BMB
reports

Identification of AGE-precursors and AGE formation in glycation-induced BSA peptides

*Waqar Ahmad, Lili Li & Yulin Deng**
School of Life Science & Technology, Beijing Institute of Technology (BIT), Beijing-10008, P.R. China

The glycation of BSA leads to protein/peptide modifications that result in the formation of AGEs. AGEs react with the amino groups of N-terminal amino acid residues, particularly arginine and lysine residues. Enhanced AGE formation exists in the blood and tissues of diabetics, as well as in aging and other disorders. The Identification of AGEs is of great importance. Mass spectrometry has been applied to identify and structurally elucidate AGEs. Here, we report on the identification of AGE- peptides and AGE-precursors based on relative mass changes as a result of specific AGE formation. HPLC-ESIMS, ESI-MS/MS, and the Mascot database were used. The relative mass changes due to the specific type of AGE formation were added to the identified peptides followed by a manual search of the glycated samples, which resulted in the identification of seven peptides for the formation of five AGEs, namely CML, pyrraline, imidazolone A, imidazolone B, and AFGP. Four glycated peptides (FPK, ECCDKPLLEK, IETMR, and HLVDEPQNLIK) were identified in the formation of AGE-precursors. [BMB reports 2008; 41(7): 516-522]

INTRODUCTION

Advanced glycation end products (AGEs) are a heterogeneous group of proteins that have been modified with glucose or carbohydrate adducts, and arise from the nonenzymatic addition of reducing sugars to the side chains of lysine and/or arginine residues in proteins (1). The free amino groups of the proteins react slowly with the reducing sugars, including glucose, via glycation or the Maillard reaction (2-4). This process is initiated by condensation reactions between the reducing sugars and free amino groups to form Schiff bases, which undergo rearrangement to form relatively stable Amadori products (3, 5). The Amadori products, through oxidation and dehydration reactions, subsequently degrade into a variety of carbonyl compounds (glyoxal, methylglyoxal, deoxyglucosones) (6, 7). These compounds are more reactive than the parent sugars with respect to their ability to react with the protein amino groups to form cross-links, stable end products called *advanced Maillard products* or advanced glycation end products (1). The exposure of proteins to high glucose levels, as observed in diabetes, and the further reaction of glucose with proteins are retained responsible for the long-term complications of this disease (8). Chronic hyperglycemia leads to micro- and macro-vascular diabetic complications through four intracellular mechanisms, including increased polyol pathway flow (9, 10), the activation of protein kinase C (11), increases in the hexosamine pathway (12), and increased AGE formation (13). AGEs accumulate slowly in long-lived proteins such as collagen, resulting in the altered structure and properties of certain tissues such as vascular walls, the kidneys, etc. (14, 15). This process is thought to be an important pathogenic factor for diabetes complications (16-20). AGE accumulation in tissue proteins has been implicated in the pathological development of aging, diabetes, atherosclerosis, as well as in diabetes complications such as retinopathy, nephropathy, and neuropathy (21, 22). The three main mechanisms by which AGEs cause tissue damage are: cross-link formation, interactions with specific cellular receptors, and intracellular glycation (13).

Serum AGE concentration is recognized as a marker for monitoring the treatment of diabetic patients, especially those with renal and/or vascular complications. However, utilizing AGE measurements in clinical practice remains limited by a lack of simple and rapid analytical procedures (23, 24). The high sensitivity and specificity of mass spectrometry and the utilization of its ionization systems for analyzing polar macromolecules are shown to be effective in the study of glycation processes (25). The LC-MS/MS approach with electrospray ionization (ESI) has proved to be highly effective in analyzing glycated amino acids and peptides (8). Therefore, HPLC-ESI-MS and ESI-MS/MS were used to study glycation-induced post-translational protein/peptide modifications with the subsequent identification of AGEs and AGE-precursors. Keeping in mind the high complexity of plasma, the direct identification of AGE-peptides is certainly a difficult task. Consequently, we started from *in-vitro* experiments, based on the *in vitro* non-enzymatic glycation of BSA followed by enzymatic digestion with trypsin. Here, we report on the glycation-induced protein/peptide modifications as well as the identification of both AGE-precursors

*Corresponding author. Tel / Fax: 86-10-6891-4607;
Email: deng@bit.edu.cn

Received 22 October 2007, Accepted 26 December 2007

Keywords: AGEs, Bovine serum albumin, Glycated peptides, Mass spectrometry

and AGEs by means of HPLC-ESI-MS, ESI-MS/MS, and the Mascot database, using a manual search method of analysis. This identification relied on the relative mass changes (△ mass) due to the formation of the AGE-precursors and AGEs.

