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Honey bee venom decreases the complications of diabetes by preventing hemoglobin glycation

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ABSTRACT

Honey bee venom (BV) and its constituents have been reported to contain a wide variety of pharmaceutical properties such as analgesic, anti-inflammatory, anti-nociceptive, and anticancer effects. Hyperglycemia in diabetes leads to increased protein glycation resulting in structural and functional alteration in proteins. Here, we investigated the effect of BV on the glycation of human hemoglobin. Hemoglobin was incubated with glucose in the presence or absence of BV for 5 weeks. The glycation extent of hemoglobin was examined by UV–visible, Circular Dichroism (CD) and fluorometry methods. Results of the present study showed that BV prevents glycation-induced increasing in β-sheet structure, decreasing in free amino groups, altering in the secondary structure and heme degradation in the hemoglobin. These results imply that BV has a significant antiglycation effect, which can restrain glycation-induced alteration in the secondary structure and function of hemoglobin. Hence, BV has the potential to be used as a natural drug to prevent diabetes complications.

1. Introduction

Honey bee (*Apis mellifera* L.) venom, which is stored by honey bees within their venom sacs for self-defense against a broad diversity of predators, has traditionally been used to treat a variety of diseases and conditions, such as arthritis, rheumatism, back pain, cancerous tumors, and skin disease [1–3].

Bee venom (BV) contains various peptides including mellitin, apamin, adolapin and mast cell degranulation peptide, which have a wide variety of pharmaceutical properties. It also contains enzymes (e.g. phospholipase A2) and non-peptide components (e.g. histamine, lipids and carbohydrates) [4–6]. Two major ingredients of BV are phospholipase A and melittin [7]. Melittin is a small protein containing 26 amino acid residues with a molecular weight of 2850 Da and is the principal toxin in BV [8]. Melittin has been reported to contain pro-inflammatory [9], anti-inflammatory [10], anti-nociceptive [11], and anticancer effects [12].

BV exhibits pharmacological actions such as analgesic, anti-arthritic, and anti-inflammatory effects attributable to bioactive compounds [13,14]. It has been used for the treatment of inflammatory diseases such as rheumatoid arthritis and relief of pain in oriental medicine [15,16]. Besides, previous studies have reported that BV induces apoptotic death in mouse melanoma cells [1]; however, the underlying mechanisms are not clear. BV therapy is done by various methods, such as Apitherapy (using live honey bee stings) [17], apipuncture (bee venom acupuncture) and direct injection of BV [18].

Hyperglycemia, a hallmark of diabetes mellitus (DM), leads to increased protein glycation resulting in structural and functional alteration in proteins [19]. Diabetic patients are susceptible to long-term complications of diabetes, such as retinopathy [20], cataract [21], atherosclerosis [22,23], neuropathy [24], and nephropathy [25] and incomplete and prolonged wound healing [26]. Protein glycation is the most important factor in the development of these complications [27], and these complications of diabetes as a later result of disordered glucose metabolism, are the main reason of morbidity and mortality for patients [28].

Glycation is a non-enzymatic reaction between sugars and a free amino group of proteins resulting in advanced glycation end-products (AGEs) [29]. Protein glycation and AGEs are accompanied by increased free radical activity that leads to the biomolecular damage in diabetes [19]. AGEs generate oxygen free radicals that may potentiate the development of atherosclerosis [30]. Moreover, AGEs can produce oxygen free radicals through an indirect process, by inducing the release of cytokines through interaction of AGEs with their cellular receptors [31].

Because of widespread occurrence of AGEs and the oxidative stress derived from them in a variety of diseases and diabetes complications, it has a great deal of interest to identify and develop AGE inhibitor that can suppress AGE formation [32]. Numerous AGE inhibitors have been developed, such as amino guanidine the most well-known AGE inhibitor. In animal models, amino guanidine ameliorated diabetic

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complications but because of side effects its practical applications are limited in clinical trials [33]. In spite of the wide range of pharmacological drugs that have been used as an antiglycating agent, there is a continuing search for new alternatives, both because of the low efficacy and side effects of them.

In order to study the effects of glycation on human hemoglobin and also to assess the effects of BV, in different concentration, on the glycation of human hemoglobin with glucose, this work describes the structural changes on hemoglobin during glycation and the effects of BV on restraining glycation.

2. Materials and methods

2.1. Reagents

Bovine serum albumin (BSA), fluorescamine and Thioflavin T (ThT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium citrate, ammonium sulfate, glucose and Coomassie brilliant blue R-250 were purchased from Merck Co. (Darmstadt, Germany). All other materials were of analytical grade. All solutions were prepared with deionized water.

