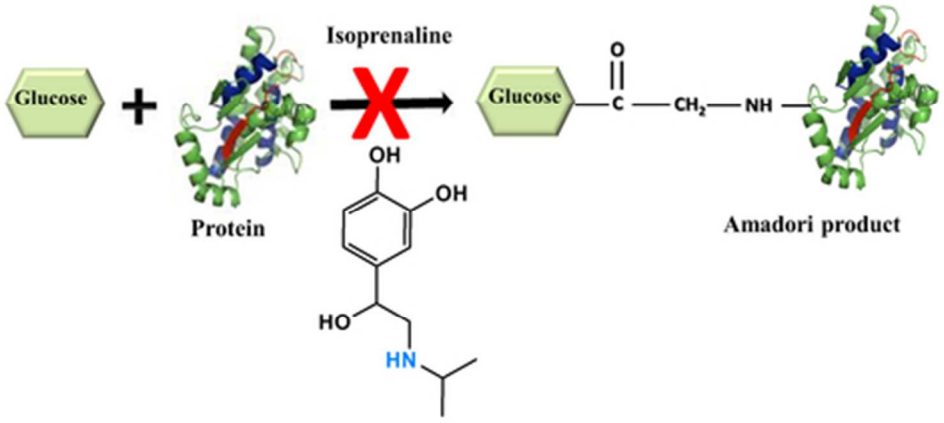
RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances



Isoprenaline inhibits Advanced Glycation Endproducts and may be suitable candidate for the treatment of glycation associated diseases.
39x17mm (300 x 300 DPI)

Title: Investigation of Antiglycation Activity of Isoprenaline

Yogesh M Kolekar[1], Vannuruswamy Garikapati[1], Sneha B Bansode[1], Santhakumari B[2], Hirekodathakallu V. Thulasiram[3], Mahesh J Kulkarni[1*]

1- Proteomics Facility, Division of Biochemical Sciences, CSIR-National Chemical Laboratory, Pune-411008, India

2- Centre for Material Characterization, CSIR-National Chemical Laboratory, Pune-411008, India

3- Division of Organic Chemistry, CSIR-National Chemical Laboratory, Pune-411008, India

Keywords; Post translational modifications, diabetes, mass spectrometry, drug discovery

*** For correspondence**

Mahesh J Kulkarni, PhD
Scientist, Proteomics Facility
Division of Biochemical Sciences
CSIR-National Chemical Laboratory
Pune-411008, India
E-mail: mj.kulkarni@ncl.res.in
Phone: +912025902541

Abstract

Advanced Glycation End products (AGEs) are implicated in pathogenesis of diabetes and its complications. In this study, we report the ability of isoprenaline to inhibit the AGE modification of protein by fluorescence spectroscopy and western blotting. Isoprenaline was more effective in inhibiting AGE modification than aminoguanidine, a well known glycation inhibitor. Further, we show that isoprenaline inhibits at Amadori product formation during glycation reaction by various techniques such as MALDI-TOF-MS, LC-MS/MS, and fructosamine assay. Mass spectrometric analysis of glycation reaction mixture incubated with isoprenaline suggested that it forms adduct with glucose and thus inhibits glycation. The finding of additional property of isoprenaline to inhibit glycation suggests that it is a potential candidate for drug repositioning for the treatment of diabetes and complication, as it is an FDA approved drug.

Keywords: mass spectrometry, diabetes, drug discovery, glycation

Introduction

Diabetes is a group of metabolic diseases characterized by elevated levels of blood sugar over a prolonged period. Poorly controlled diabetes is associated with serious complications such as cardiovascular disease, stroke, kidney failure, foot ulcers and damage to the eyes. Advanced Glycation End products (AGEs) have been implicated as one of the major causal factors in pathogenesis of diabetes and its complications. AGEs are formed as a result of a series of non-enzymatic reaction between reducing sugars and proteins.[1-2] In diabetes, the levels of AGEs are substantially increased due to hyperglycaemic condition. Especially plasma proteins are known to undergo AGE modification and interact with the Receptor for AGEs (RAGE). The AGE-RAGE interaction leads to oxidative stress and activation of proinflammatory pathways. AGE-RAGE axis is involved in the development of many degenerative diseases such as diabetes, atherosclerosis, chronic renal failure and Alzheimer's disease.[2-4] Therefore, reducing AGE levels has been considered as a therapeutic strategy in the treatment and management of diabetes. Although widely used FDA approved drugs like metformin, aspirin, diclofenac, hydralazine, rifampicin known to possess antiglycation activity, they are not directly used for the reduction of AGEs.[4-8] Many efforts have been made to seek new AGE inhibitors; however aminoguanidine and pyridoxamine are the only drugs that were evaluated in clinical trials but their adverse effects made them less favourable in clinical applications.[9] Previously we have reported anti glycation activity of rifampicin, hydralazine and protriptyline by using MALDI-TOF-MS based insulin glycation assay.[7,8,10,11] The present study deals with demonstration of antiglycation activity of isoprenaline and its mode of action by various physicochemical approaches like fluorescence spectroscopy, MALDI-TOF-MS, LC-MS/MS etc.

Results and discussion

Fluorescence inhibition and IC_{50} studies

AGEs display a characteristic fluorescence (Ex 370/Em 440 nm) that can used to quantify the extent of AGE-modification.[12] Using AGE fluorescence assay isoprenaline was found to be a potent AGE inhibitor as reflected by decreased AGE fluorescence (A, Fig. 1). Isoprenaline was more effective in decreasing AGE fluorescence compared to aminoguanidine at the same concentrations. AGE fluorescence based assay was used to determine the IC_{50} of aminoguanidine.[13] Therefore, using this assay, the IC_{50} of isoprenaline was determined, which was found to be lower (0.07mM) than aminoguanidine (6.99 mM) (B, C Fig. 1). The lower IC_{50} of isoprenaline suggests that it is a more potent glycation inhibitor than aminoguanidine.

The isoprenaline and aminoguanidine induced decrease in AGE fluorescence of BSA was also reflected in western blotting by anti-AGE antibodies (D, Fig. 1). *In vitro* glycation of BSA showed increase in the extent of AGE modifications, which was not considerably inhibited by aminoguanidine. Densitometric analysis of Western blot with Anti-AGE antibodies showed significant reduction in AGE modifications in isoprenaline treated glycated BSA than with aminoguanidine.