RESULTS AND DISCUSSION

The incubation of BSA with glucose is shown to induce protein modifications that eventually result in the formation of AGEs (15, 26). Enhanced AGE formation was found to exist in the blood and tissues of diabetics as well as in various pathophysiological states, such as atherosclerosis, Alzheimer's disease, end-stage renal disease, rheumatoid arthritis, and liver cirrhosis (27). AGEs induce chemical modifications and cross-linking of tissue proteins, affecting structure, function, and turnover, and ultimately contributing to gradual declines in tissue function and to the pathogenesis of diabetic complications. Various AGE structures such as imidazolone A and B, pyrraline, 1-alkyl-2-formyl-3,4-glycosyl-pyrrole (AFGP), crossline, carboxyethyl-lysine (CEL), carboxymethyl-lysine (CML), deoxyglucosone-derived lysine dimmer (DOLD), fructosyl-lysine (FL), glyoxalderived lysine dimmer (GOLD), methylglyoxal-derived lysine dimmer (MOLD), and pentosidine were detected in the cellular and extracellular proteins of physiological systems (23, 28, 29). The identification of AGEs based on their relative mass changes (△ mass) was previously reported (30).

We undertook this study to investigate glycation-induced protein/peptide modification with subsequent AGE formation originating from the enzymatic digestion (trypsin) of glycated BSA. Briefly, control BSA and BSA treated with D-glucose (1.0 and 2.5 M) were digested with trypsin. The reason for choosing these high glucose concentrations, which are quite different from physiological levels, was to enhance the yield of glycation with subsequent AGE formation. Digestion was completed without any derivatization of the sulfhydryl groups, to remain as close as possible to the conditions present at the systemic level. The digestion mixtures of BSA (control) were analyzed using LC-ESI/MS and ESI-MS/MS experiments with incubation for 0 days, whereas the LC-ESI/MS experiments were performed to analyze the glycated BSA incubated for 14 and 28 days. The MS/MS data of the control BSA (0 days incubation) were exported to the online Mascot database in order to rationalize the data and to identify the various peptides and their amino acid sequences in the online database (Fig. 1). For the control BSA digested with trypsin, a total of 27 peptides and their amino acid sequences were matched in the online database search query (Table 1). Mass increments of 58.03, 108.02, 144.03, 142.03, 162.02, and 270.07 were reported for the formation of N^{ε}-(carboxymethyl)-lysine (CML), pyrraline, imidazolone A, imidazolone B, Schiff bases or Amadori products (AP), and 1-alkyl-2-formyl-3,4-glycosyl-pyrrole (AFGP), respectively (30). A data set of theoretical peptide masses was generated by simply adding the corresponding mass increments for these specified AGEs and AGE precursors to the known peptides of the non-glycated control BSA (Table 1). These theoretical peptide masses corresponded to the formation of the respective AGEs. A thorough manual search to compare these theoretical masses with the HPLC-ESIMS experimental data was conducted in both the glycated and control (non-glycated) BSA samples. The LC-MS parameters to edit the chromatograms for this manual search of the experimental data (HPLC-ESIMS) were: (1) type: *extracted ion chromatogram (EIC)*, (2) scan mode: *all*, (3) width: *±0.5*, (4) polarity: *positive [+]* and (5) background: *none*. The △ m/z that corresponded to any of the specified AGE formations was carefully examined in the chromatograms and spectra of both the control and glycated BSA samples. In this manner, the glycation-induced formation of five AGEs was observed via the cross-linking with seven peptides (two for CML, two for pyrraline, one each for imidazolone A and B, and one for AGFP). Similarly, four glycated peptides were identified in the formation of AGE-precursors (Schiff base or AP). The degrees of incidence for these identified AGEs and AGE-precursors (Schiff base and AP), as a function of glucose concentration and incubation time, are given in Table 2, where '-' stands for absence and '+' for presence. An increasing number of '+' stands for increasing peak intensity as observed in the various glycated BSA samples.

