Identification of Cleaved Haptoglobin in the Serum of Bee Venom-Hypersensitive Patients

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Background: Bee venom has been used as a therapeutic compound for various human diseases in oriental medicine; however, it can induce anaphylaxis in hypersensitive patients during treatment. Anaphylaxis is an acute allergic reaction that occurs after allergen exposure. IgE is released from immune-related cells such as mast cells and basophils during anaphylaxis. Various inflammatory mediators are also released into the bloodstream during the acute response.

Objectives: We aimed to identify specific proteins from bee venom-hypersensitive patients.

Methods: We analyzed the blood serum of control and bee venom-hypersensitive patients using two-dimensional (2D) electrophoresis.

Results: An interesting protein spot with a molecular size of 10 kDa was identified at an isoelectric point (p.I.) of 5.5. Spots detected both before and after sweet bee venom therapy were not proteins induced by sweet bee venom. The 10 kDa protein was identified as the cleaved form of haptoglobin through liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Statistical analysis indicated that the presence of the spot was highly significant in the bee venom-hypersensitive group.

Conclusion: The findings suggest that cleaved haptoglobin may be a significant diagnostic protein for anaphylaxis. In addition, a high incidence of bee venom hypersensitivity may be associated with the haptoglobin genotype.

Keywords: Bee venom, Anaphylaxis, 2D electrophoresis, Haptoglobin, *Hp2* genotype, Diagnostic protein

INTRODUCTION

Anaphylaxis is an acute allergic reaction caused by an allergen infiltrating the bloodstream of an individual [1]. Both classical and alternative pathways are known as typical physiological pathways of anaphylaxis [2]. Immunoglobulin E (IgE)-mediated anaphylaxis has been described as a classical pathway [3]. IgE is synthesized in response to allergens such as foods, medications, or insect venom [4]. Allergen-specific IgE specifically binds to the high-affinity IgE receptor (FcεRI) on mast cells and basophils [5]. These cells may rapidly release histamine and tryptase into the bloodstream to induce an acute anaphylactic response. When an individual with IgE antibodies is exposed to the same allergen again, the secreted factors stimulate smooth muscle contraction or increased vascular permeability, inducing the functional dysregulation

of the respiratory or cardiovascular system [6,7]. The IgG-dependent pathway described in animal models represents a second, alternative pathway. Macrophages instead of mast cells, platelet-activating factor (PAF) instead of histamine, and IgG rather than IgE, are involved in this alternative pathway [8].

Bee venom contains various physiologically active components including melittin, apamin, mast cell-degranulating (MCD) peptide, adolapin, phospholipase A2 (PLA2), and hyaluronidase [9]. Bee venom has been used therapeutically in Asia for the treatment of inflammatory diseases, such as rheumatoid arthritis, cataracts, spinal inflammation, degenerative arthritis, acute pyrolytic arthritis, paralysis, and spinal nerve damage [10]. Recent studies have suggested that bee venom exerts anti-inflammatory activity by reducing IgE levels, cytokine release, and NF-κB activity [11,12].

A reduction in NF- κ B activity can alter the expression of inflammatory COX-2 and iNOS genes [13]. Bee venom has also been found to downregulate inflammatory cytokines, such as IL-1 β , IL-6, TNF- α , and TGF- β 1, in a rheumatoid arthritis animal model, suggesting the medicinal effect of bee venom [14]. However, individuals who are sensitive to bee venom may experience symptoms such as vomiting, chills, abdominal cramps, and difficulty in breathing [15]. Therefore, although the therapeutic potential of bee venom has been scientifically demonstrated, life-threatening immune responses such as anaphylaxis are still an issue [16].

Two-dimensional (2D) electrophoresis/liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis is widely used to identify proteins of interest [17]. Several diagnostic marker proteins have been identified following 2D analysis in some disease models [18]. Apolipoprotein A1 precursor, calgranulin B, and apolipoprotein E were identified as possible marker proteins for lung cancer, and increased gp96 protein and decreased annexin I may indicate liver cancer. Apolipoprotein A1 (M.W. 28 kDa) levels range from 0.9 to 2.1 g/L in the serum of normal individuals; however, it is markedly reduced to 0.31-0.35 g/L in the serum of patients with early ovarian cancer, suggesting that decreased apolipoprotein A1 may be a useful marker for ovarian cancer [19]. On the other hand, apolipoprotein AII with a molecular weight of 9 kDa is present at high concentrations in the serum of prostate cancer patients [20].

Anaphylaxis is defined as a serious allergic reaction that is rapid in onset and may cause death. Bee venom also triggers an anaphylactic response in hypersensitive individuals. In this study, we analyzed human serum obtained from bee venom-hypersensitive patients using 2D/LC-MS/MS.