2.2. Bee venom

A. mellifera venom was collected by means of an electric shocker apparatus composed of a shocker and a collector unit. The shocker unit produces a light electric shock once every few seconds. Honeybees were stimulated with light electric shock and sting in beehives. The collector unit is a network of wires with small gaps and a glass plane between them. Every 25 min, the shocker unit turned off and the dried bee venom material on collector panel was collected by scraping. Dried venom was dissolved in distilled water and centrifuged at 12,000 RPM for 10 min to remove insoluble materials. The resulting solutions were used as a stock for preparing different concentrations of BV solutions. In this study BV was used in three different concentrations (10, 20 and 40 μg/ml).

2.3. Preparation and in vitro glycation of hemoglobin

Human hemoglobin was prepared from freshly drawn blood according to the Riggs [34]. After dialysis, protein concentrations were estimated by the Bradford's method in which bovine serum albumin (BSA) was used as standard [35]. Hemoglobin (10 mg/ml) was incubated in the presence and absence of glucose (40 mM), as a glycating sugar, in a 50 mM phosphate buffer with pH 7.4. Also BV was used in different concentrations (10, 20 and 40 μg/ml) as an antiglycating agent. Incubation was carried out at 37 °C and 40 RPM for 5 weeks in a shaker incubator. At the end of each week, sampling was conducted to store at −70 °C until processing.

2.4. Determination of free amino groups using fluorescamine

1 μl protein solution (10 mg/ml), 100 μl Na_2HPO_4 (100 mM), 45 μl distilled water and 50 μl fluorescamine reagent solution (1 mM fluorescamine in acetonitrile) were mixed and incubated for 10–15 min in the dark in a 96-well plate. The fluorescence intensity of fluorescamine was measured at excitation/emission wavelengths of 390/490 nm using a Carry spectrofluorometer. The percentage of free amino groups was calculated according the relation:

$$\text{percentage of free amino groups} = \frac{\text{flourescence emission of hemoglobin in desired condition}}{\text{flourescence emission of control hemoglobin}} \times 100.$$

2.5. Determination of fibrilar state with Thiofavin T

The fibrilar state of incubated hemoglobin was determined as previously described [35]. Briefly, solutions of 0.2 mg/ml glycated hemoglobin and control samples were incubated at 25 °C with Thioflavin T (10 μg/ml) for 40 min. The sample fluorescence was measured at excitation/emission wavelengths of 450/490 nm.

2.6. Amount of heme degradation

Fluorescence emission of heme degradation product was recorded as previously described [36]. Fluorescence was detected at excitation 460 nm and emission 570 nm using a Carry spectrofluorometer.

2.7. Soret band statue

The UV/vis spectrum was recorded at room temperature on a Shimadzu spectrophotometer. The scope of the scanning wavelength was from 380 to 440 nm.

2.8. Circular Dichroism studies

Circular Dichroism measurement was carried out between 190 and 260 nm, at the far UV region with an Aviv CD spectropolarimeter (USA) at 25 °C. Bandwidth was 1 nm. The CD measurement was performed using a 0.1 cm path length quartz cuvette at the final protein concentration of 0.2 mg/ml. The CD software of CDNN was used to predict the secondary structure of the protein according to the statistical method.

2.9. Statistical analysis

Data were obtained from three independent experiments with similar patterns. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, and results were expressed as mean ± SD.

3. Results and discussion

3.1. Determination of free amino groups using fluorescamine

Fig. 1 shows the percentages of reacted amino groups of samples incubated in different condition using fluorescamine as a fluorescent reagent, which has a fluorescent characteristic upon reacting with free amino groups in proteins [37]. In all samples, before incubation and

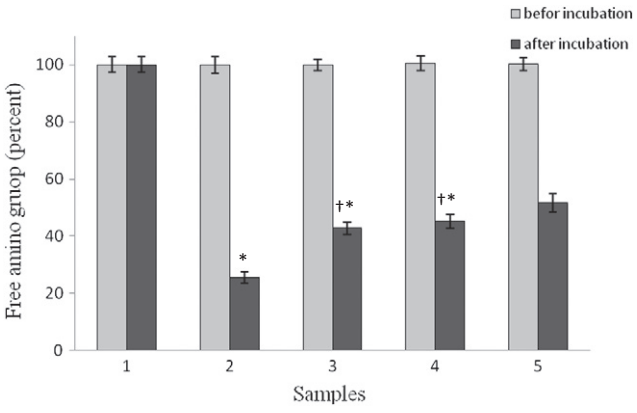

Fig. 1. Percentage of free amino groups after incubating of hemoglobin samples for 5 weeks in 50 mM phosphate buffer, pH 7.4 at 37 °C with 1) no additives (control), 2) 40 mM glucose, 3) 40 mM glucose and 10 μg/ml bee venom, 4) 40 mM glucose and 20 μg/ml bee venom and 5) 40 mM glucose and 40 μg/ml bee venom. * represented $P < 0.001$ compared to control group. † represented $P < 0.001$ compared to group no. 2.