Circular dichroism

Protein glycation is associated with change in protein conformation and function.[14] The circular dichroism spectroscopy analysis showed both isoprenaline and aminoguanidine treatment resulted in decrease of beta sheet percentage in glycated BSA (A, Fig. 2). Isoprenaline was able to protect the conformation of BSA at concentration as low as 0.1 mM. At higher concentration (> 0.2 mM), isoprenaline interferes in CD analysis.

Probable mechanism of action of isoprenaline:

AGE fluorescence and western blotting have suggested that isoprenaline is capable of reducing AGE modification of proteins. Thus, to understand the mechanism by which isoprenaline reduces AGE modification of proteins, MALDI-TOF-MS based Insulin glycation assay, fructosamine assay, LC-MS analysis of glycated BSA and isoprenaline-glucose adduct were studied.

Isoprenaline inhibits Schiff's base or Amadori modification

We have previously reported MALDI-TOF-MS based insulin glycation inhibition assay, where in insulin (m/z 5808) was allowed to react with glucose to form Amadori modified insulin (m/z 5970) and the intensity of Amadori modified insulin was monitored in presence or absence of inhibitors.[7,8] This assay was used to evaluate whether isoprenaline inhibits at Amadori modification during glycation reaction. Figure 3C shows that isoprenaline was able to inhibit Amadori modification, as the intensity of Amadori modified insulin decreased in presence of isoprenaline. The extent of inhibition was more in presence of isoprenaline than aminoguanidine (Fig. 3). Further, isoprenaline's ability to inhibit Amadori modification was studied by high resolution accurate mass spectrometer (Q-Exactive, Orbitrap mass spectrometer). BSA was glycated in absence or presence of isoprenaline or aminoguanidine and formation of Amadori modified peptides after tryptic digestion was monitored by LC-MS/MS. A representative MS/MS spectrum of Amadori modified peptide is depicted in Supplementary Fig. 1. As expected glycated BSA had maximum number (23) of Amadori modified peptides. While glycated BSA treated with aminoguanidine and isoprenaline had 16 and 10 Amadori modified peptides, respectively (A, Fig. 4, Table 1). Next, the extent of glycation was determined by calculating the ratio of area under curve (AUC) of few modified to unmodified peptides (Table 2, Supplementary Fig 2.), as well as by comparing the area under curve for some of the glucose sensitive peptides (GSPs)[16] in glycated BSA, glycated

BSA treated with isoprenaline or aminoguanidine (B, Fig. 4, Supplementary Fig. 3 and 4). Based on these results, it was observed that the extent of glycation was higher in glycated BSA, followed by glycated BSA treated with aminoguanidine and isoprenaline.

The lysine residues of the amadori modified peptides were analyzed for their solvent accessibility using PDB structure of BSA. The solvent accessible lysine residues are highlighted in red color (C, Fig. 4). The glycated BSA showed nine surface exposed glycated lysine residues (K28, 36, 117, 151, 183, 299, 386, 401 and 580), whereas the glycated BSA treated with aminoguanidine showed five surface exposed glycated lysine residues (K28, 36, 183, 386 and 580). Interestingly only three surface lysine residues were identified to be glycated in the glycated BSA treated with the isoprenaline (K36, 568 and 580). However, in case of glycated BSA, several additional lysine residues were found to Amadori modified in mass spectrometric analysis but were not observed as solvent accessible. As glycation promotes beta sheet formation, during this process, exposure of buried lysine residues could be possible.[17]

Fructosamine assay

Mass spectrometric analysis suggested that isoprenaline inhibits at the Amadori product formation. This was also corroborated with fructosamine assay. Fructosamine is one of the early stage products of glycation reaction.[18] It has been used commonly to measure short-term control of blood sugar in diabetic patients. In glycated BSA fructosamine levels were found to be 834.32 µmol/L, whereas glycated BSA treated with 10mM aminoguanidine or 10 mM isoprenaline showed decreased fructosamine level (714.62 µmol/L and 520.84 µmol/L, respectively) (D, Fig. 4).

Isoprenaline forms adduct with glucose

Further LC-MS analysis of glycation reaction mixture incubated with isoprenaline (m/z 212.1281) suggested that it forms glucose adduct (m/z 374.1809) (A, Fig. 5). The probable

mechanism of isoprenaline-glucose adduct formation is depicted in Fig 5B. Although the drug-glucose adduct formed was relatively less, formation of adduct could be one of the probable ways by which isoprenaline inhibits glycation, along with its ability to induce conformation changes in albumin.

As AGEs are associated with various diseases including aging, diabetes, cancer, neurodegenerative diseases, it is important to keep their levels minimal. Thus, there has been great effort in discovering molecules that inhibit glycation and AGE formation. Drug repositioning is emerging as a powerful approach in the area of drug discovery, as it has several advantages including reduced time and cost necessary for clinical trials. In this study we report the discovery of isoprenaline as a potent effective AGE inhibitor amongst several screened drugs. Mass spectrometric analysis and fructosamine assays suggested that it inhibits at Amadori product formation by forming adduct with glucose. Isoprenaline is an FDA approved drug used for the treatment of bradycardia and asthma. It has also been patented as an eye drop for AGE inhibition for the treatment of cataract.[19] Thus isoprenaline is a potential candidate for repositioning for the treatment of diabetes and its complications.

Experimental

Chemicals

All the chemicals were procured from Sigma-Aldrich unless otherwise mentioned. AGE antibody, protein A-HRP conjugate were purchased from Merck Millipore (India).

In vitro glycation of bovine serum albumin (BSA)

In vitro glycation of pure BSA was done as described,[20] with slight modifications. Briefly, the reaction was carried out by incubating 225 µl of 50 mg/ml BSA in 0.1 M phosphate buffer (pH 7.4) and 75µl of 2 M D-glucose containing 5 mM sodium azide as a bacteriostat with or without 10mM (final concentration) of isoprenaline and aminoguanidine drugs at

37 °C for 15 days. BSA glycation was monitored by fluorescence spectroscopy, excitation at 370 and emission at 440nm by using a (Thermo, Varioskan Flash Multimode Reader) spectrofluorometer.