MATERIALS AND METHODS

1. Human blood samples and ethics

Blood samples were collected from volunteers who visited an oriental medical clinic located in Chuncheon, Kangwon, Republic of Korea. Blood (5 mL) was obtained from each individual using an 18-gauge syringe. The blood was centrifuged to separate the serum and kept at -20°C until further analysis. This study was conducted after approval from the Institutional Review Board (IRB) of Kangwon National University (KWNUIRB-2019-05-011-022). Trichloroacetic acid (TCA) solution was added to precipitate the proteins overnight before centrifugation. Dithiothreitol (DTT), 10% in acetone) was added to stabilize the protein pellets. Urea lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, and 0.4% 3-10 ampholyte) was added to the protein pellets for 2D analysis.

2. Two-dimensional electrophoresis

A tube gel (urea, 30% acrylamide/bis-acrylamide 29:1 solution, 3-10 ampholyte, 100 μL of 10% ammonium persulfate (APS), and 40 μL of TEMED) was prepared for isoelectric focusing. Protein samples were loaded into the tube gel after heat denaturation and separated in the tube gel for 10 h at 1000 V. The tube gel was stabilized in transfer buffer (0.5 M Tris-HCl, 10% sodium dodecyl sulfate (SDS), and bromophenol blue). Then, the tube gel was loaded onto a 15% acrylamide gel, and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate the proteins according to size. Electrophoresis was performed for 1 h at 80 mA. Subsequently, the acrylamide gel was stained with Coomassie Brilliant Blue R-250 (in 50% methanol and 10% glacial acid) and destained with a solution containing 50% methanol and 10% glacial acid.

3. Identification of protein spots by LC-MS/MS

Protein spots were cut off, destained with acetonitrile, and dissolved in methanol. LC-MS/MS analysis was performed at Proteomtech, Inc. (Seoul, Korea). In brief, a nano ACQUITY UPLC and LTQ-orbitrap-mass spectrometer were used for the analysis (Thermo Electron, San Jose, CA, USA). The column used was a BEH C18 1.7 μm , 100 μm $\times$ 100 mm column (Waters, Milford, MA, USA). Information on the oligopeptides was obtained from LS-MS/MS analysis. The oligopeptides were compared with those in the NCBIprot protein database by searching the mascot database (www.matrixscience.com).

4. Statistical analysis

Protein spots were captured by designating areas that were 1 cm wide and 2 cm long with ImageJ software (Wayne Rasband, Bethesda, MD, USA), and the intensity was quantified. All statistical analyses were performed with EXCEL software (Microsoft, Redmond, WA, USA). An F-test was performed to examine the variance in the population under the null hypothesis. A t-test was performed to examine the t-distribution under the null hypothesis. $p < 0.05$ was considered statistically significant.

RESULTS

1. Blood donor medical information

This study was conducted after approval by the Institutional Review Board (IRB) of Kangwon National University. Blood samples were collected from volunteers among patients who visited an oriental medical clinic located in Chuncheon, Kangwon. We analyzed the blood serum from eight control patients and eight bee venom-hypersensitive patients (Table 1). The primary reasons for visiting the clinic were skeletal

musculoskeletal disorders and joint pain. The control group included two males and six females with an average age of 53 years; they had no history of allergic reactions from treatment with bee venom pharmacopuncture. In contrast, the bee venom-hypersensitive group showed noticeable

allergic reactions during or after treatment with bee venom pharmacopuncture. Their allergic reactions varied and included flushing, itching, and edema. Itching involved the skin and mucous membranes. Particularly, patients P-02, P-04, and P-09 showed severe allergic reactions during

Table 1. Medical information on the control and anaphylaxis groups

Group	ID	Age/Sex	Symptoms	Post-sBVT
Control	C-02	39M	Finger arthritis (-DIP PIP pain)	-
	C-03	29F	Cervical pain	-
	C-04	57F	Back pain	-
	C-06	44F	Back pain	-
	C-09	60F	Back pain	-
	C-11	71F	Knee pain	-
	C-14	62F	Finger arthritis (-DIP PIP pain)	-
	C-16	42F	Rump pain	-
Patient	P-01	54F	Knee joint pain	Flushing, itching
	P-02	47M	Cervical sprain	Flushing, itching
	P-03	58F	Back pain	Flushing, itching-
	P-04	54F	Back pain, knee pain	Flushing, itching
	P-05	33F	Stomachache	Flushing, itching
	P-08	49F	Wrist tendinitis	Flushing, itching, edema
	P-09	62F	Asthma, cough, dyspnea Bear's gall allergy	Flushing, itching, edema
	P-10	70F	Finger arthritis (-DIP PIP pain)	Flushing, itching, edema

*sBVT = sweet bee venom therapy.