Identification of CML

Carboxymethyl-lysine (CML), a glyco-oxidation product, was structurally characterized. CML is not reactive and not fluorescent, and derives from the oxidative degradation of Amadori products (31). CML and pentosidine are present within the collagen of diabetic subjects, where high levels were correlated with retinopathy and nephropathy (32). Only two glycated peptides were identified in our search for CML formation. The data

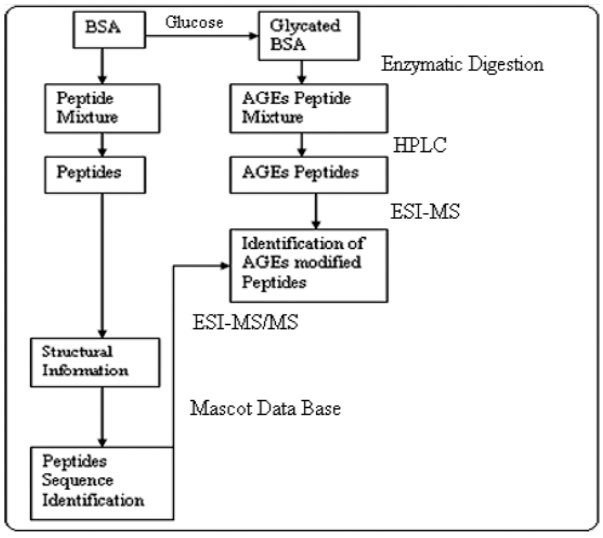

Fig. 1. Flow chart of the analytical strategy adopted for AGE Identification.

Table 1. Theoretical mass list obtained after addition of relative masse change (△ mass) for AGEs and AGEs-precursors with peptides identified for control BSA

Control BSA		CML (+58.03)	Pyrraline (+108.02)	Imidazolon A (+144.03)	Imidazolon B (+142.03)	Schiff Base/AP (+162.02)	AFGP (+270.07)
m/z	Peptide Sequence						
347.38	VTK	405.41	455.58	491.41	489.41	509.4	617.45
391.22	FPK	449.25	499.42	535.25	533.25	553.24	661.29
395.26	LVTDLTK	453.29	503.46	539.29	537.29	557.28	665.33
464.36	YLYEIAR	522.39	572.56	608.39	606.39	626.38	734.43
507.89	QTALVELLK	565.92	616.09	651.92	649.92	669.91	777.96
511.76	CCTESLVNR	569.79	619.96	655.79	653.79	673.78	781.83
545.35	VASLR	603.38	653.55	689.38	687.38	707.37	815.42
571.96	KQTALVELLK	629.99	680.16	715.99	713.99	733.98	842.03
582.41	LVNELTEFAK	640.44	690.61	726.44	724.44	744.43	852.48
588.4	ECCDKPLLEK	646.43	696.6	732.43	730.43	750.42	858.47
625.46	FKDLGEEHFK	683.49	733.66	769.49	767.49	787.48	895.53
649.3	IETMR	707.33	757.5	793.33	791.33	811.32	919.37
653.52	HLVDEPQNLIK	711.55	761.72	797.55	795.55	815.54	923.59
660.35	TPVSEK	718.38	768.55	804.38	802.38	822.37	930.42
681.95	SLHTLFGDELCK	739.98	790.15	825.98	823.98	843.97	952.02
700.57	TVMENFVAFVDK	758.6	808.77	844.6	842.6	862.59	970.64
720.68	RHPEYAVSVLLR	778.71	828.88	864.71	862.71	882.7	990.75
740.68	LGEYGFQNALIVR	798.71	848.88	884.71	882.71	902.7	1010.75
784.43	*DAFLGSFLYEYSR*	842.46	892.63	928.46	926.46	946.45	1054.5
784.71	*DAFLGSFLYEYSR*	842.74	892.91	928.74	926.74	946.73	1054.78
812.87	GLVLIAFSQYLQQCPFDEHVK	870.9	921.07	956.9	954.9	974.89	1082.94
820.85	KVPQVSTPTLVEVSR	878.88	929.05	964.88	962.88	982.87	1090.92
834.49	MPCTEDYLSLILNR	892.52	942.69	978.52	976.52	996.51	1104.56
847.59	LSQKFPK	905.62	955.79	991.62	989.62	1009.61	1117.66
912.57	RPCFSALTPDETYVPK	970.6	1020.77	1056.6	1054.6	1074.59	1182.64
461.82	AEFVEVTK	519.85	570.02	605.85	603.85	623.84	731.89
922.69	AEFVEVTK	980.72	1030.89	1066.72	1064.72	1084.71	1192.76
945.51	HPYFYAPELLYYANK	1003.54	1053.71	1089.54	1087.54	1107.53	1215.58
1023.2	RHPYFYAPELLYYANK	1081.23	1131.4	1167.23	1165.23	1185.22	1293.27

