Anti-allergic activity of Glycopeptide isolated from *Perilla frutescens* Britton

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Abstract

The anti-allergic activity of Perilla glycopeptide (Pe-GP) isolated from dried *Perilla frutescens* Britton leaves was investigated. Pe-GP was found to be effective in inhibiting histamine release from IgE-sensitized rat peritoneal mast cells induced by a specific antigen. The histamine release induced by several substances such as concanavalin A, compound 48/80, mastoparan, and ionophore A23187, was also found to be inhibited by Pe-GP. These results suggest that Pe-GP mainly interrupts a pathway of histamine release after calcium influx. Anti-allergic activity of Pe-GP was investigated by ear-swelling response in mice that had been passively sensitized with IgE via intravenous injection. Pe-GP, injected intraperitoneally before exposure to the antigen, was found to inhibit the allergic response in a dose-dependent manner (5-100 mg/kg). Pe-GP shows promise as an anti-allergic substance for medical use as well as in health foods.

Key words Glycopeptide from *Perilla frutescens* Britton, Anti-allergic activity, Histamine release inhibition from mast cells.

Abbreviations BSA, bovine serum albumin; DEAE, Diethylaminoethyl; DNP, 2,4-dinitrophenyl; EGTA, o,o'-bis (2-aminomethyl) ethyleneglycol-N,N,N',N',-tetra acetic acid; FBS, fetal bovine serum; GTP, guanosine 5'-triphosphate; HPLC, high-performance liquid chromatography; IgE, immunoglobulin E; Pe-GP, Perilla Glycopeptide; Perilla, *Perilla frutescens* Britton; PS, phosphatidylserine; TNP, 2,4,6-trinitrophenyl.

Introduction

Perilla frutescens Britton (Perilla), which is widely used in the Japanese diet, is also used as an ingredient in Chinese medicine, because of its diuretic, sedative, detoxic, and antibacterial actions. In order to investigate these effects of Perilla, ether extract and ethanol extract from its leaves have been studied. Perilla aldehyde was found as a component of the major volatile extracts, and found to exhibit sedative and antibacterial actions.[1,2] In addition, Perilla has been reported to exhibit anti-allergic and anti-neoplastic actions, and its active ingredients have also attracted attention. Two major lipophilic components of Perilla leaves, rosmarinic acid and α-linolenic acid, have been reported to be anti-inflammatory and anti-allergic substances.[3-5] A diet rich in α-linolenic acid is known to reduce the production of leukotriene B_4 and slow-reacting substance of anaphylaxis, which participate in allergic reaction.[6] Recently, the water extract from Perilla leaves has been reported to inhibit the production of the tumor necrosis factor in mice[7] and the

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production of nitric oxide in murine mesangial cells.[8] However, the major compositions of the water extract from Perilla leaves have not yet been determined.

In a previous study, we isolated Perilla Glycopeptide (Pe-GP) (molecular weight: 6.0 kDa) from the hot water extract of Perilla leaves, using hyaluronidase inhibitory activity as an index. We found that Pe-GP inhibits hyaluronidase activity in a concentration-dependent manner (IC_{50}=0.42 mg/ml), inhibits histamine release from mast cells, and exhibits protein kinase C inhibitory activity, and even anti-human immunodeficiency virus activity.[9]

The objectives of this study are to elucidate the inhibitory effect of Pe-GP on the mechanism of histamine release from mast cells and evaluate the anti-allergic activity of Pe-GP *in vivo*.

Materials and Methods

Preparation of Pe-GP : Dried Perilla leaves (200 g) were pulverized and then suspended in distilled water (2 l), followed by boiling for 15 min to obtain the hot water extract. Subsequently, the extract was sequentially subjected to cetylpyridinium chloride precipitation, DEAE ion-exchange chromatography, silica gel chromatography, and hyaluronidase-immobilized affinity chromatography according to the method described by Asada *et al.* to purify Pe-GP as a single band on sodium dodecylsulfate-polyacrylamide gel electrophoresis.[9]

Inhibitory assays of histamine release from mast cells

1. Histamine release by the antigen-antibody reaction : Wistar rats (7 to 12 -weeks old, CLEA Japan, Inc., Shizuoka, Japan) were bred at Laboratory Animal Center, Toyama Medical and Pharmaceutical University. The antigen and antibody used were 2,4-dinitrophenyl (DNP)-BSA (11.3 DNPs per BSA molecule) and anti-DNP mouse monoclonal IgE antibody (Sera-lab, England), respectively.