IC_{50} of Isoprenaline and Aminoguanidine

The percent inhibition of AGE formation was calculated by formula: inhibition = (1 – *Fi* / *Fc*) x 100, Where *Fi* = fluorescence intensity of glycated BSA treated with inhibitor and *Fc* = fluorescence intensity of glycated BSA without inhibitor. The apparent *IC50* was determined by plotting the per cent glycation inhibition versus inhibitor concentration.

Western blot analysis using anti AGE antibody

BSA, glycated BSA, glycated BSA with 10mM of Aminoguanidine or 10mM of Isoprenaline was incubated at 37 °C for 15 days. 2µg of each protein sample was separated on 12% SDS-PAGE and transferred onto the PVDF membrane. The membranes were blocked with 5% skimmed milk powder dissolved in TBS (20 mM Tris-HCl (pH 7.5), 0.15 M NaCl). The proteins were probed by AGE antibodies the antibody dilution was adopted according to the manufacturer instructions. Protein `A´ HRP conjugate was used in 1:5000 dilutions. Immunoreactive bands were visualized using WesternBright Quantum western blotting detection kit (Advansta) and documented on Dyversity system (Syngene, UK).

Circular dichroism spectroscopy measurements

0.05 mg ml^{-1} of BSA, glycated BSA, glycated BSA treated with 10mM of Aminoguanidine or 10mM of Isoprenaline was used to acquire the CD spectra. All the spectra were measured at room temperature on a JASCO J-815 Chiro-Optical Spectrometer (Jasco Inc., Easton, MD, USA). The spectra results from averaging three scans and were corrected for respective blanks. Results are expressed as molar ellipticity, $[\Theta]$ (deg cm^2 $dmol^{-1}$), based on a mean amino acid residue weight (MRW).The molar ellipticity was determined as $[\Theta]\ \lambda = (\Theta\ 100\ MRW)/ (cl)$, where l, is the light path length in centimeters, 'c' is the protein concentration in

mg/ml, and Θ is the measured ellipticity in degrees at the relevant wavelength (250-190nm). The CD spectra of the protein samples were analysed to calculate the content of secondary structure using CDPro software that has three algorithms: CONTINLL, CDSSTR and SELCON3.[21]

Insulin-MALDI based assay

The reaction for insulin glycation with Isoprenaline was incubated in 200µl of 0.1 M phosphate buffer (pH 7.4) containing 10mM Isoprenaline, insulin 50µl (1.8 mg/ml) and 50µl of glucose (250mM). The reaction was monitored for 7 days at 37 °C till the relative intensity decreased to 50% on MALDI-TOF-TOF (AB SCIEX TOF/TOF™ 5800). The reaction mixture was mixed with sinapinic acid and analysed on MALDI-TOF-TOF system in linear mode using Anchor Chip 384 targets as described in.[8]

Fructosamine assay

Fructosamine level was measured by the NBT Labkit (Chemelex, S.A.). 300 µl of 0.75 mM NBT was added to a 96-well microplate containing 30 µl of 150 µg BSA, glycated BSA, glycated BSA with 10mM of Aminoguanidine and glycated BSA with 10mM of Isoprenaline. The reduction of NBT by fructosamine group was measured at 520 nm immediately after additions considered as (Abs1) the incubation at 37 °C for 15 min considered as (Abs2) the absorbance was monitored by using an UV 1800 spectrophometer (Shimadzu). The fructosamine level was calculated by using the Labkit (Chemelex, S.A.). Fructosamine level=A2-A1 (ΔA)/absorbance of calibrant X concentrations of calibrant (µmol/L)

LC-HR/AM Q-Exactive Orbitrap Analysis (Full MS/dd-MS2)

Tryptic digestion

Prior to LC MS/MS analysis, 100µg of protein was reduced with 100mM dithiotreitol (DTT) at 60 °C for 15 min, then alkylated with 200mM iodoacetamide in dark at room temperature for 30 min. Proteins were digested by adding porcine trypsin in the ratio of 1:50 (final

enzyme : proteins) at 37 °C overnight. The digestion reaction was stopped by adding concentrated HCL and incubated for 10 min at 37 °C before vertex and centrifugation.

Chromatographic Separation

Peptides digest (1.5µg) was separated on Hypersil Gold C18-RP HPLC column (150X2.1mm, 1.9µm) with 98% of mobile phase A (100% water, 0.1% FA) and 2% of mobile phase B (100% ACN, 0.1% FA) at 350 µl/min flow rate with a 45 min linear gradient of 2% to 40% mobile phase B.

Mass Spectrometry acquisition (Full MS/dd-MS2)

All samples were analysed on Orbitrap mass MS (Q-Exactive). The instrument was tuned and calibrated before analysis. The tune parameters include: spray voltage 4200V, capillary temperature 320 °C, heater temperature 200 °C, S-lens RF value 55, sheath and auxiliarygases pressure were 30 and 8 psi respectively. The samples were acquired in positive ionization (HESI) mode in data-dependent manner using a top-5 method with scan range from 350 -1800 m/z. MS spectra were acquired at a resolution of 70,000 with maximum injection time (IT) of 120 ms and automatic gain control (AGC) value of 1 × e6 ions and MS/MS spectra were acquired at 17,500 resolution with maximum IT of 120ms and AGC value of 1 × e5 ions. Precursor's selectivity was performed at an isolation width of 3m/z, under fill ratio of 0.3% and dynamic exclusion time of 15 sec. The peptide fragmentation was performed in HCD cell using normalized high energy collision induced dissociation at 30 eV.

Database search and PTM analysis

The mass spectrometric data was processed using Proteome discoverer 1.4 (Version 1.4.0.288, (Thermo Fisher Scientific, Bremen, Germany). SEQUEST HT search engine was used for peptide identification. The data was searched against UniProt Bovine Serum Albumin (P02769) sequence database. Carbamidomethylation of cysteine (C) and oxidation at methionine (M) was considered as fixed and variable modification respectively.

Additionally glycation modifications at lysine position were searched as dynamic variable modifications. The search was performed using the following parameters: Peptide and fragment mass tolerance were 10ppm, 0.5Da respectively with minimum of 2 missed cleavages and 1% false discovery rate (1% FDR). The identified glycation modified peptides were selected based on the criteria described earlier.[22] The Glycation modification list includes carboxymethyllysine (CML) (+58.005 Da); carboxyethyllysine (CEL) (+72.021 Da), Amadori (+162.02 Da); FL-1H_2O (+144.042 Da); FL-2H_2O (+126.032 Da). The extent of glycation was monitored by extraction ion chromatogram (XIC) of modified peptides using Xcalibur (Thermo xcalibur 2.2 SP1.48).