control hemoglobin after incubation, all of the amino groups were free of glucose. The percentages of free amino groups were found to decrease significantly in the presence of 40 mM glucose as a glycating agent. Estimation of the number of free lysine residues in hemoglobin after incubation with glucose showed a 65% decrease in the percentage of free amino groups. In addition, Fig. 1 shows that BV at the concentration of 40 μg/ml increased the percentage of free amino groups up to 50%, which is 2 times more than the number of free amino groups in the presence of glucose. Also, data showed that BV increases the amount of free amino groups of glycated hemoglobin in a dose-dependent manner that means BV prevents binding of glucose and reactive di-carbonyls to proteins because the addition of these materials to proteins decreases the amount of free amino groups in proteins.

3.2. Determination of fibrilar state with Thiofavin T

ThT is a cationic benzothiazole dye, which has been previously shown to interact with amyloid structures in proteins and exhibits enhanced fluorescence when bound to amyloid fibrils [38]. This quality of ThT was used in the detection of β-sheet structures in proteins at ex vivo and in vitro conditions [39]. As shown in Fig. 2, increasing the ThT fluorescence intensity of the samples indicates the formation of amyloid fibrils. The fibrillar state of all glycated hemoglobin is enhanced compared to that of the hemoglobin control. Incubation of hemoglobin in the presence of glucose for 5 weeks results an increase of 60% of β-sheet formation for glycated hemoglobin relative to the control; whereas the ThT fluorescence intensity decreased in the presence of BV. This decreasing depends on the concentration of BV, which means that using more concentration of BV leads to less amyloid fibril formation. Previous studies have proven the anti-arthritis, pain-releasing and anti-cancer effects of BV [8]. Our results can be considered the first report on antiglycation properties of BV.

3.3. Amount of heme degradation

Fig. 3 shows the emission diagram at one week intervals of the fluorescent product formed during the glycation of hemoglobin. The fluorescence emission increased with increasing the time of incubation in samples that incubated in the presence of glucose, which shows the progressive degradation of heme. The most reasonable hypothesis for destruction of heme by glucose is that oxidative stress induced during glycation is the initiating agent in the degradative mechanism [40–42]. Also, the fluorescence emission of control hemoglobin increased after 5 weeks of incubation, which means a little heme degradation. The amount of heme degradation in control hemoglobin is

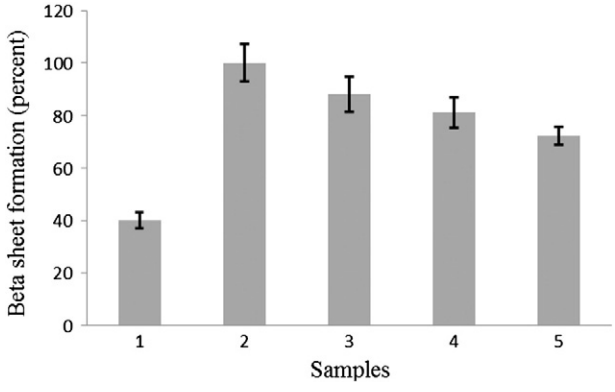

Fig. 2. Beta sheet formation after incubating of hemoglobin samples for 5 weeks in 50 mM phosphate buffer, pH 7.4 at 37 °C with 1) no additives (control), 2) 40 mM glucose, 3) 40 mM glucose and 10 μg/ml bee venom, 4) 40 mM glucose and 20 μg/ml bee venom and 5) 40 mM glucose and 40 μg/ml bee venom. * represented $P < 0.001$ compared to control group. † and # respectively represented $P < 0.001$ and $P < 0.05$ compared to group no. 2.