A mass increment of 58.03, 108.02, 144.03, 142.03, 162.02, and 270.07 for the formation of (CML, Pyrraline, Imidazolone A, Imidazolone B, Schiff base or Amadori product (AP), and AFGP, respectively, were added to peptides identified (mascot data base) for control bsa

search revealed a m/z of 711.3 that was prominent in the EIC and mass spectra of the glycated BSA (2.5 M D-glucose) incubated for 28 days, but absent in the non-glycated control BSA as well as the glycated BSA (2.5 M D-glucose) incubated for 14 days. The m/z at 711.3 corresponded to the peptide **H**LVDEPQNLI**K** (a. a. 402-412) plus a CML (+58.03). Similarly, a m/z at 1081.2 corresponded to the peptide R**H**PYFYAPELLYY AN**K** (a. a. 168-183) plus a CML (+58.03), which was absent in the control BSA sample as well as the glycated BSA (1.0 and 2.5 M D-Glucose) incubated for 14 days, but present in the glycated BSA samples incubated for 28 days (Table 2). This indicates that CML formation was dependent on both the glucose concentration and incubation time, and therefore, supports the pre-existing hypothesis that both the process of glycation and subsequent AGE formation are reactant concentration and reaction time dependent. CML formation is characterized by peptide crosslinking at lysine or histidine residues; therefore, the two glycated peptides (R**H**PYFYAPELLYYAN**K** and **H**LVDEPQN LI**K**) identified for CML formation were expected to have undergone crosslinking at their lysine (**K**) or histidine (**H**) residues.

Identification of pyrraline

Pyrraline has been identified as an advanced glycation end product both *in vitro* and *in vivo*. Higher levels of pyrraline were found in the urine of diabetics than in that of control subjects by an HPLC method (31). Two peptides (m/z 768.37 and 808.1) were identified for crosslinking and the subsequent formation of pyrraline in the glycated samples. A *m/z* at 808.1 was detected in the EIC and mass spectra of the glycated BSA (2.5 M D-glucose) incubated for 28 days, but was absent in the EIC of the control BSA and the BSA incubated for 14 days with 2.5 M D-glucose. The m/z of 808.1 corresponded to the peptide TVMENFVAFVD**K** (a. a. 569 - 580) plus pyrraline (+108.02). Similarly, a second modified peptide TPVSE**K** (m/z 768.0) was only present in the glycated BSA (1.0 and 2.5 M D-glucose) incubated for 28 days, and was absent in the control BSA as well as the glycated BSA (1.0 and 2.5 M D-glucose) incubated for 14 days (Table 2). Pyrraline-peptide crosslinkages also occur at either lysine or histidine residues. However, crosslinking was expected to have occurred at the lysine residue (**K**) of the two glycated peptides (TVMENFVAFVD**K**

Table 2. The comparative incidence of AGEs and AGE-precursors in control and glycated BSA

AGE-precursors and AGEs	Control BSA (0 Day)	Glycated BSA			
		14 Days		35 Days	
		(G 1.0M)	(G 2.5M)	(G 1.0M)	(G 2.5M)
HLVDEPQNLI**K** |(711.55) **CML**	-	-	-	+	+ +
R**H**PYFYAPELLYYAN**K** |(1081.23) **CML**	-	-	-	+	+ +
TPVSE**K** |(768.55) **Pyrraline**	-	-	-	+ +	+ +
TVMENFVAFVD**K** |(808.77) **Pyrraline**	-	-	-	-	+ +
IETM**R** |(791.33) **Imidazolone B**	-	-	-	+ + +	+ +
LGEYGFQNALIV**R** |(884.71) **Imidazolone A**	-	-	-	-	+ + +
SL**H**TLFGDELC**K** |(952.02) **AFGP**	-	+ +	+ +	+ +	+ +
FPK |(553.24) **Schiff Base/AP**	-	+ + +	+	-	-
ECCDKPLLEK |(750.42) **Schiff Base/AP**	-	+ + +	+ +	-	-
IETMR |(811.32) **Schiff Base/AP**	-	-	+ + +	-	-
HLVDEPQNLIK |(815.54) **Schiff Base/AP**	-	+ + +	+	-	-

The incidence of AGE-precursors and five identified AGEs are compared in non-glycated and glycated BSA samples incubated for 0, 14 and 28 days. [Absence (-), Presence (+)]

and TPVSE**K**), as both peptides lack a histidine residue.