Molecular modelling of BSA

Three dimensional structure of bovine serum albumin (BSA) was downloaded from the RCSB protein data bank (PDB ID: 3V03). Solvent accessible residues in BSA were identified using Swiss PDB viewer (http://spdbv.vital-it.ch/) with solvent accessibility index > 30%. Lysine residues that are exposed and identified as glycated in mass spectrometric analysis were marked on the surface model of BSA using PyMol software (PyMol Molecular Graphics System, Version 1.2r3 pre, Schrodinger LLC). Surface model of glycated BSA, glycated BSA + Aminoguanidine and glycated BSA + Isoprenaline were compared to analyze the effect of these compounds on the glycation of surface exposed residues.

Isoprenaline– Glucose conjugate adduct formation

To elucidate the AGE inhibition mechanism of Isoprenaline, the Isoprenaline and Amino guanidine (10mM) were incubated with glucose (0.5mM) in phosphate buffer pH 7.4 at 37 °C for 3 days. The reaction was analysed on Q-Exactive Orbitrap to find the drug glucose conjugate adduct formation.

Acknowledgements

This work was financially supported by CSIR-Network project CSC0111. YMK thanks CSIR, India for post doctoral Research Associateship.

References

1 R. Singh, A. Barden, T. Mori and L. Beilin, *Diabetologia*., 2001, 44, 126-141.

2 M. J. Kulkarni, A. M. Korwar, S. Mary, H. S. Bhonsle and A. P. Giri, *Proteomics Clin. Appl.,* 2013, 7, 155-170.

3 K. B. Batkulwar, S. B. Bansode, G. V. Patil, R. K. Godbole, R. S. Kazi, S. Chinnathambi, D. Shanmugam and M. J. Kulkarni, *Proteomics*., 2015, 15(2-3), 245-259.

4 S. Rahbar, R. Natarajan, K. Kumar, K. K. Yerneni, S. Scott, N. Gonzales and N. J. Nadler, Clin. *Chim. Acta.*, 2000, 301, 65-77.

5 M. Sensi, M. PricciGrazla De Rossi, S. Morano and U. Mario, *Clin. Chem.*, 1989, 35, 384-387.

6 M. A. M. Van Boekel, P. J. P. C. Van den Bergh and H. J. Hoenders, *Bioch.Bioph. Acta.*, 1992, 1120, 201-204.

7 S. B. Golegaonkar, H. S. Bhonsle, R. Boppana, and M. J. Kulkarni, *Eur. J. Mass Spectrom.,*(Chichester, Eng). 2010, 16, 221-226.

8 S. K. Kesavan, S. Bhat, S. B. Golegaonkar, M. G. Jagadeeshaprasad, A. B. Deshmukh, H. S. Patil, S. D. Bhosale, M. L. Shaikh, H. V. Thulasiram, R. Boppana and M. J. Kulkarni,*Sci. Rep.* 2013, 3, 2941.

9 M. E. William, *Curr. Diab. Rep.*, 2004, 4, 441-446.

10 S. B. Bansode, A.K. Jana, K. B. Batkulwar, S. D. Warkad, R. S. Joshi, N. Sengupta and M. J. Kulkarni, PLoS One., 2014, 20,9(8), e105196.

11 M. M. Joglekar, S. N. Panaskar, A. D. Chougale, M. J. Kulkarni and A. U. Arvindekar, *Mol.Biosyst*., 2013, 9(10), 2463-2472.

12 K. Nomoto, M. Yagi, U. Hamada, J. Naito and Y. Yonei, *Anti. Aging. Med*., 2013, 10(5), 92-100.

13 L. Séro, L. Sanguinet, P. Blanchard, B. T. Dang, S. Morel, P. Richomme, D. Séraphin and S. Derbré, *Molecules*., 2013, 18, 14320-14339.

14 E. Herczenik and M. F. B. G. Gebbink, *The FASEB J*., 2008, 22, 7, 2115-2133.

15 S. P. Wolff and R. T. Dean, *Biochem. J*., 1987, 245, 243-250.

16 M. Zhang, W. Xu, Y. Deng, *Diabetes*., 2013, 62(11), 3936-3942.

17 A. Lapolla, D. Fedele, R. Reitano, N. C. Aricò, R. Seraglia, P. Traldi, E. Marotta and R. Tonani, *J. Am. Soc. Mass Spectrom*., 2004, 15(4), 496-509.

18 A. Ardestani and R. Yazdanparast, *Int. J. Biol. Macromol*., 2007, 41, 572-578.

19 Y. Konishi, A. Mullick, US Pat., 12, 293 339, 2007

20 U. Kanska and J. Boratyński, *Arch. Immunol. Ther. Exp.,*(Warsz) 2002, 50, 61-66.

21 N. Sreerama and R.W. Woody, *Anal. Biochem.,*2000, 287, 252-260.

22 H. S. Bhonsle, A. M. Korwar, S. K. Kesavan, S. D. Bhosale, S. B. Bansode and M. J. Kulkarni, *Eur. J. Mass Spectrom*., 2012, 18(6), 475-481.

Figure and legends

Figure 1. (A) AGE fluorescence (Ex/Em 370/440 nm) spectra of BSA, glycated BSA or glycated BSA treated with 10mM aminoguanidine or 10 mM isoprenaline; IC_{50} values for AGE inhibition (B) Isoprenaline and (C) aminoguanidine; (D) Western blotting of BSA, glycated BSA or glycated BSA treated with 10mM aminoguanidine or 10mM of isoprenaline.

