Glycoprotein Derived from the Hot Water Extract of Mint Plant, *Perilla frutescens* Britton

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Glycoprotein showing inhibitory activity against mast cell degranulation and hyaluronidase activity was purified from the hot water extract of mint plant (*Perilla frutescens* Britton). The purified inhibitor gave a single band detected with Coomassie brilliant blue staining and periodic acid-Schiff staining on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis. The molecular mass was estimated to be 6.0 kDa on SDS−PAGE. The inhibitor did not become inactivated when boiled for 30 min or digested with trypsin, V8 protease, or proteinase K but was inactivated by $NaIO_4$ oxidation. The inhibitor prevented mast cell degranulation and hyaluronidase activity (IC_{50} = 0.42 mg/mL) in a dose-dependent manner. The inhibitor also inhibited the protein kinase C activity. It is possible to purify and characterize a glycoprotein with putative pharmacological properties from mint plants.

Keywords: *Mint plant; Perilla frutescens Britton; glycoprotein; inhibitor; hyaluronidase; mast cell degranulation; protein kinase C*

INTRODUCTION

Mint plant (*Perilla frutescens* Britton) is widely used as a spice and food. The extracts of *Perilla harba* have been used as a diuretic, a sedative, an antidote, and an antifebrile in Chinese traditional medicine. To elucidate these effects, many ether extracts and ethanol extracts of Perilla leaves were investigated, and perillaldehyde, a sedative and an anti-fungal agent (Honda et al., 1986; Kurita et al., 1979), and limonene, a selective inhibitor of isoprenylation of ras-like small G proteins (Crowel et al., 1991), were found as the main components of the volatile extracts.

Recently, much attention is being denoted to anti-allergic substances of perilla leaves. Yamazaki et al. (1992) reported that the water extract of perilla leaves inhibited the endogenous production of tumor necrosis factor (TNF) in mice. Inhibition of the overproduction of TNF suppressed both acute and chronic inflammation. Although rosmarinic acid (Gracza et al., 1985; Okuda et al., 1986) and α-linolenic acid (Tsuyuki et al., 1978) are known as anti-inflammatory and anti-allergic substances of perilla leaves, the main components in the water extract that show an inhibition of TNF overproduction remain to be defined. Therefore, we attempted to isolate water-soluble active substances from the hot water extract of perilla leaves.

In this paper, the isolation and characterization of a glycoprotein with putative pharmacological properties from the hot water extract of perilla leaves are described.

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MATERIALS AND METHODS

Materials and Plant Material. Hyaluronidase (from bovine testis), hyaluronic acid potassium salt, and proteinase K were purchased from Sigma Chemical Co. (St. Louis, MO). 2,4-Dinitrophenyl−bovine serum albumin (DNP−BSA) was kindly given by Dr. Sumiko Suzuki from the National Institute of Public Health, Japan. Anti-DNP mouse monoclonal IgE antibody was purchased from Sera Lab Co. (Belton, England). The protein kinase C Biotrak enzyme assay system RPN-77A was purchased from Amersham International plc. (Bucks, England). Partially purified protein kinase C (PKC) for the RPN-77A assay system was obtained from rat brain. Staurosporine was purified from the culture broth of *Streptomyces livani* as described by Oka et al. (1986). All other chemicals were of analytical grade.

Mint plant, *Perilla frutescens* Britton, was cultured in Tohya-cho, Hokkaido, Japan, by the Hokuren Federation of Agricultural Cooperatives. Its green leaves were dried up for 10 days in the shade.