Identification of AFGP

The search for glycation-induced peptide modifications with subsequent AFGP formation only exposed one peptide (m/z 951.9). An *m/z* at 951.9 was observed in the EIC and mass spectra of the glycated (1.0 M D-glucose) BSA samples incubated for 14 and 28 days, respectively, but it was absent in the EIC of the control BSA. The m/z of 951.9 corresponded to the peptide SL**H**TLFGDELC**K** (a. a. 89 - 100) plus AFGP (+144.03). Similar to CML and pyrraline, AFGP also forms crosslinkages at lysine or histidine residues; therefore, the likely site for crosslinking was either the histidine (**H**) or lysine residue (**K**).

Identification of imidazolone A

Imidazolone A is formed upon the reaction of the sugar degradation product 3-deoxyglucosone with an arginine side chain. A data search on the glycated BSA revealed only one peptide (LGEYGFQNALIV**R**) for glycation-induced peptide modification and crosslinking, with subsequent imidazolone A formation. A m/z at 884.5 was found in the EIC and mass spectra of the glycated BSA (2.5 M D-glucose) incubated for 28 days, but was absent in the EIC of both the control and glycated BSA (2.5 M D-glucose) incubated for 14 days (Table 2). The m/z of 884.71

corresponded to the peptide LGEYGFQNALIV**R** (a. a. 421 - 433) plus imidazolone A (+144.03). The peptide (LGEYGFQN ALIV**R**) was expected to be derived from a crosslinkage at the arginine residue (**R**).

Identification of imidazolone B

The search to identify imidazolone B formation with subsequent crosslinking among the glycated (1.0 and 2.5 M D-glucose) samples incubated for 14 and 28 days revealed only one peptide (IETM**R**). The m/z of 790.9 corresponded to the peptide IETM**R** (a. a. 205 - 209) plus imidazolone B (+142.03). The *m/z* at 790.9 was prominent in the EIC and mass spectra of the glycated BSA (2.5 M D-glucose) incubated for 28 days, but was absent in the EIC of the control BSA and glycated BSA (2.5 M D-glucose) incubated for 14 days. Similar to imidazolone A, imidazolone B formation and subsequent crosslinkage occurs at arginine residues; therefore, the glycated peptide (IETM**R**) was expected to have undergone crosslinking at the arginine residue (**R**).

Identification of schiff base or amadori products

Amadori-albumin, a major glycated protein, was shown to be involved in experimental hyperglycemia-induced microvascular complications (33, 34), and is associated with early and advanced nephropathy and early retinopathy status in Type 1 diabetic patients (35). Fig. 2 shows the absence of *m/z* at 750.5 in the EIC of the control BSA (A) and the EIC of the BSA incubated for 28 days with 2.5 M D-glucose (C); however, it was present in the EIC (B) and mass spectra (D) of the glycated BSA (2.5 M D-glucose) incubated for 14 days. The m/z of 750.5 corresponded to the peptide ECCD**K**PLLE**K** (a. a. 300 - 309) plus a Schiff base or AP (+162.02). Schiff base or AP formation was predominantly observed in the glycated samples (1.0 and 2.5 M D-glucose) incubated for 14 days, in contrast to the 28 day-old glycated samples and the remainder of identified AGEs (Table 2). These findings confirm that Schiff bases and APs are formed as early glycation products. Similarly, two glycated peptides

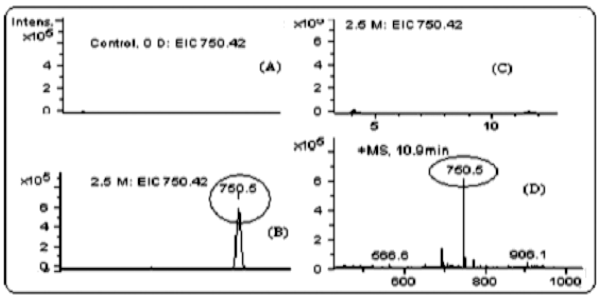

Fig. 2. Identification of Schiff Bases or APs; (A) EIC of control BSA (m/z 750.42), (B) EIC of glycated BSA (2.5 M D-glucose) incubated for 14 days (m/z 750.42), (C) EIC of glycated BSA (2.5 M D-glucose) incubated for 14 days (m/z 750.42), and (D) mass spectra of glycated BSA (2.5 M D-glucose) incubated for 14 days.