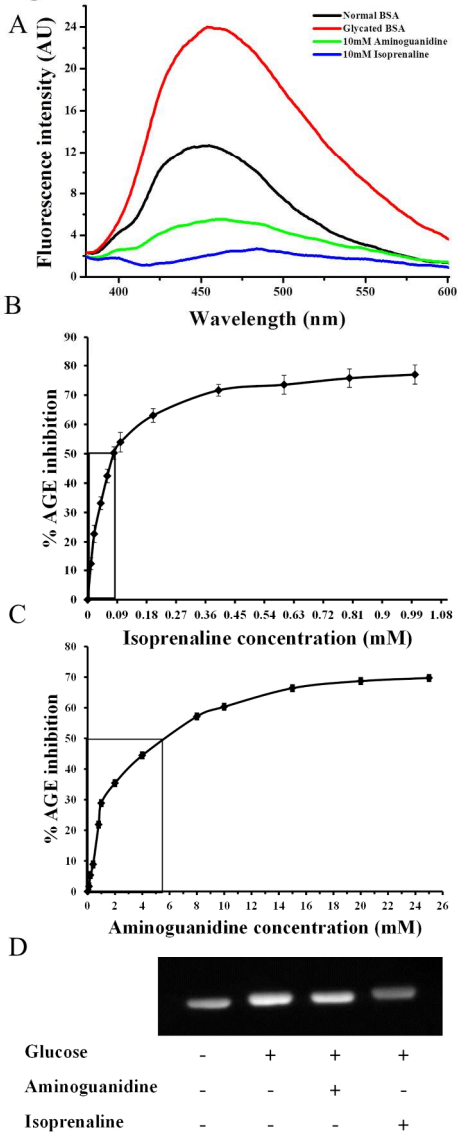
Figure 2. CD Pro analysis of native BSA, Glycated BSA, or glycated BSA treated with 0.1mM isoprenaline, or 0.2mM isoprenaline or 10mM aminoguanidine

Figure 3. MALDI-TOF-MS based insulin glycation inhibition, A. Glycated insulin B. Glycated insulin treated with 10mM Aminoguanidine C. Glycated insulin treated with 10mM Isoprenaline.

Figure 4. (A) LC-MS/MS analysis depicting the number of AGE modified peptides in glycated BSA, glycated BSA treated with 10mM of aminoguanidine or 10mM of Isoprenaline; (B) AUC for glucose sensitive peptides of glycated BSA, glycated BSA treated with aminogaunidine or Isoprenaline; (C) Glycation modifications depicting surface exposed lysine residues, 1) glycated BSA 2) glycated BSA treated with aminoguanidine 3) glycated BSA treated with Isoprinaline; (D) Fructosamine levels of glycated BSA, glycated BSA treated with aminoguanidine or Isoprinaline.

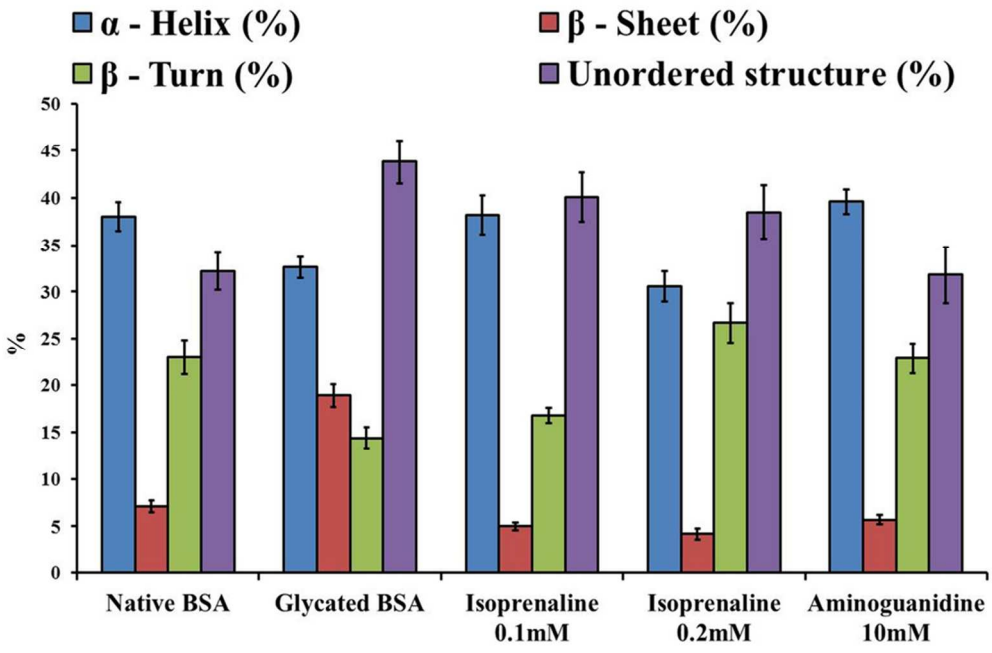
Figure 5. (A) LC-MS analysis of isoprenaline glucose adduct formation. (B) The probable mechanism of isoprenaline glucose adduct formation.

Fig. 1



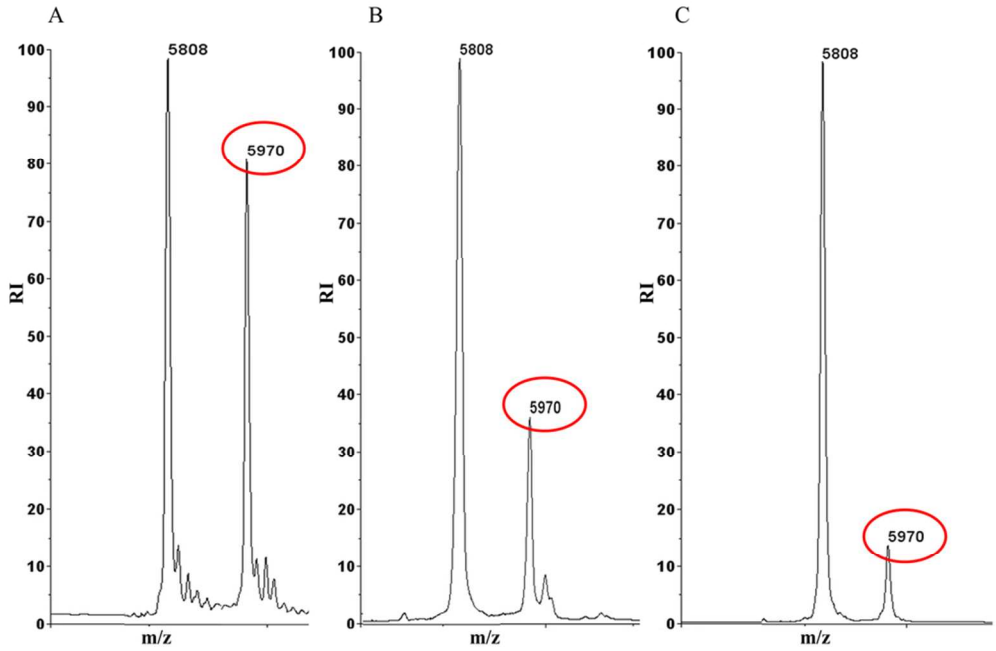
126x307mm (300 x 300 DPI)