The test was conducted according to the following method, as described by Nakagomi *et al.*[10] Mast cells collected from the abdomen of the rats were suspended in a Tyrode solution, superposed to BSA-saline (d=1.068) and subjected to centrifugation. Subsequently, the mast cells were collected from the bottom layer, and incubated with anti-DNP IgE for 1 hour at 37℃. After the cells were washed, 2 ml of 0.1 % BSA-Tyrode was added to suspend the cells, to thereby prepare an IgE-sensitized mast cell suspension having approximately 10^6 cells/ml. The purity of mast cells was about 90 % as observed with an Olympus phase-contrast microscope. A 20 μl of mast cells were added to 20 μl of the sample, and the mixture was incubated for 10 minutes at 37℃, followed by further incubation with 10 μl of DNP-BSA (200 ng/ml) and phosphatidylserine (PS, 10 μg/ml, Sigma, St.Louis, MO, USA) for 10 minutes at 37℃. After the cells were centrifuged at 3000 rpm for 5 minutes, the histamine in the supernatant was analyzed by post-column derivatization HPLC using o-phthalaldehyde (Wako Pure Chemical Industries, Osaka, Japan). The amounts of histamine released were expressed as the value relative to that from 0.05 % Triton X-100-treated cell supernatant, which corresponds to 16.6 $\pm$ 0.8 nmol of released histamine per 2 x 10^4 cells in a tube (mean $\pm$ S.E., n=6). Inhibition of histamine release was calculated according to the following formula:

Inh (%) = $100 \times \{1-(T-Bl)/(C-Bl)\}$

T : histamine content in the cell supernatant with a test sample

C : histamine content in the cell supernatant without a test sample

Bl : histamine content in the cell supernatant without an inducer or a test sample

The histamine content that was spontaneously released from the mast cells was measured to be 2-3 % (Bl in formula).

2. Histamine release by various mast cell degranulation inducers : Mast cell degranulation inducers were prepared so as to react in the concentrations shown below, which were found to induce 55-65 % histamine release from mast cells:

Calcium ionophore A23187 (Calbiochem, La Jolla, CA, USA): 1 μM, Compound 48/80 (Sigma): 1 μg/ml, Concanavalin A (Wako): 10μg/ml + PS (Sigma): 10 μg/ml, Mastoparan (Peptide Institute, Osaka, Japan): 10 μM. The assay was carried out in the same manner as described above except for IgE sensitization.

3. Histamine release in the absence of extracellular calcium : o,o'-Bis (2-aminomethyl) ethyleneglycol-N, N, N', N',-tetra acetic acid (EGTA) was purchased from Dojindo Laboratories, Kumamoto, Japan. 1 mM EGTA and 0.1 % glucose in PBS was intraperitoneally injected

in the rats, and mast cells were collected. The cells were washed twice with PBS containing 1 mM EGTA and 0.1 % glucose, followed by another washing with PBS containing 0.1 % glucose but not containing EGTA. Assay was performed in a conventional manner, with compound 48/80 (1μg/ml) serving as the inducer.

Preparation of type I allergy model mice and quantification of ear swelling response[11,12] : BALB/C mice (6-weeks old, CLEA Japan) were bred at Laboratory Animal Center, Toyama Medical and Pharmaceutical University. Monoclonal antibodies were prepared in the following manner. Anti-2,4,6-trinitrophenyl (TNP) IgE producing hybridoma IGELa2 (provided from American Type Culture Collection) was cultured in RPMI-1640 medium (GIBCO BRL, Rockville, MD, USA) containing 10 % FBS (GIBCO). 2 x 10^7 cells of IGELa2 were administered to the murine peritoneal cavity and IgE was prepared from the murine ascites according to the method described by Nagai *et al.*[13] The mice were sensitized with the anti-TNP IgE (0.1ml/10g bodyweight) monoclonal antibody solution, via intravenous injection in the tail. Then, 24 hours after injection, 10μl of 4 % picryl chloride (Nacalai Tesque, Osaka, Japan) acetone solution was administered to the left ear of each mouse. Following exposure to the antigen, the thickness of the ear was measured chronologically by use of a dial thickness gauge (Ozaki Seisakusho Co., Tokyo, Japan). The amount of ear swelling was calculated, with the thickness of the ear before exposure to the antigen serving as an index. Three hours prior to exposure to the antigen, Tranilast (Kissei Pharmaceutical Co., Ltd.) and Pe-GP were administered either intraperitoneally or orally. The mice were separated into groups of 3 to 6.