(IETM**R** and **H**LVDEPQNLI**K**) were found to first form as AGE precursors, and then later to convert into imidazolone B and CML, respectively. Structural isomers such as Schiff bases and Amadori products can not be distinguished with LC-MS; therefore, the single glycated peptide observed for both the Schiff bases and Amadori products indicates the formation of any one of the two. However, Schiff bases are quite labile and undergo rearrangement to form relatively stable Amadori products. Thus, the glycated peptides were anticipated to be Amadori products.

A total of nine modified peptides (FPK, ECCDKPLLEK, IETMR, HLVDEPQNLIK, TPVSEK, TVMENFVAFVDK, RHPYFYAPELLY YANK, SLHTLFGDELCK, and LGEYGFQNALIVR) were identified for the formation of CML, pyrraline, AFGP, imidazolones A and B, as well as AGE-precursors (Schiff bases or APs). The HPLC-ESIMS experiments were performed to analyze glycated BSA samples incubated for 14 and 28 days; therefore, site-localizations for lysine (K) and histidine (H) could not be established for the formation of CML, pyrraline, and AFGP. In most cases, AGE formation was predominantly observed in the 28 day-old incubated samples glycated with 1.0 and 2.5 M D-glucose, respectively. On the other hand, early glycation products (Schiff bases or APs) were identified in the 14 day-old incubated samples, which support the hypothesis that glycation-induced AGE formation is both glucose concentration and incubation time dependent.

For the first time we are reporting the identification of five AGEs and four AGE precursors (Schiff bases or APs) in glycated samples of BSA by a new approach; although it was laborious and time consuming, it allowed for a comprehensive analysis of glycation-induced protein/peptide modifications with subsequent identification of AGE formation. We believe that manual searches to identify AGEs within control and glycated samples, based on relative mass changes due to corresponding AGE formation, is simple, economical, and more reliable, as each and every peptide is analyzed individually, which can not be claimed for algorithm or mathematical model based methods. Keeping in mind the importance of AGEs from a pathophysiological standpoint, this relatively simple method will help in dealing with the vast data generated (by mass spectrometry) for the identification of AGE-peptides and other post translational protein/peptide modifications. We expect that the strategy employed in this *in-vitro* study for identifying AGEs and AGE precursor formation will provide a foundation for the identification of AGEs within *in-vivo* study models of diabetes and its associated complications, as well as in aging, atherosclerosis, and various other pathophysiological conditions.

MATERIALS AND METHODS

All reagents and solvents were used as purchased without further purification. BSA was purchased from Merck, Germany. Trypsin, glucose, dithiothreitol (DTT), and trifluoroacetic acid (TFA) were purchased from Sigma Chemical Co, USA. HPLC grade acetonitrile was purchased from Fisher Scientific, New Jersey, USA. All other materials were purchased locally.

Glycation of bovine serum albumin

Bovine serum albumin (BSA) was incubated with different concentrations of glucose using the procedure of Lapolla, *et al*., 2004, with slight modifications. Briefly, pure, defatted BSA (100 mg/ml in 0.01 M phosphate buffer, pH 7.4) containing 5 mM toluene (as a bacteriostatic) was incubated with 1.0 and 2.5 M glucose at 37°C for 0, 14, and 28 days. After incubation, the BSA was separated from the solution by extensive dialysis against 0.001 M phosphate buffer (MW cut-off 10,000 Da), and finally lyophilized and stored at -75°C. A control BSA was incubated under the same conditions without the addition of glucose.

Tryptic digestion

One milligram of BSA was dissolved in 1.3 ml of 50 mM NH_4HCO_3 buffer solution (pH 8.3). After the addition of 15 µl of a solution of 45mM dithiothreitol, the mixture was heated at 50°C for 15 min, followed by incubation with trypsin (200 µl of a 100 ng/µl solution, substrate to enzyme ratio = 50:1 w/w) for 24 hours at 37°C. The reaction was stopped with the addition of 80 µl of 10% TFA.

Chromatographic analysis

The chromatographic separation was carried out using HPLC (Agilent Technologies) coupled with quadruple trap mass-spectrometry. After digestion with trypsin, the digests were diluted 1:1 with 50% acetonitrile solution containing 0.1% TFA. The chromatographic separation was brought about by injecting 40 µl of the sample solution into a Vydac protein and peptide C18 column (particle size 5 um, 250 × 4.6 mm i.d.) (W.R. Grace & Co., USA). Solvent A consisted of distilled water with 0.1% TFA, and solvent B was comprised of acetonitrile with 0.1% TFA. The gradient profile for solvent B was as follows: 15%, 1 min; 15-45%, 33 min; 45%, 5 min; 45-95%, 10 min; 95%, 15 min. Elution was carried out at a flow rate of 0.2 ml/min.