Assay for the Inhibitory Activity of Degranulation from Rat Peritoneal Mast Cells. The inhibitory effect on mast cell degranulation was measured as the inhibitory activity of histamine release from mast cells. Mast cells were collected from the peritoneal cavity of Wistar rats and sensitized with anti-DNP mouse monoclonal IgE antibodies as described by Nakagomi et al. (1990) and Asada et al. (1997). An antigen (DNP−BSA, 200 ng/mL) and phosphatidylserine (10 μg/mL) were used as inducers of histamine release. After the degranulation reaction, the histamine content in the supernatant solution was analyzed by high-performance liquid chromatography (HPLC) by a postcolumn labeling fluorescent reaction with *o*-phthalaldehyde (Sigma) as described by Arakawa et al. (1986) with slight modifications. The percentage inhibition of histamine release from rat peritoneal mast cells was calculated as follows:

$$\text{inhibition (\%)} = 100 \times \{1 - (T_0 - B_l)/(C_0 - B_l)\} \quad (1)$$

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where T_0 is the histamine content in the solution with test sample, C_0 is the histamine content in the solution without test sample, and B_l is the histamine content in the solution without cells.

Determination of Inhibitory Activity on Activated Hyaluronidase. The inhibitory effect of Perilla extract on activated hyaluronidase was determined by the method described in the paper by Asada et al. (1997). Hyaluronidase (2.83 mg/mL) and hyaluronic acid (1.80 mg/mL) were dissolved in 0.1 M acetate buffer (pH 4.0). Sodium chloride was used as an activator of the hyaluronidase. The quantitative analysis of *N*-acetylamino sugar was determined by the modified Morgan-Elson method (Reissig et al., 1955). The percent of inhibition was calculated as follows:

$$\text{inhibition (\%)} = 100 \times \{1 - (S - B)/(C - B)\} \qquad (2)$$

where B is the absorbance without an enzyme, S is the absorbance with an inhibitor, and C is the absorbance without an inhibitor.

Preparation of PKC and Assay for Inhibitory Activity on PKC. PKC was partially purified from rat brain as follows. Buffer A was composed of 20 mM Tris-HCl buffer (pH 7.5) containing 2 mM EDTA, 0.5 mM EGTA, 50 mM 2-mercaptoethanol, 100 mg/mL leupeptin, and 2 mM phenylmethanesulfonyl fluoride (PMSF). Rat brain was homogenized with Potter-Elvehjem type Teflon homogenizer in buffer A containing 0.25 M sucrose and ultracentrifuged at 100000*g* for 30 min. The supernatant was applied on to the diethylaminoethyl (DEAE)−Sepharose (Pharmacia Biotech) column (1.0 cm × 1.3 cm) equilibrated with buffer A containing 10% glycerol. After being washed, the bound proteins were eluted with the same buffer containing 0.1 M NaCl. All procedures above were carried out at 4 °C. Assay of PKC activity was performed by using a PKC assay system (RPN-77A, Amersham) following the manufacturer's instruction. Ten microliters of diluted enzyme and 15 μL of inhibitor solutions were used for the assay.

Analysis of Neutral Sugar Components. The sample was hydrolyzed with 1 mL of 4 N hydrochloric acid at 100 °C for 4 h in a sealed glass tube. After removal of hydrochloric acid by evaporation, free amino groups were re-acetylated by acetic anhydride. Released monosaccharides were labeled by 2-aminopyridine (PA) and separated by high-performance liquid chromatography (HPLC) using a TSK gel AXI column according to a previous report (Suzuki et al., 1991). Eluted PA-labeled monosaccharides were identified by comparing the retention time with standard PA-monosaccharides, and the molar ratio was calculated based on the peak area, where that of galactose was expressed as 1.0.

Preparation of Hyaluronidase-Immobilized Toyopearl. AF-Tresyl Toyopearl (TOSOH, Japan) (2 g) was suspended in 20 mL of 0.1 M $NaHCO_3$ and 0.5 M NaCl solution (solution A). Hyaluronidase solution (200 mg/5 mL of solution A) was added to the suspension, and then the suspension was left standing at 4 °C for 24 h. After sequential washing with 0.5 M NaCl solution and with 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl, the suspension was left standing at 4 °C for 4 h. Hyaluronidase-immobilized Toyopearl was equilibrated with 0.1 M acetate buffer (pH 4.0) containing 0.1 M NaCl and then used in the purification steps of the inhibitor by column chromatography.