Results

Inhibitory activity of Pe-GP on the mechanism of histamine release from mast cells

In a previous study, we have published a preliminary report on the inhibitory activity of Pe-GP on degranulation of mast cells.[9] In the present study, we confirmed the anti-allergic activity of Pe-GP *in vitro* and investigated which pathway of histamine release from mast cells is interrupted by Pe-GP. First, the inhibitory response of Pe-GP to histamine release from IgE-sensitized mast cells caused by the antigen-antibody reaction was confirmed (Fig. 1A). Pe-GP was found to inhibit histamine release from mast cells in a concentration-dependent manner, and when administered in amounts of 5 to 50 μg/ml, Pe-GP was found to inhibit 70 % of histamine release. We confirmed by the Trypan blue exclusion test that 1 mg /ml of Pe-GP is not toxic to mast cells.

We also observed inhibition of histamine release from mast cells by Pe-GP induced by several inducers that act on various mechanisms, to thereby investigate the mechanism whereby Pe-GP inhibits histamine release from mast cells. Pe-GP was found to inhibit histamine release induced by all the inducers tested, although the effective concentrations and the inhibitory effects show

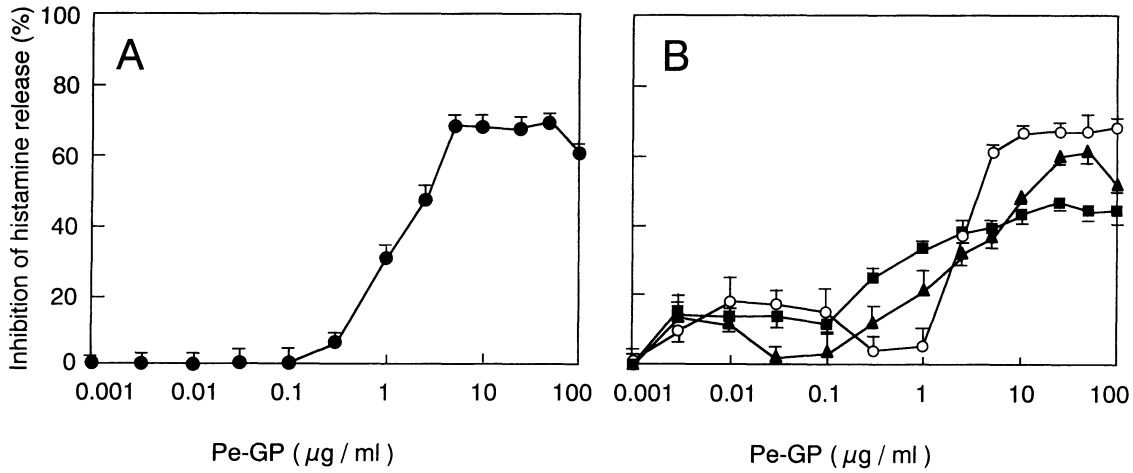

Fig. 1 Inhibition of histamine release from rat peritoneal mast cells by Pe-GP induced by 200 ng/ml of antigen (● in A), 10 μg/ml of concanavalin A (▲ in B), 10 μM mastoparan (■ in B) or 1 μM ionophore A23187 (○ in B).
Vertical bars indicate S.D. (n=6).

slight variance (Fig. 1B). Pe-GP having a concentration of 50 μg/ml was found to inhibit 62 % of histamine release from mast cells induced by concanavalin A, 44 % of histamine release induced by mastoparan, and 67 % of histamine release induced by calcium ionophore A23187.