Mass spectrometric analysis

The mass spectrometric analyses (ESI-MS and ESI-MS/MS) were performed on an Agilent 1100 LC/MSD Trap (Agilent Technologies) operating in positive ion mode. The instrumental parameters were: a nebulizer pressure of 25 psi, auxiliary gas flow at 8 ml/min, an auxiliary gas temperature of 310°C, a capillary voltage of 3.5 kV, and a skimmer voltage of 5 V. The MS and MS/MS analyses were performed favoring the fragmentation of doubly charged ions.

Peptide sequences

The peptide sequence of the control BSA digested with trypsin was matched in an online database search query by exporting the MS/MS data of the control BSA to the online Mascot database (http://www.matrixscience.com/).

Acknowledgements

The authors are thankful to the Higher Education Commission of Pakistan (HEC) for funding the PhD scholarship of Waqar Ahmad.

REFERENCES

1. Brownlee, M., Cerami, A., and Vlassara H. (1988) Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N. Engl. J. Med.* **318**, 1315-1321.
2. Nwabuisi, C. (2002) Prophylactic effect of multi-herbal extract 'Agbo-Iba' on malaria induced in mice. *East Afr. Med. J.* **79**, 343-346.
3. Reynolds, T. M. (1963) Chemistry of nonenzymatic browning. I. The reaction between aldose and amines. *Adv. Food Res.* **12**, 1-52.
4. Huh, J. W., Yang, S. J., Hwang, E. Y., Choi, M. M., Lee, H. J., Kim, E. A., Choi, S. Y., Choi, J., Hong, H. N. and Cho, S. W. (2007) Alteration of the quaternary structure of human UDP-glucose dehydrogenase by a double mutation. *J Biochem. Mol. Biol.* **40**, 690-696.
5. Reynolds, T. M. (1965) Chemistry of nonenzymatic browning. II. *Adv. Food Res.* **14**, 167-283.
6. Kato, H., Cho, R. K., Okitani, A. and Hayase, F. (1987) Responsibility of 3-deoxyglucosone for the glucose-induced polymerization of proteins. *Agric. Biol. Chem.* **51**, 683-689.
7. Park, J., Kim, S., Oh, J. K., Kim, J. Y., Yoon, S. S., Lee, D. and Kim, Y. (2005) Identification of differentially expressed proteins in imatinib mesylate-resistant chronic myelogenous cells. *J. Biochem. Mol. Biol.* **38**, 725-738.
8. Baynes, J. W. (2004) The clinical chemome: a tool for the diagnosis and management of chronic disease. *Clin. Chem.* **50**, 1116-1117.
9. Lee, A. Y., Chung, S. K. and Chung, S. S. (1995) Demonstration that polyol accumulation is responsible for diabetic cataract by the use of transgenic mice expressing the aldose reductase gene in the lens. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 2780-2784.
10. Chen, L., Yang, Y., Han, J., Zhang, B. Y., Zhao, L., Nie, K., Wang, X. F., Li, F., Gao, C., Dong, X. P. and Xu, C. M. (2007) Removal of the glycosylation of prion protein provokes apoptosis in SF126. *J. Biochem. Mol. Biol.* **40**, 662-669.
11. Koya, D. and King, G. (1998) Protein kinase C activation and the development of diabetic complications. *Diabetes.* **47**, 859-866.
12. Kolm-Litty, V., Sauer, U., Nerlich, A., Lehmann, R. and Schleicher, E. D. (1998) High glucose-induced transforming growth factor fetal production is mediated by the hexosamine pathway in porcine glomerular mesangial cells. *J. Clin. Invest.* **101**, 160-169.
13. Sing, R., Barden, A., Mori, T. and Beilin, L. (2001) Advanced glycation endproducts: a review. *Diabetologia.* **44**, 129-146.
14. Kohn, R. R., Cerami, A. and Monnier, V. M. (1984) Collagen aging *in vitro* by nonenzymatic glycosylation and browning. *Diabetes.* **33**. 57-59.
15. Wu, J. T. (1993) Advanced glycosylation end products: a new disease marker for diabetes and aging. *J. Clin. Lab. Anal.* **7**, 252-255.
16. Beisswenger, P. J., Makita, Z., Cuphrey, T. J., Moore, L. L.,