RESULTS

Purification of Glycoprotein from the Hot Water Extract of Perilla Leaves. The active substance showing inhibitory activity against mast cell degranulation and hyaluronidase activity was purified. In each step, hyaluronidase inhibitory activity was measured. A scheme of purification is shown in Figure 1. Dried green Perilla leaves (200 g) were powdered, suspended in 2 L of distilled water, and boiled for 15 min. The extract contained 72.4 g of water-soluble compounds.

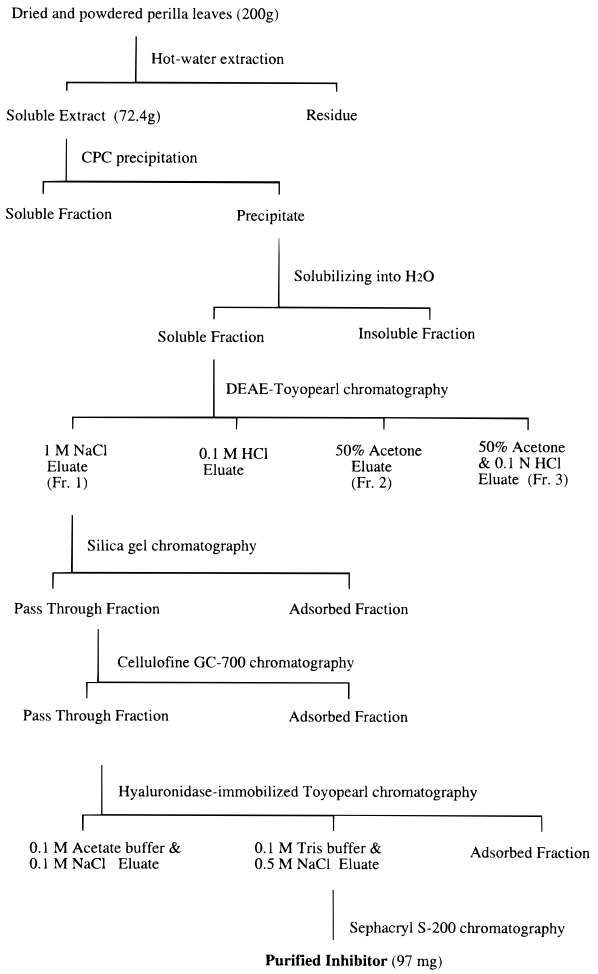

Figure 1. Scheme for extraction and isolation of a mast cell degranulation inhibitor from mint plant. Details are shown in Results.

The extract was filtered to remove leaves and precipitated with the addition of cetylpyridinium chloride (CPC), a precipitant for polysaccharides, and let stand at 37 °C for 16 h. After washing the precipitate with 15% ethanol and then 80% ethanol, the precipitate was dissolved in water and was loaded on to a DEAE-Toyopearl (TOSOH, Japan) column (4.4 cm × 26 cm) equilibrated with 50 mM borate buffer (pH 7.4). After washing the column with the same buffer, the fractions were eluted with 1 M NaCl in 50 mM borate buffer (pH 7.4), 0.1 N HCl, and 50% acetone or 50% acetone containing 0.1 N HCl as shown in Figure 2. The active components were separated into three fractions. Fraction 1 was eluted with 1 M NaCl solution, fraction 2 was eluted with 50% acetone, and fraction 3 was eluted with 50% acetone containing 0.1 N HCl, respectively. Further purification of fraction 1 was carried out. Fraction 1 was concentrated with Centriprep-3 concentrator (cutoff M_w is 3000) (Amicon) and then dialyzed against water. By the addition of MeOH, the concentrated solution was adjusted to a 50% MeOH solution and charged on a column (3.0 cm × 40 cm) of silica gel previously equilibrated with 50% MeOH. The active fractions eluted with 50% MeOH solution were pooled, evaporated, and applied on a column (2.8 cm × 93 cm) of Cellulofine GC-700 (Chisso, Japan) equilibrated with 0.01% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). The nonadsorbed eluate was further purified by using an affinity column (1.6 cm ×