Fig. 1. Analysis of patient sera using 2D electrophoresis. Serum proteins were prepared from the control and bee venom-hypersensitive groups. A protein spot was identified in the anaphylaxis group with a p.I. of 5.5 and molecular size of 10 kDa.

bee venom pharmacopuncture therapy. Moreover, patient P-09 had a history of asthma and showed complex allergic reactions to Fel Ursi Pharmacopuncture solution (FUPS), which is used for the treatment of stress or insomnia. Antihistamines and steroid drugs were used to treat bee venom hypersensitivity for approximately three weeks. The average age of the patients in the hypersensitive group was 53 years old, which included one male and seven females.

2. Identification of a specific protein spot in the bee venom-hypersensitive group

Blood was collected from voluntary donors, and the serum was separated by centrifugation and subjected to 2D analysis. First, serum proteins were separated in gel tubes with an ampholyte solution (pH 3-10) for 1D isoelectric focusing. Several main bands were observed in the pH 5-7 range. After rotating the gel tubes, 2D SDS-PAGE was performed

to separate the proteins according to size. We found an interesting protein spot in the bee venom-hypersensitive group compared with the control group (Fig. 1). Molecular characterization of the protein spot showed an approximate p.I. of 5.5 and a molecular size of 10 kDa. There were one or three protein spots on the gels analyzed. We performed a 2D analysis of 16 serum samples obtained from the control and hypersensitive volunteers (Fig. 2). Interestingly, most spots were detected in the bee venom-hypersensitive group and were rarely observed in the control group. These results indicated that the spots were associated with bee venom sensitivity. Furthermore, we analyzed serum samples obtained from a patient before and after sweet bee venom therapy (Fig. 3). Spots were detected on the gels of both serum samples, suggesting that the spots were not proteins induced by sweet bee venom.

	C-02	C-03	C-04	C-06	C-09	C-11	C-14	C-16
Spot								
Intensity	1907.82	918.941	621.799	382.921	3066.648	1658.92	1801.012	1581.497
	P-01	P-02	P-03	P-04	P-05	P-08	P-09	P-10
Spot								
Intensity	4708.861	28640.397	10309.983	3740.033	2600.305	9858.033	25762.69	27449.69

Fig. 2. Expression level of protein spots between the control and anaphylaxis groups. The protein spot was detected in the bee venom-hypersensitive group using 2D analysis. The image was captured by ImageJ software and the intensity was quantitated.

P-03

Fig. 3. 2D analysis of serum before and after sBV therapy. The protein spots were identified in both 2D analyses.

Fig. 4. Identification of the cleaved form of haptoglobin through LC-MS/MS. (A) The protein spot was trypsinized into small oligopeptides and subjected to LC-MS/MS analysis. Oligopeptide information on the spot identified it as haptoglobin based on the m/z value. (B) The haptoglobin protein is synthesized as a single polypeptide chain containing a signal peptide of 18 amino acids and both the α 2 and β subunits. The red-colored oligopeptide sequence shows the exact match on the haptoglobin protein.

3. Identification of cleaved haptoglobin through LC-MS/MS analysis

Protein spots were cut from the acrylamide gels, trypsinized to produce oligopeptides, and subjected to LC-MS/MS analysis. The charge-to-mass (m/z) value was obtained and compared with that in the NCBIproteome database by searching the mascot database (www.matrixscience.com). A total of 41 candidate proteins were identified including haptoglobin, which had the highest protein score (Fig. 4A). The oligopeptide sequence “RTEGDGVYTLNNEKQ” was observed with a high m/z value. Moreover, 4-5 oligopeptides matched the amino acid sequences of haptoglobin. Haptoglobin is a protein consisting of 406 amino acids with a predicted protein size of 46 kDa (Fig. 4B). We observed an approximately 10 kDa protein in 2D analysis. The matched amino acid sequences coincided with 101 amino acids in the full haptoglobin sequence and were scattered throughout the entire haptoglobin sequence. Therefore, the protein spot may be a cleaved form of haptoglobin.

4. Statistical analysis of the protein spot

We performed statistical analysis to determine whether the spots observed in the bee venom-hypersensitive group were statistically significant. The protein spots were captured by designating areas that were 1 cm wide and 2 cm long with ImageJ software and quantified by measuring the intensity. The F-test was used to examine the variance in the intensity data of the protein spots, which were assumed to be heteroscedastic. We also performed t-test analysis with the heteroscedastic populations in the control and bee venom-

Table 2. Statistical analysis of the quantification of cleaved haptoglobin between the control and patient groups

	Control	Patient
Average	1492.4	14133.7
Variance	728497.7	126538642.9
Count	8	8
F-test: <i>p</i> -value	0.0000003	
T-test: <i>p</i> -value	0.0157207	

hypersensitive groups. The *p*-value (0.015) from t-test analysis indicated that the presence of the spots was significant in the bee venom-hypersensitive group compared with the control group (Table 2).