Among the inducers tested, only compound 48/80 is known to induce degranulation of mast cells in the absence of extracellular calcium ions. In our assay, 1 μg/ml of compound 48/80 was found to promote degranulation of mast cells and release about 60 % of total histamine, irrespective of the presence or absence of extracellular calcium ions. We investigated degranulation inhibition by Pe-GP when extracellular calcium was trapped with EGTA. As shown in Fig.2, the presence or absence of extracellular calcium (1mM) does not seem to affect the inhibitory activity of Pe-GP on the histamine release from mast cells.

Inhibitory effects of Pe-GP on ear swelling response in type I allergy model mice

Type I allergy model mice were prepared by passively sensitizing mice via intravenous injection of anti-TNP IgE. The amount of ear swelling reached its maximum 1 hour after exposure, subsequently declined, and remained fairly constant between 8 hours and 24 hours after exposure. In addition, oral administration of tranilast, an anti-allergic agent, in an amount of 50 mg/kg three hours prior to exposure to the antigen was found to reduce ear swelling 1 hour after exposure to the antigen 29±2.1 (mean ±S.E.) % in relation to the control group. These findings confirm that this response is an immediate type I allergic response proceeding via mast cells.

Pe-GP was injected intraperitoneally to type I allergy model mice 3 hours prior to exposure to the antigen, and the amount of ear swelling was measured 1 hour after exposure to the antigen. Ear swelling was reduced by 29±7.2 % in the group of mice to which 5 mg/kg of Pe-GP had been administered, by 53±12% in the group to which 50 mg/kg had been administered, and by 67±22% in the group to which 100 mg/kg had been administered, and each of the groups showed a significant difference of at least 99% relative to the control group ($p<0.01$, Fig. 3). No significant difference was observed in the inhibitory effects on the ear swelling response between the group to which 50 mg/kg of Pe-GP had been administered and the group to which 100 mg/kg of Pe-GP had been administered. Although oral administration of Pe-GP in an amount of 100 mg/kg reduced ear swelling by 9.5±8.0%, the obtained value was low with a wide variance.

Discussion

When IgE-sensitized mast cells are brought into contact with a specific antigen, the IgE on the IgE receptors are crosslinked by the antigen, and the IgE receptors on the mast cells aggregate. Subsequently, phospholipase

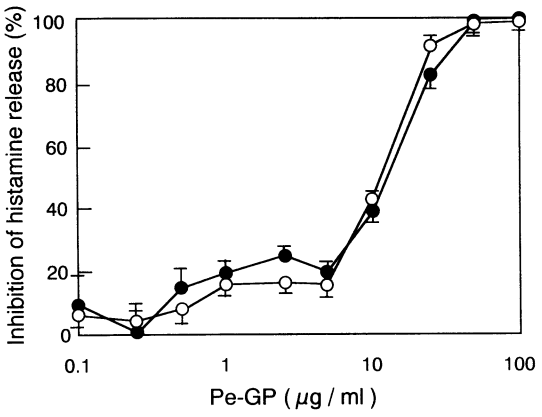

Fig. 2 Inhibition of histamine release from rat peritoneal mast cells by Pe-GP induced by 1 μg/ml of compound 48/80.

Pe-GP was incubated for 10 min with mast cells in the presence (●) or absence (○) of 1 mM Ca^{2+} followed by addition of 1 μg/ml of compound 48/80.
Vertical bars indicate S.D. (n=6).

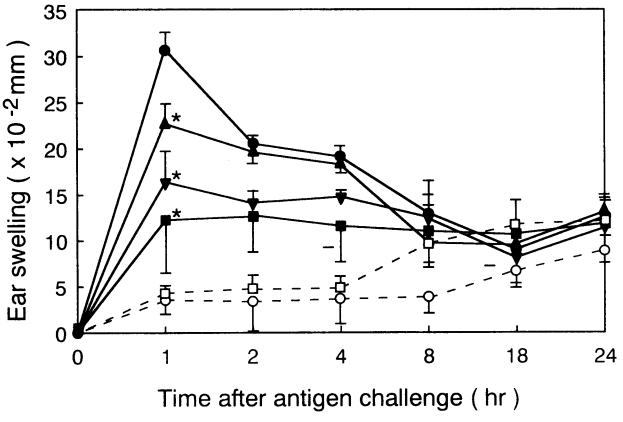

Fig. 3 Inhibitory effect of Pe-GP on ear swelling response in passively sensitized mice.