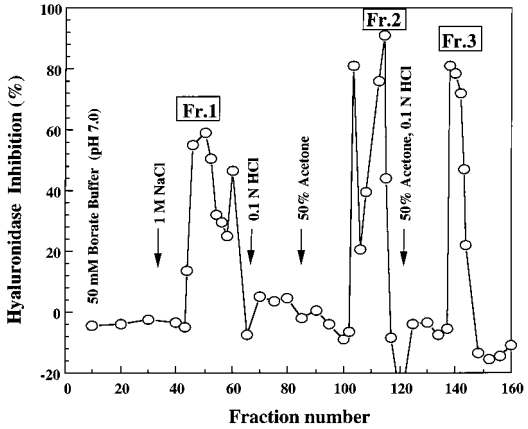

Figure 2. DEAE-Toyopearl column chromatography. Chromatography was performed by stepwise elution as described. Open circles: hyaluronidase inhibitory activity. The 10 mL fractions were collected.

15 cm) of hyaluronidase-immobilized Toyopearl equilibrated with 0.1 M acetate buffer (pH 4.0) containing 0.1 M NaCl. After washing the column with the same buffer, fractions were eluted with 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl. The active fractions were gel filtered on a Sephacryl S-200 (Pharmacia Biotech) column (2.8 cm × 90 cm) equilibrated with 50 mM phosphate buffer (pH 7.4). The active fractions were pooled and evaporated to produce 97 mg of a preparation of the inhibitor.

Characteristics of the Purified Inhibitor. The purified preparation was soluble in water. It was positive both for the phenol−H_2SO_4 reaction (Dubois et al., 1956) and the carbazole−H_2SO_4 reaction (Bitter et al., 1962). The total carbohydrate content was estimated to be 80.4% by the phenol−H_2SO_4 reaction using glucose as a standard. The content of total uronic acid was estimated to be 48.1% by the carbazole−H_2SO_4 reaction using sodium glucuronate as a standard. When the inhibitor was analyzed on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis using a 4−20% polyacrylamide gradient gel, it gave a single band at equally migrated positions that were detected with Coomassie brilliant blue (CBB) staining and periodic acid-Schiff (PAS) staining (Figure 3).

These findings indicated that the purified preparation was a glycoprotein. The molecular mass was estimated to be 6.0 kDa on SDS−PAGE. The inhibitor did not become inactive when boiled for 30 min, digested with trypsin at 37 °C for 30 min, digested with V8 protease (Sigma) at 37 °C for 20 h, or digested with proteinase K [EC 3.4.21.64] (Sigma) at 37 °C for 3 h, but it was inactivated by $NaIO_4$ oxidation at 4 °C for 7 days. Pectinase digestion using polygalacturonase from *Aspergillus japonicus* [EC 3.2.1.15] (Sigma) at pH 4.5 at 30 °C for 24 h according to a previous report (Rexova-Benkova and Mrackova, 1978) also did not decrease the inhibitory activities against hyaluronidase. Composition of neutral sugars is shown in Table 1(the molar ratio of galactose is calculated as 1.00). Though uronic acid could not be detected in this condition, glucose content was high in the carbohydrate moiety.

Inhibitory Effects against the Mast Cell Degranulation, Hyaluronidase, and PKC. The inhibitory effect of the purified glycoprotein on mast cell degranulation is shown in Figure 4A. The mast cell degranulation was 26% inhibited at the concentration of 0.5 mg/mL.