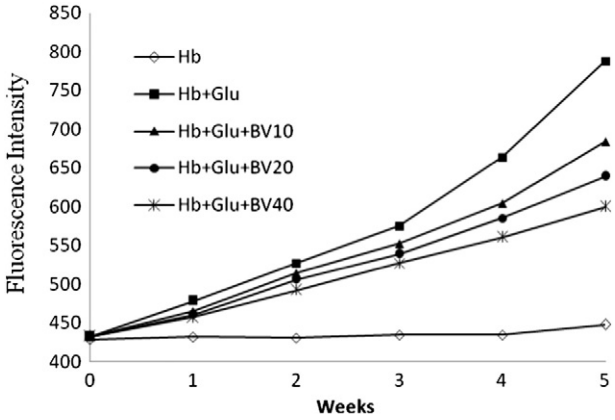

Fig. 3. Fluorescence intensity after excitation at 460 nm of hemoglobin samples incubated for 5 weeks in 50 mM phosphate buffer, pH 7.4 at 37 °C with 1) no additives (Hb), 2) 40 mM glucose (Hb + Glu), 3) 40 mM glucose and 10 μg/ml bee venom (Hb + Glu + BV10), 4) 40 mM glucose and 20 μg/ml bee venom (Hb + Glu + BV20) and 5) 40 mM glucose and 40 μg/ml bee venom (Hb + Glu + BV40).

negligible, compared to glycated hemoglobin. The rate of heme degradation of glycated hemoglobin in final weeks is faster than that in early weeks, which means that heme degradation during glycation is a time-dependent process. As it is shown in Fig. 3, BV inhibits the glycation-induced heme degradation in hemoglobin. Previous reports have shown that BV has significant antioxidant properties [43], and it is possible that BV decreases glycation through inhibition of glycoxidation. As the concentration of BV increased, the fluorescence intensity as well as the amount of heme degradation decreased in a concentration dependent manner. BV in the concentration of 40 μg/ml decreases the amount of heme degradation of hemoglobin up to 50% after incubation for 5 weeks in the presence of glucose.

3.4. Soret band statue

UV–vis spectroscopy is a very fundamental and simple spectroscopic technique to study the conformational changes of heme proteins; the Soret band of the heme can provide useful information on their secondary structure [44]. The absorption spectral changes of hemoglobin due to incubation in the presence and absence of glucose and BV for 5 weeks of incubation time are shown in Fig. 4. The absorption intensity of hemoglobin at soret band not only decreases significantly during

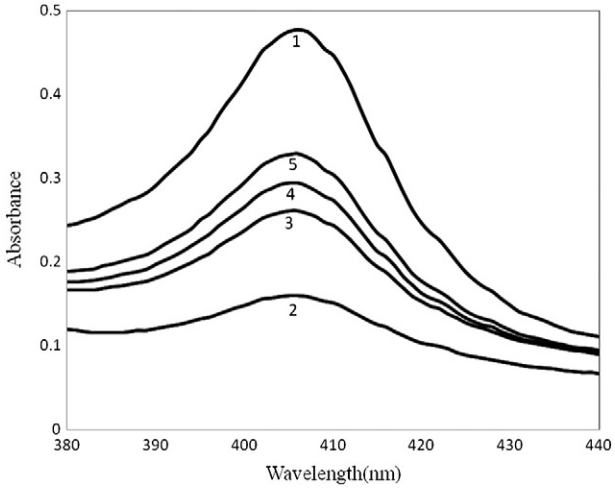

Fig. 4. UV–vis spectra of hemoglobin samples incubated for 5 weeks in 50 mM phosphate buffer, pH 7.4 at 37 °C with 1) no additives (control), 2) 40 mM glucose, 3) 40 mM glucose and 10 μg/ml bee venom, 4) 40 mM glucose and 20 μg/ml bee venom and 5) 40 mM glucose and 40 μg/ml bee venom.

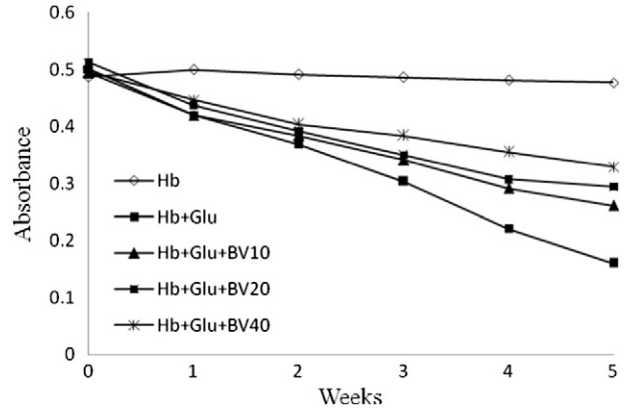

Fig. 5. Absorbance of soret band's peak in hemoglobin samples incubated for 5 weeks in 50 mM phosphate buffer, pH 7.4 at 37 °C with 1) no additives (Hb), 2) 40 mM glucose (Hb + Glu), 3) 40 mM glucose and 10 μg/ml bee venom (Hb + Glu + BV10), 4) 40 mM glucose and 20 μg/ml bee venom (Hb + Glu + BV20) and 5) 40 mM glucose and 40 μg/ml bee venom (Hb + Glu + BV40).

