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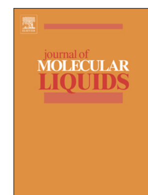

Honey bee venom decreases the complications of diabetes by preventing hemoglobin glycation

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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.

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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:

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

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

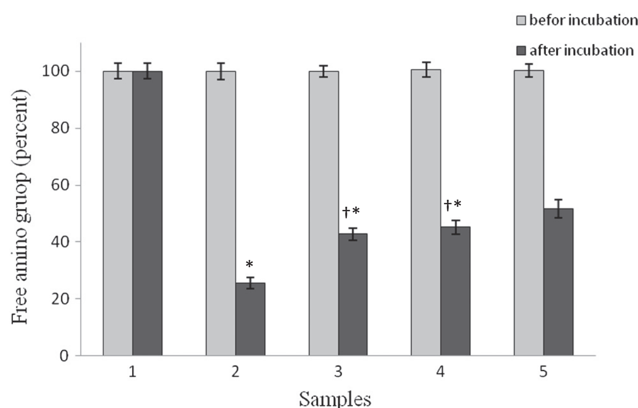

Fig. 1. Percentage of free amino groups after incubating of hemoglobin samples for 5 weeks in 50 mM phosphate buffer, pH 7.4 at 37 °C with 1) no additives (control), 2) 40 mM glucose, 3) 40 mM glucose and 10 μg/ml bee venom, 4) 40 mM glucose and 20 μg/ml bee venom and 5) 40 mM glucose and 40 μg/ml bee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

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