Fig. 2

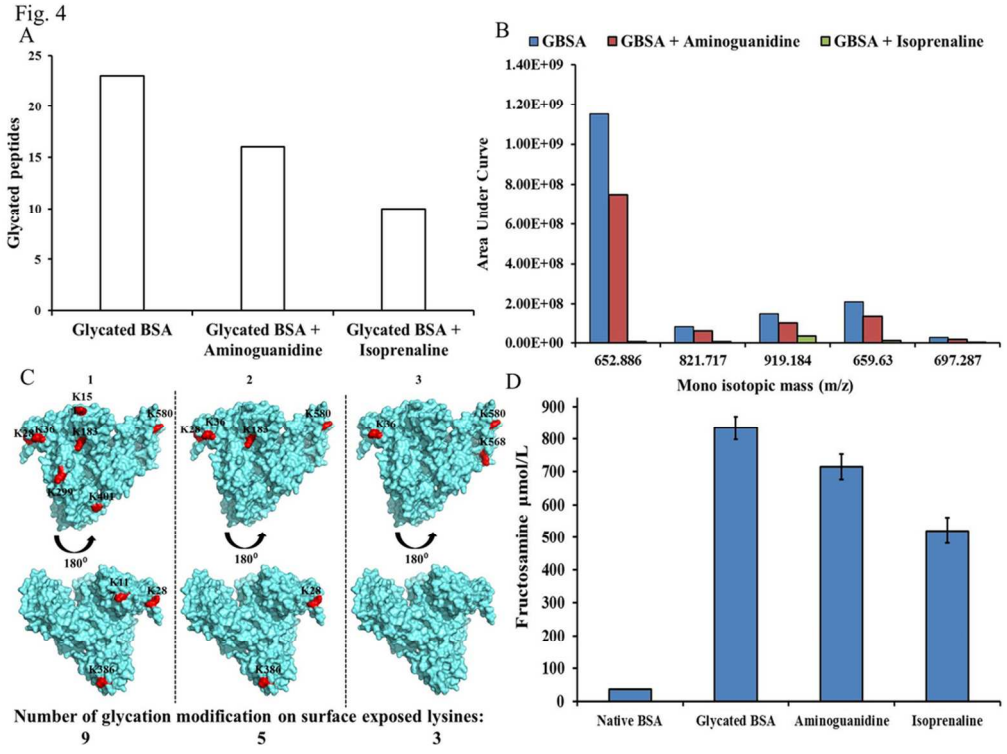


89x63mm (300 x 300 DPI)

Fig. 3

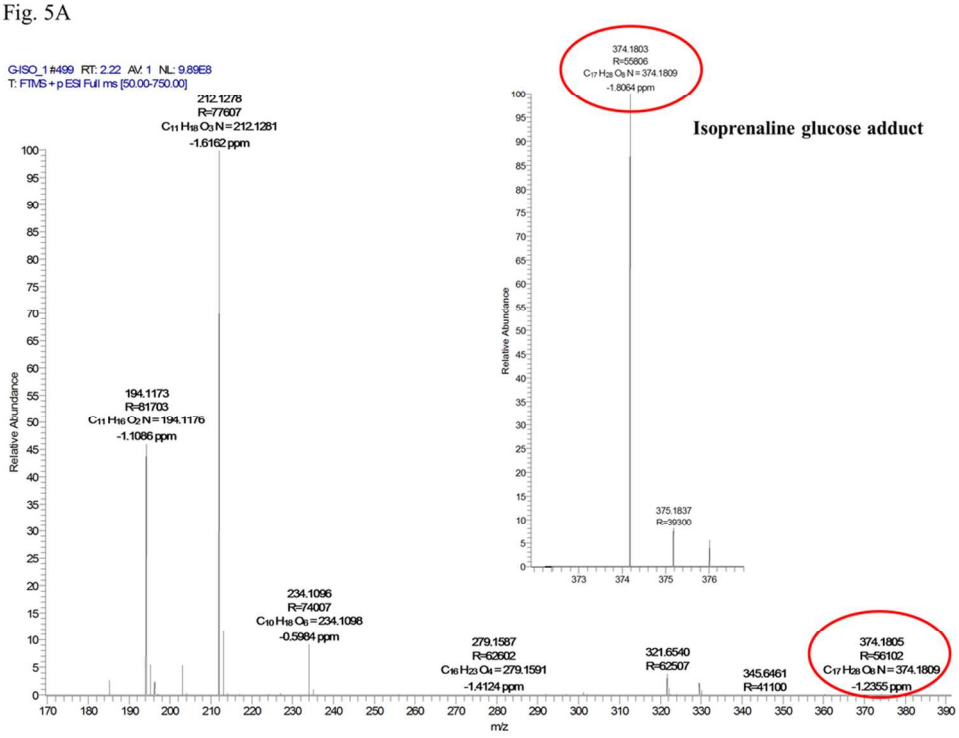


86x59mm (300 x 300 DPI)



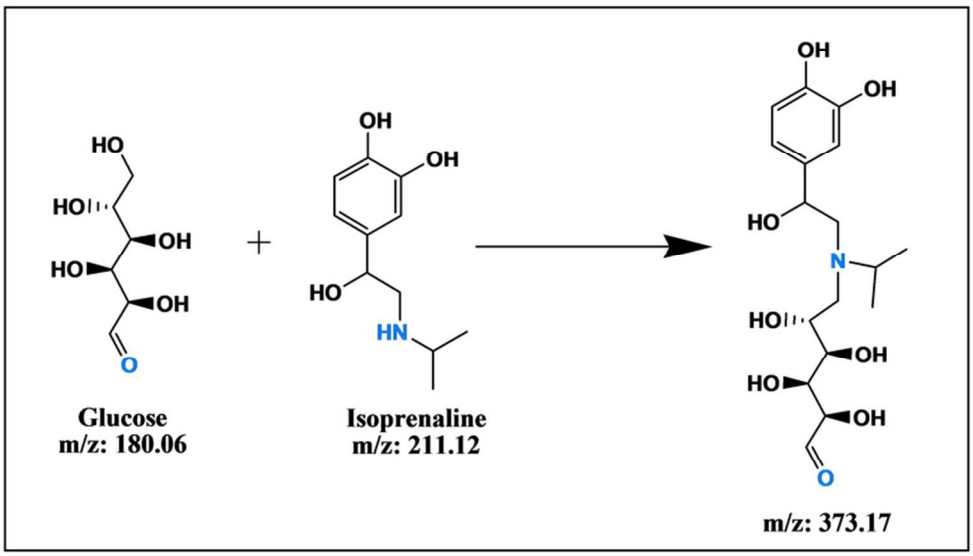
95x71mm (300 x 300 DPI)