Pe-GP was injected intraperitoneally 3 hr prior to exposure to the antigen at a dose of 0 (●, ○), 5(▲), 50 (▼) or 100 (■, □) mg/kg to IgE-sensitized (closed figures) or unsensitized (open figures) mice.
* indicates $p<0.01$, and vertical bars indicate S.E. (n=3).

C is activated, and phosphatidylinositol is decomposed into inositol 1,4,5-triphosphate and diacylglycerol. Inositol 1,4,5-triphosphate releases calcium ions from intracellular calcium storage sites, and diacylglycerol activates protein kinase C. Simultaneously, extracellular calcium ion influx occurs, resulting in an increase in the concentration of intracellular calcium ions. Consequently, actin filaments contract, and intracellular granules including chemical mediators such as histamine are released to the outside of the cells.[15] Concanavalin A induces degranulation by aggregating IgE receptors on mast cells.[16] Compound 48/80[17] and mastoparan[18] induce degranulation by acting on GTP binding proteins and thereby activating phospholipase C. Calcium ionophore A23187 induces degranulation by taking extracellular calcium directly into the cells and thus bypassing the first half of the degranulation reaction.[19]

Pe-GP was found to inhibit, in a concentration-dependent manner, histamine release from mast cells induced by all the tested mast cell degranulation inducers such as concanavalin A, compound 48/80, mastoparan and ionophore A23187. The finding that Pe-GP inhibits histamine release induced by all the tested inducers suggests that Pe-GP acts on or after calcium influx. Compound 48/80 has been reported to utilize the intracellular calcium in the endoplasmic reticulum to release histamine in mast cells.[20] The finding that Pe-GP inhibits its histamine release induced by compound 48/80 in the absence of extracellular calcium suggests that Pe-GP inhibits histamine release induced by intracellular calcium. These findings suggest that Pe-GP mainly interrupts a pathway of histamine release after calcium influx. However, since we have not yet determined whether Pe-GP can penetrate cell membranes, it may be the case that Pe-GP does not act intracellularly but physically adheres to cell surfaces, thereby preventing the action of degranulation inducers, or physically blocking granule release.

Pe-GP exhibits an anti-allergic action against an immediate allergic reaction in type I allergy model mice to which anti-TNP IgE had been injected intravenously. It was strongly suggested that this allergic response in mice proceeds via mast cells, therefore Pe-GP was thought to exhibit an anti-allergic activity by inhibiting the mast cell functions both *in vivo* and *in vitro*. Since intraperitoneal injection of Pe-GP reduces ear swelling, Pe-GP is expected to exhibit anti-allergic effects when present in a sufficient amount in blood. A possible reason why oral administration of Pe-GP failed to reduce ear swelling is insufficient blood level, stemming from an underdose of the sample, digestion and decomposition of the sample, or difficulty in absorption from the gastrointestinal tract.

As the structure of Pe-GP becomes elucidated in more detail, the mechanism of anti-allergic activity of Pe-GP *in vivo* and *in vitro* will be studied further. Use of Pe-GP as an anti-allergic agent seems worthy of consideration, as does the use of *Perilla frutescens* BRITTON as a health food.

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和文抄録

シソ（*Perilla frutescens* BRITTON）葉熱水抽出液から分離精製されたシソ糖ペプチド（Pe-GP, 6 kDa）の抗アレルギー活性を検討した。Pe-GP は，ラット腹腔内より単離して IgE で感作したマスト細胞において，特異抗原で刺激したときに誘発されるヒスタミン遊離反応を抑制した。同様に Pe-GP は concanavalin A, compound 48/80, mastoparan, ionophore A23187 などの誘発するヒスタミン遊離反応も抑制した。これらの結果から Pe-GP はヒスタミン遊離機構のうちカルシウム動員以降の経路を主として抑えると考えられる。Pe-GP の生体内における抗アレルギー活性は，IgE を静注して受動感作したマウスの耳介浮腫反応により検討した。抗原塗布前にあらかじめ腹腔内に投与しておくと，Pe-GP は5-100 mg/ml の範囲で濃度依存的に浮腫を抑制し抗アレルギー活性を示した。Pe-GP の抗アレルギー活性を有する医薬品としての開発が，健康食品と同様，期待される。

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