DISCUSSION

Efforts have been made to develop disease-targeted therapeutic drugs in the biopharmaceutical market for several decades [21]. Recently, concerns regarding diagnosis in the early stage of diseases have increased [22]. Many phenotypic changes appear as cellular, biochemical, or molecular changes. Specifically, molecular changes can be detected in the blood, saliva, or sputum; thus, these changes can be quantified and can be considered as useful biomarkers [23]. The speed and accuracy of diagnostic technology should be high to identify specific marker proteins for a target disease [24].

Anaphylaxis is a serious hypersensitivity reaction with immunologic or non-immunologic mechanisms [25]. Anaphylaxis has been defined as a side effect of exposure to

an allergen, and various mediators are released into the bloodstream in response to the allergen [26]. The mediators include factors released from mast cells or basophilic storage vesicles, as well as cytokines from immune cells [27]. Considering that many factors are secreted into the bloodstream during anaphylaxis, it is important to study serological data [28]. Previously, the concentrations of the fibrinogen alpha chain and C3 and C4-A complement proteins were reported to be altered in the sera of patients with bee venom anaphylaxis [29]. In another study, the concentration of mast cell tryptase (MCT) was increased in the serum in severe anaphylaxis [27]. Moreover, platelet activation factor (PAF), chymase, carboxypeptidase A3, dipeptidyl peptidase I (DPPI), basogranulin, and CCL-2 were increased during anaphylaxis, suggesting that they may be useful biomarkers for diagnosis [30]. However, the relationship between different mediators and diagnostic markers is not fully understood [31].

In this study, we identified the cleaved form of haptoglobin as a specific protein marker in bee venom-hypersensitive patients, which may be used as a diagnostic marker protein. Haptoglobin belongs to the family of acute-phase plasma proteins and functions as a plasma detoxifier by combining with hemoglobin. The full-length haptoglobin protein is produced as a pre-pro-protein of 58 kDa, containing a signal peptide of 18 amino acids and both α and β subunits. Functional haptoglobin is synthesized from a single polypeptide chain with cleavage into α and β chains by c1r-like serine protease [32]. The alleles of the haptoglobin gene (Hp gene) consist of the Hp1 and Hp2 variants, and both alleles have been found in populations worldwide [33]. However, there are some reports indicating that the Hp2 allele is predominant in Asian populations, whereas the Hp1 allele is predominant in Western populations [34]. The α 1 chain derived from the Hp1 allele is a subunit of 9 kDa covering one CCP domain, whereas the α 2 chain derived from the Hp2 allele is a subunit of 14 kDa covering two CCP domains [32]. We obtained a high number of hits on amino acids 116-154 in the α 2 chain of haptoglobin, suggesting that the identified protein spot was the α 2 chain of cleaved haptoglobin. However, we also obtained hits on the 297-312, 325-346, and 379-402 regions in the β chain of full-length haptoglobin. These results suggest that the cleaved form of haptoglobin might be a mix of partial α 2 chains and partial β chains. Therefore, the incidence of bee venom-related anaphylaxis may be higher among individuals with the Hp2 allele. The expression of haptoglobin is reduced in congenital haptoglobin deficiency, hemolytic anemia, acute hepatitis, and severe liver disorders [35]. Mutations in the haptoglobin gene can cause kidney failure in diabetes, as well as Crohn's disease or Parkinson's disease [36]. The expression

of haptoglobin can be induced by several growth hormones and pro-inflammatory cytokines; however, its concentration can greatly decrease during accelerated hemolysis, suggesting that haptoglobin might be a marker of the acute phase of hemolysis [37]. Although spots were detected both before and after sweet bee venom therapy, we do not know whether the cleaved form of haptoglobin was the result of exposure to bee venom or was previously present in the blood of the hypersensitive patients. Nevertheless, the spots were not proteins induced by sweet bee venom.

CONCLUSIONS

This is the first report of an abnormal cleaved form of haptoglobin as a specific protein in bee venom-hypersensitive patients, which might predict the high incidence of hypersensitivity in populations with different genotypic backgrounds. It showed an association with the occurrence of anaphylaxis during bee venom therapy. Further studies on the cleaved form of haptoglobin in populations with different genotypic backgrounds are needed to help clarify its diagnostic utility.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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