Fig. 5A



95x71mm (300 x 300 DPI)

Fig. 5B



74x45mm (300 x 300 DPI)

Table 1. List of Amadori modified peptides of *in vitro* glycated BSA, glycated BSA treated with isoprenaline (10mM) or aminoguanidine (10mM).

Glycated BSA										
Sr. No	**Mod. site**	**Peptide sequence**	**MH+**	**m/z**	**CS**	**RT**	**XCorr**	**MC**	**Modification**	**AUC**
1	183	RHPYFYAPELLYYAN**K***YNGVFQE**C**CQAEDK	3935.746	787.955	+5	25.07	6.42	2	Amadori	5.04E+07 ± 20071
2	580	TVMENFVAFVD**K***C**C**AADDKEA**C**FAVEGPK	3470.531	868.388	+4	26.94	6.05	2	Amadori	5.98E+07 ± 22342
3	346	NYQEA**K***DAFLGSFLYEYSR	2463.143	821.719	+3	26.21	4.95	1	Amadori	8.42E+07 ± 30953
4	437	**K***VPQVSTPTLVEVSR	1801.988	601.334	+3	17.40	3.51	1	Amadori	2.99E+07 ± 14022
5	318	SH**C**IAEVE**K***DAIPENLPPLTADFAEDKDV**C**K	3673.719	919.185	+4	21.74	5.41	2	Amadori	1.50E+08 ± 42489
6	587	**C**CAADD**K***EA**C**FAVEGPK	2089.844	697.286	+3	14.09	3.49	1	Amadori	2.79E+07 ± 14544
7	28	DTH**K***SEIAHR	1355.653	452.555	+3	2.77	2.15	1	Amadori	9.37E+06 ± 13408
8	266	VH**K***E**C**CHGDLLE**C**ADDRADLAK	2774.213	925.409	+3	14.30	4.68	2	Amadori	5.37E+07 ± 39711
9	386	EYEATLEE**C**CA**K***DDPHA**C**YSTVFDK	3200.297	800.829	+4	17.86	4.23	1	Amadori	3.16E+07 ± 12925
10	140	L**K***PDPNTL**C**DEFK	1738.818	580.277	+3	17.51	3.07	0	Amadori	3.04E+07 ± 79816
11	36	F**K***DLGEEHFK	1411.670	471.228	+3	12.74	2.13	1	Amadori	7.46E+07 ± 31968
12	548	**K***QTALVELLK	1304.764	652.885	+2	19.29	2.86	1	Amadori	1.15E+09 ± 28202
13	299	L**K***E**C**CDKPLLEK	1694.828	847.917	+2	28.95	2.53	1	Amadori	2.16E+08 ± 47561
14	401	L**K***HLVDEPQNLIK	1708.944	854.975	+2	16.00	3.17	1	Amadori	3.78E+07 ± 27453
15	463	**C**C**C**T**K***PESER	1328.543	443.519	+3	3.59	1.89	0	Amadori	1.98E+07 ± 54866
16	197	YNGVFQE**C**CQAED**K***GA**C**LLPK	2649.158	883.724	+3	19.00	2.05	1	Amadori	3.96E+07 ± 13231
17	117	ETYGDMAD**C**CE**K***QEPER	2279.865	760.626	+3	11.30	1.34	1	Amadori	2.29E+06 ± 11890
18	304	E**C**CD**K***PLLEK	1453.653	485.222	+3	10.58	1.67	0	Amadori	1.07E+07 ± 29797
19	151	LKPDPNTL**C**DEF**K***ADEKK	2310.118	578.285	+4	17.15	1.99	2	Amadori	5.71E+07 ± 30900
20	130	QEPERNE**C**FLSH**K***DDSPDLPK	2703.217	541.449	+5	14.18	2.15	2	Amadori	1.36E+07 ± 23347
21	256	AEFVEVT**K***LVTDLTK	1854.992	928.000	+2	26.98	3.50	1	Amadori	5.04E+07 ± 20071
22	374	LA**K***EYEATLEE**C**CAK	1976.880	988.943	+2	14.13	4.04	1	Amadori	5.98E+07 ± 22342
23	130	NE**C**FLSH**K***DDSPDLPK	2063.920	516.735	+4	14.94	1.89	1	Amadori	8.42E+07 ± 30953

Glycated BSA treated with Isoprenaline										
1	346	NYQEA**K***DAFLGSFLYEYSR	2463.136	821.716	+3	26.50	3.26	1	Amadori	5.04E+06 ± 8633
2	256	AEFVEVT**K***LVTDLTK	1854.992	927.999	+2	27.34	2.81	1	Amadori	1.35E+07 ± 22150
3	587	**CC**AADD**K***EA**C**FAVEGPK	2089.846	697.287	+3	14.47	2.42	1	Amadori	2.72E+06 ± 10972
4	548	**K***QTALVELLK	1304.767	652.887	+2	19.90	2.16	1	Amadori	6.94E+06 ± 12061
5	580	TVMENFVAFVD**K*CC**AADDKEA**C**FAVEGPK	3486.516	872.384	+4	23.56	2.46	2	Amadori	4.34E+06 ± 78917
6	318	SH**C**IAEVE**K***DAIPENLPPLTADFAEDKDV**C**K	3673.716	919.184	+4	22.65	5.06	2	Amadori	3.36E+07 ± 35344
7	568	ATEEQL**K***TV**M**ENFVAFVDK	2377.155	793.056	+3	26.35	3.98	1	Amadori	2.59E+07 ± 45698
8	36	F**K***DLGEEHFK	1411.671	471.228	+3	13.67	1.41	1	Amadori	2.46E+06 ± 34145
9	266	VH**K***E**CC**HGDLLE**C**ADDRADLAK	2774.213	555.648	+5	14.65	2.96	2	Amadori	1.32E+07 ± 17338
10	374	LA**K***EYEATLEE**CC**AK	1976.878	659.630	+3	14.57	2.68	1	Amadori	1.25E+07 ± 23067