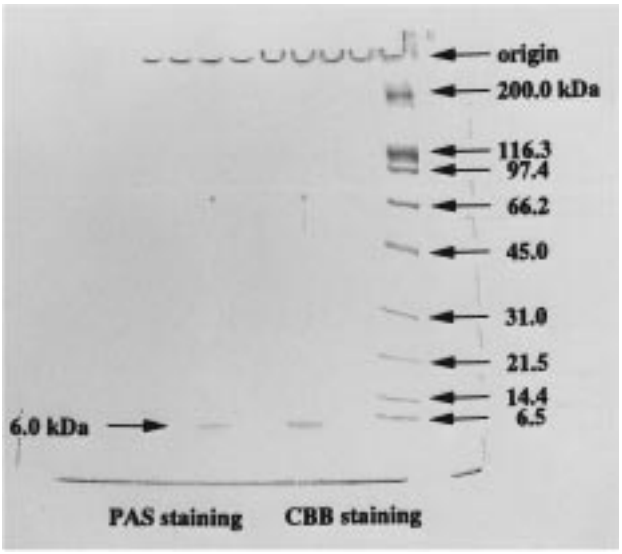

Figure 3. SDS−PAGE analysis of the purified inhibitor. Lane 1 (from left side), PAS staining; lane 2, CBB staining; lane 3, Bio-Rad low molecular weight prestained markers. Relative molecular weights ($\times 10^{-3}$) of protein standards are given at the right side of the gel.

Table 1. Molecular Ratio of Neutral Sugars in the Carbohydrate Moiety of the Purified Inhibitor

neutral sugars	molecular ratio	neutral sugars	molecular ratio
xylose	1.44	mannose	1.69
ribose	trace	fucose	1.77
galactose	1.00	rhamnose	1.66
glucose	9.24		

Because it has been reported that most mast cell degranulation inhibitors show hyaluronidase inhibitory activity (Kakegawa et al., 1985a,b), the inhibitory effect on hyaluronidase activity was measured. As shown in Figure 4B, the glycoprotein also inhibited hyaluronidase activity in a dose-dependent manner. The glycoprotein strongly prevented activated hyaluronidase (IC_{50} = 0.42 mg/mL) as compared to its inhibitory activity on mast cell degranulation.

Because the activation of PKC is involved in the mechanism of mast cell degranulation (Izushi et al., 1992; Ozawa et al., 1993; Hirasawa et al., 1995a), the inhibitory effect of the purified glycoprotein on PKC was measured using partially purified PKC. The glycoprotein also inhibited the PKC activity as shown in Figure 5. The magnitude of the inhibitory activity was weaker than that of staurosporine (Tamaoki et al., 1986), a powerful inhibitor of protein kinases. However, the concentration that inhibits the PKC activity is consistent with the effective concentration against mast cell degranulation.

DISCUSSION

We attempted to isolate the water-soluble active substances of Perilla leaves. As a result, we isolated a novel glycoprotein with inhibitory activities of mast cell degranulation and hyaluronidase.

The molecular mass of the purified preparation was estimated to be 6.0 kDa on SDS−PAGE analysis. Because the preparation was positive for CBB staining and PAS staining on SDS−PAGE, it was deduced to be glycoprotein. Proteolytic digestion with trypsin, V8 protease, or proteinase K did not decrease the inhibitory