glycation but also has a red shifting. The decreasing of Soret band intensity and the red shifting show that a geometric distortion has occurred in the structure of glycated hemoglobin [45]. These results mean that glycation leads to conformational changes in hemoglobin. BV inhibits both reductions in absorption intensity and red shifting. Fig. 5 represents the absorption intensity of hemoglobin in different condition of incubation. It was shown that the absorption intensity decreased in glycated hemoglobin; this process depends on time and at the ending weeks has a faster rate. Absorption of the Soret band of glycated hemoglobin increased significantly in the presence of BV and aspirin. The Soret band intensity depends on the BV concentration, the higher the BV concentration, the greater the absorption of glycated hemoglobin.

3.5. Circular Dichroism studies

Glycation-induced conformation changes in the secondary structure of hemoglobin were studied by circular dichroic (CD) spectra analysis. Fig. 6 shows the far-UV CD spectrum of hemoglobin between 200 and 250 nm in the presence and absence of BV and glucose, which is an evidence that glycation leads to a systematic alteration in the secondary structure of hemoglobin. Glycation leads to a significant decrease in negative ellipticity in the region 210–230 nm, which means loss of helical structure of the protein. However, the far-UV CD data for

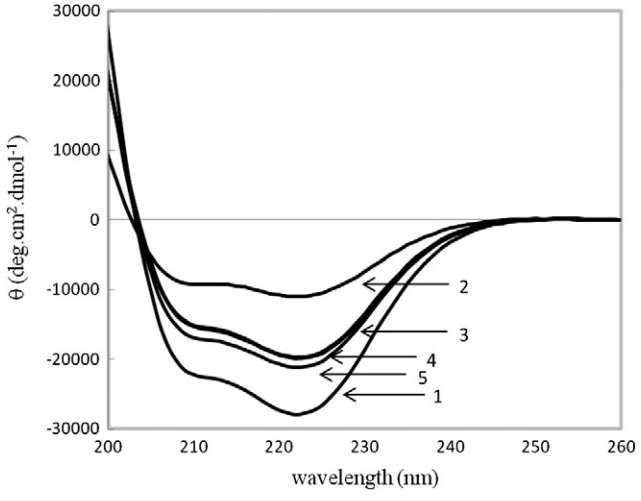

Fig. 6. Circular Dichroism (CD) spectra of hemoglobin samples incubated for 5 weeks in 50 mM phosphate buffer, pH 7.4 at 37 °C with 1) no additives (control), 2) 40 mM glucose, 3) 40 mM glucose and 10 μg/ml bee venom, 4) 40 mM glucose and 20 μg/ml bee venom and 5) 40 mM glucose and 40 μg/ml bee venom.

Table 1
Effect of bee venom on the percentage of secondary structure of hemoglobin incubated for 5 weeks in 50 mM phosphate buffer, pH 7.4 at 37 °C.

Sample	α-Helix %	β-Sheet %	Random coil %
Hemoglobin	70.3	13.5	16.2
Hemoglobin + glucose	41.4	31.2	27.4
Hemoglobin + glucose + bee venom 10 μg/ml	49.2	29.9	20.9
Hemoglobin + glucose + bee venom 20 μg/ml	52.6	27.1	20.3
Hemoglobin + glucose + bee venom 40 μg/ml	55.3	24.6	20.1

hemoglobin incubated with different concentration of BV showed an increase in negative ellipticity compared to the glycated hemoglobin. Our data show that glycation induces structural changes in hemoglobin, such as reduced α-helix content and increased β-sheet and random coil content, which are in good agreement with previous reports [46]. Also, Table 1 shows the percentages of different types of secondary structure of hemoglobin in different condition of incubation. As it is shown in Table 1, the α-helicity of glycated hemoglobin decreased dramatically from 70.3 to 41.4% as a function of incubation time, while β-conformation increased from 13.5 to 31.2%. However, BV significantly inhibits alterations in the percentage of different types of secondary structure induced by glycation.

4. Conclusion

In conclusion, glycation leads to structural and functional alteration in proteins in diabetic people. Because of the significant role of proteins, their modifications should be prevented. Since previous studies have shown that glycated hemoglobin has not only structural changes but also a functional alteration such as reduced peroxidase activity and high oxygen affinity [19]. Then, our results indicate that BV has a significant antiglycation effect and it can prevent glycation-induced alteration in the secondary structure and function of hemoglobin, thus BV could be developed as a natural drug against glycation-associated complications in diabetes.

Conflicts of interest statement

The authors declare that there are no conflicts of interest associated with this work.

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