Jean, S., Brinck-Johnsen, T., Bucala, R. and Vlassara, H. (1995) Formation of immunochemical advanced glycosylation end products precedes and correlates with early manifestations of renal and retinal disease in diabetes. *Diabetes* **44**, 824-829.
17. Wu, J. T. (1993) Review of diabetes: identification of markers for early detection, glycemic control, and monitoring clinical complications. *J. Clin. Lab. Anal.* **7**, 293-300.
18. Korbet, S. M., Makita, Z., Firanek, C. A. and Vlassara, H. (1993) Advanced glycosylation end products in continuous ambulatory peritoneal dialysis patients. *Am. J. Kidney Dis.* **22**, 588-591.
19. Makita, Z., Vlassara, H., Cerami, A. and Bucala, R. (1992) Immunochemical detection of advanced glycosylation end products *in vivo*. *J. Biol. Chem.* **267**, 5133-5138.
20. Makita, Z., Radoff, S., Rayfield, E. J., Yang, Z., Skolnik, E., Delaney, V., Friedman, E. A., Cerami, A. and Vlassara, H. (1991) Advanced glycosylation end products in patients with diabetic nephropathy. *N. Engl. J. Med.* **325**, 836-842.
21. Vlassara, H. (1994) Recent progress on the biologic and clinical significance of advanced glycosylation end prod. *J. Lab. Clin. Med.* **124**, 19-30.
22. Thorpe, S. R and Baynes, J. W. (1996) Role of the Maillard reaction in diabetes mellitus and diseases of aging. *Drugs Aging.* **9**, 69-77.
23. Yim, M. B, Yim, H. S, Lee, C., Kang, S. O. and Chock, P. B. (2001) Protein glycation: creation of catalytic sites for free radical generation. *Ann. N.Y. Acad. Sci.* **928**, 48-53.
24. Makita, Z., Vlassara, H., Rayfield, E., Cartwright, K., Friedman, E., Rodby, R., Cerami, A. and Bucala, R. (1992) Hemoglobin-AGE: a circulating marker of advanced glycosylation. *Science* **258**, 651-653.
25. Gaskel, S. J. (1986) Mass spectrometry in biomedical research, John Wiley & Sons, New York, U.S.A.
26. Brownlee, M., Vlassara, H. and Cerami, A. (1984) Non-enzymatic glycosylation and the pathogenesis of diabetic complications. *Ann. Intern. Med.* **101**, 527-537.
27. Sebekova, K., Kupcova, V., Schinzel, R. and Heidland, A. (2002), Markedly elevated levels of plasma advanced glycation end products in patients with liver cirrhosis-amelioration by liver transplantation. *J. Hepatol.* **36**, 66-71.
28. Thornalley, P. J, Battah, S., Ahmed, N., Karachalias, N., Agalou, S., Babaei-Jadidi, R. and Dawnay, A. (2003) Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. *Biochem. J.* **375**, 581-592.
29. Niwa, T. (1997) Mass spectrometry in the search for uremic toxins. *Mass Spectrom. Rev.* **16**, 307-332.
30. Zhang, Y., Cocklin, R. R., Bidasee, K. R. and Wang, M. (2003) Rapid determination of advanced glycation end products of proteins using MALDI-TOF-MS and PERL script peptide searching algorithm. *J. Biomol. Tech.* **14**, 224-230.
31. Furth, A., J. (1997) Glycated proteins in diabetes. *Br. J. Biomed. Sci.* **54**, 192-200.
32. Lapolla, A., Traldi, P. and Fedele, D. (2005) Importance of measuring products of non-enzymatic glycation of proteins. *Clin. Biochem.* **38**, 103-115.
33. Chen, S., Cohen, M. P., Lautenslager, G. T., Shearman, C. W. and Ziyadeh, F. N. (2001) Glycated albumin stimulates TGF-beta 1 production and protein kinase C actvity in glomerular endothelial cells. *Kidney Int.* **59**, 673-681.
34. Chen, S., Chen, M. P. and Ziyadeh, F. N. (2000) Amadori-glycated albumin in diabetic nephropathy: patophysiologic connections. *Kidney Int. Suppl.* **77**, S40-S44.
35. Schalkwijk, C. G., Chatuverdi, N., Twaafhoven, H., van Hinsbergh, V. W. and Stehouwer, C. D. (2002) Amadori albumin correlates with microvascular complications and precedes nephropathy in type 1 diabetes. *Eur .J. Clin. Invest.* **32**, 500-506.