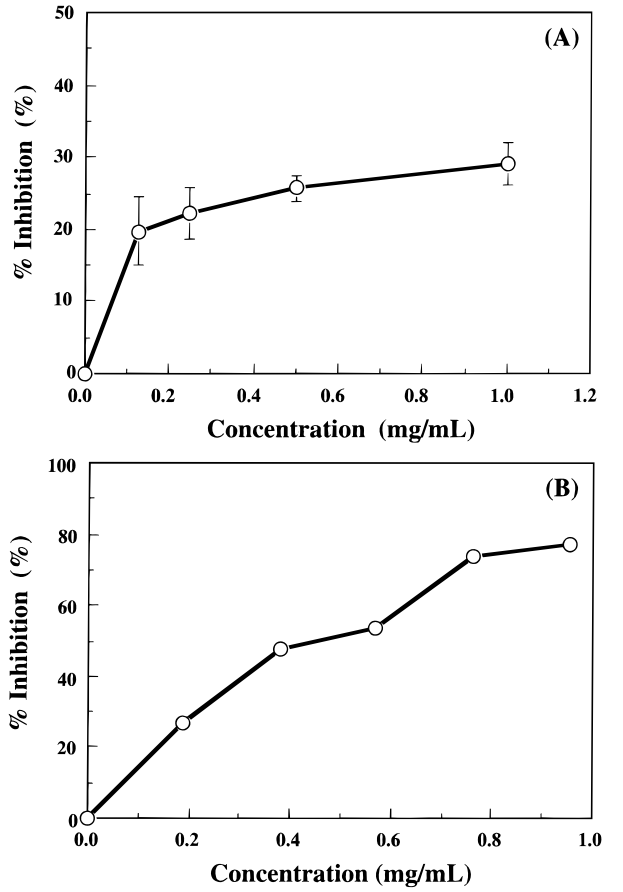

Figure 4. (A) Inhibitory effect of the purified inhibitor on mast cell degranulation. (B) Inhibitory effect of the purified inhibitor on activated hyaluronidase.

Figure 5. Inhibitory effect of the purified inhibitor on protein kinase C (PKC) activity.

activities against mast cell degranulation and hyaluronidase. On the other hand, the inhibitory activities were lost by $NaIO_4$ oxidation. Because compositions of gulutaminic acid including glutamine and aspartic acid containing asparagine also decreased with degradation of carbohydrate after the oxidation, further experiments are required for the elucidation of role of carbohydrate moiety. Carbohydrate compositions were analyzed. The carbohydrate moiety consisted mainly of glucose with uronic acids. Sawabe et al. (1992) reported pectic substances, and polygalacturonic acid inhibited both activated hyaluronidase and histamine release from mast cells. However, because pectinase digestion of our inhibitor had no effect on the mast cell degranulation activity, the purified preparation was not thought to be a pectic substance despite the presence of uronic acids. In the course of the screening of the mast cell degranulation inhibitor, we also found that alginic acids, polymerized compounds of mannuronic acid and guluronic acid, inhibited activated hyaluronidase and mast cell degranulation activity (Asada et al., 1997). Therefore, the role of uronic acids was deduced to be important for the inhibitory activities of the inhibitor.

It has been suggested that histamine release from mast cells is primarily regulated by Ca^{2+} and PKC. Therefore, the inhibitory effect of the purified glycoprotein on PKC was measured. As a result, the glycoprotein also inhibited the PKC activity although the potency of the inhibitory activity was not as strong as staurosporine, a powerful inhibitor of protein kinases. Because the concentration and the magnitude that inhibit mast cell degranulation are almost the same as those for PKC inhibitory activity, the inhibition of PKC activity may be important for the action point of the inhibitor.

On the other hand, a considerable amount of hyaluronidase is present in rat peritoneal mast cells. Kakegawa et al. (1985c) has suggested that hyaluronidase is one of the target enzyme directly controlling the degranulation of mast cells. The purified glycoprotein strongly inhibited the hyaluronidase activity in a dose-dependent manner. Therefore, we have deduced that the inhibition of hyaluronidase activity is also important for the action point of the glycoprotein against the degranulation of mast cells.

Recently, the effect of Perilla was drawing attention as a functional food. We attempted to isolate the water-soluble active substances of Perilla leaves. As a result, we isolated a novel glycoprotein with putative pharmacological properties. However, the activities of the glycoprotein against mast cell degranulation, hyaluronidase, and PKC were not so strong. Further experiments in vivo are underway whether the glycoprotein shows anti-allergic activity. It is possible to purify and characterize a glycoprotein with putative pharmacological properties from mint plants.

ABBREVIATIONS USED

SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TNF, tumor necrosis factor; DNP, 2,4-dinitrophenol; DNP−BSA, 2,4-dinitrophenyl−bovine serum albumin; PKC, protein kinase C; DEAE, diethylaminoethyl; CPC, cetylpyridinium chloride; CBB, Coomassie brilliant blue; PAS, periodic acid-Schiff; MAP kinase, mitogen-activated protein kinase.

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