Glycated BSA treated with aminoguanidine										
1	580	TVMENFVAFVD**K*CC**AADDKEA**C**FAVEGPK	3470.520	868.385	+4	26.77	6.31	2	Amadori	4.04E+07 ± 60140
2	183	RHPYFYAPELLYYAN**K***YNGVFQE**CC**QAEDK	3935.768	984.697	+4	25.28	6.75	2	Amadori	1.62E+07 ± 10204
3	346	NYQEA**K***DAFLGSFLYEYSR	2463.139	821.717	+3	26.31	5.10	1	Amadori	6.24E+07 ± 14931
4	266	VH**K***ECCHGDLLECADDRADLAK	2774.214	925.409	+3	14.71	4.72	2	Amadori	3.36E+07 ± 80061
5	587	**CC**AADD**K***EA**C**FAVEGPK	2089.847	697.287	+3	14.51	3.20	1	Amadori	1.79E+07 ± 61888
6	386	EYEATLEE**CC**A**K***DDPHA**C**YSTVFDK	3200.299	800.830	+4	17.94	4.11	1	Amadori	2.22E+07 ± 67252
7	437	**K***VPQVSTPTLVEVSR	1801.987	601.334	+3	17.49	3.68	1	Amadori	1.98E+07 ± 10180
8	318	SH**C**IAEVE**K***DAIPENLPPLTADFAEDKDV**C**K	3673.721	919.185	+4	22.28	5.13	2	Amadori	1.36E+08 ± 325001
9	140	L**K***PDPNTL**C**DEFK	1738.817	580.277	+3	17.53	3.02	0	Amadori	2.15E+07 ± 19161
10	548	**K***QTALVELLK	1304.765	652.886	+2	19.62	2.84	1	Amadori	7.65E+08 ± 13907
11	36	F**K***DLGEEHFK	1411.672	471.229	+3	13.97	2.12	1	Amadori	4.82E+07 ± 17859
12	130	NE**C**FLSH**K***DDSPDLPK	2063.920	516.735	+4	15.07	1.77	1	Amadori	4.55E+06 ± 30820
13	401	L**K***HLVDEPQNLIK	1708.945	854.976	+2	15.96	2.70	1	Amadori	2.37E+07 ± 98943
14	28	DTH**K***SEIAHR	1355.651	452.555	+3	2.5	2.16	1	Amadori	7.04E+06 ± 23819
15	463	**CC**T**K***PESER	1328.544	443.519	+3	3.42	2.00	0	Amadori	1.36E+07 ± 23415
16	304	E**CC**D**K***PLLEK	1453.654	485.222	+3	11.50	1.59	0	Amadori	7.07E+06 ± 82741

Static modification: Carbamidomethyl (57.02146 Da), CS-Charge state, RT-Retention time, MC-Missed cleavage

Table 2. Area under curve ratio of few modified to unmodified peptides in glycated BSA, glycated BSA treated with isoprenaline (10mM) or aminoguanidine (10mM).

Glycated BSA							
Sr. No	**Mod. Site**	**Sequence**	**MH+**	**m/z**	**CS**	**RT**	**AUC Ratio (Modified/Control)**
1	548	**K***QTALVELLK	1304.764	652.885	2	19.29	9.01 ± 2.75
		KQTALVELLK	1142.716	571.861	2	19.48	
2	580	TVMENFVAFVD**K***CCAADDKEACFAVEGPK	3470.531	868.388	4	26.94	16.95 ± 3.4
		TVMENFVAFVDKCCAADDKEACFAVEGPK	3308.468	827.872	4	27.01	
3	318	SHCIAEVE**K***DAIPENLPPLTADFAEDKDVCK	3673.719	919.185	4	21.74	1.70 ± 0.08
		SHCIAEVEKDAIPENLPPLTADFAEDKDVCK	3511.667	1171.22	3	21.82	
4	266	VH**K***ECCHGDLLECADDRADLAK	2774.213	925.409	3	14.3	1.40 ± 0.06
		VHKECCHGDLLECADDRADLAK	2612.159	871.391	3	14.47	
Glycated BSA treated with Isoprenaline							
1	548	**K***QTALVELLK	1304.767	652.887	2	19.9	0.01 ± 0.0025
		KQTALVELLK	1142.716	571.861	2	20.1	
2	587	CCAADD**K***EACFAVEGPK	2089.846	697.287	3	14.47	0.03 ± 0.0002
		CCAADDKEACFAVEGPK	1927.793	964.4	2	14.63	
3	580	TV**M**ENFVAFVD**K***CCAADDKEACFAVEGPK	3486.516	872.384	4	23.56	0.47 ± 0.025
		TV**M**ENFVAFVDKCCAADDKEACFAVEGPK	3324.466	831.872	4	23.78	
4	318	SHCIAEVE**K***DAIPENLPPLTADFAEDKDVCK	3673.716	919.184	4	22.65	0.61 ± 0.05
		SHCIAEVEKDAIPENLPPLTADFAEDKDVCK	3511.667	1171.22	3	22.6	
Glycated BSA treated with aminoguanidine							
1	548	**K***QTALVELLK	1304.765	652.886	2	19.62	6.56 ± 1.0
		KQTALVELLK	1142.716	571.861	2	19.59	
2	580	TVMENFVAFVD**K***CCAADDKEACFAVEGPK	3470.531	868.388	4	26.94	11.23 ± 1.5
		TVMENFVAFVDKCCAADDKEACFAVEGPK	3308.468	827.872	4	27	
3	318	SHCIAEVE**K***DAIPENLPPLTADFAEDKDVCK	3673.719	919.185	4	22.28	1.78 ± 0.05
		SHCIAEVEKDAIPENLPPLTADFAEDKDVCK	3511.667	1171.22	3	22.44	
4	587	CCAADD**K***EACFAVEGPK	2089.847	697.287	3	14.51	0.15 ± 0.002
		CCAADDKEACFAVEGPK	1927.793	964.4	2	14.49	