58). Serum albumin concentrations have been found to be in inverse relation with HbA1c (59). This suggests that insulin deficiency and hyperglycemic condition are associated with hypoalbuminemia. Our SDS-PAGE profiles also exhibited low albumin levels in samples from diabetic patients. Serum albumin plays an important role when it comes to analyzing the illness. Studies have revealed that hypoalbuminemia is a constant characteristic in critically ill patients (60,61). Our argument was consistent with the results from other important studies on albumin concentration since low serum albumin levels had been found in hyperglycemic patients (62). Analyzing SDS-PAGE profiles of sera samples from both normal and diabetic patients in this study showed that albumin levels in diabetic samples were lower. This clearly supports the previous studies where hypoalbuminemia has been related to hyperglycemia.

Glycation at three subsequent temperatures (i.e., 25°C, 37°C, and 55°C) provided information about the comparative glycation sites in non-diabetic and diabetic HSA. These observations were similar to those for the glycation on HSA (21) and the protein exhibited more glycation sites in the normal blood than in diabetic blood. A slight increase of relative migration distance was detected in the non-diabetic glycated bands at subsequent temperatures. Least glycation was found at 37°C compared to 25°C and 55°C. BSA also showed lower susceptibility to glycation compared to non-diabetic HSA. Diabetic samples glycated subsequently at temperatures 25°C, 37°C and 55°C also exhibited increasing relative migration distance similar to non-diabetic trend. However, comparative analysis of glycated albumin between non-diabetic and diabetic revealed a higher relative migration distance in non-diabetics, which was most likely induced by the addition of glucose residues to HSA.

Protease resistance induced by the process of glycation in collagen (63-65) and amyloid (66) has been already investigated. In diabetes, disintegration of function of proteasomes along with glycation may favor the accumulation of protease resistant protein aggregates. AGEs are partially resistant to proteolytic digestion, while healthy proteasomal processing of cellular proteins is an efficient and complete process which releases bioavailable amino acids and oligopeptides (67). Similar results were produced by our study concerning chymotryptic digestion of both non-diabetic and diabetic HSA since more proteolytic sensitivity was observed in non- diabetic HSA samples. This was indicative of a lower proteolytic susceptibility in diabetic HSA samples.

Conclusion

Conclusively, albumin may be a significant clinical biomarker for diabetes due to characteristic differences

such as proteolytic susceptibility and in vitro glycation efficiency between diabetic and non-diabetic HSA. Hypoalbuminemia was also observed in diabetic patients, which was suggestive of a relationship between hyperglycemia and hypoalbuminemia. Our study, in particular, explored the physiological and laboratory characteristics of glycated albumin and HSA. The peculiar contrariety between diabetic and non-diabetic HSA and their innuendos was briefly discussed. Albumin in its glycated form was found to be a significant biomarker for diagnosing diabetes; however, it was recommended that further studies be conducted to thoroughly explore it.

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Authors' Contribution

HH analysed data and wrote first draft. RA conceived the idea, checked results and drafts, and finally approved.

Conflict of Interests

None.

Ethical Issues

None.

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None.

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