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Hyaluronidase Inhibitor in the Fruit of *Citrus reticulata* BLANCO

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Fifty percent of ethanolic extracts from the immature fruits of *Citrus reticulata* BLANCO, *C. tamurana* hort. ex TANAKA, *C. natsudaidai* HAYATA and *C. unshiu* MARKOVICH showed a significant inhibitory effect on the activation of inactive hyaluronidase induced by compound 48/80. This effect decreased with the maturation of the fruit in all cases. Among the citrus fruits tested, the extract from the immature *C. reticulata* BLANCO showed the strongest inhibitory effect. The data of gel permeation chromatography and gas chromatography-mass spectrometry showed the hyaluronidase inhibitor in the fruit to be a pectin with a molecular weight of 1.1×10^5 . The content of D-galacturonic acid in the pectin was determined to be 58% using the carbazole method. The pectin showed almost the same inhibitory effect (IC_{50} : 91 μ g/ml) as a commercial pectin from oranges or a current anti-allergic agent, disodium cromoglycate.

Hyaluronidase has been suggested to be one of the target enzymes controlling the degranulation of mast cells, and the inhibitory effect on the activation of inactive hyaluronidase has been reported by Kakegawa *et al.* (*Chem. Pharm. Bull.* 33 [1985]) to be an index of anti-allergic activity. Therefore, it is expected that the pectin in *C. reticulata* BLANCO might possess anti-allergic potential.

Keywords — Citrus fruit; *Citrus reticulata* BLANCO; hyaluronidase inhibitor; pectin; polysaccharide; D-galacturonic acid; anti-allergic effect

Introduction

Citrus fruits have long been used as aromatic stomachics, carminatives, expectorants, antitussives, *etc.* Recently, the inhibitory effects of these fruits on 48 h homologous passive cutaneous anaphylaxis and on histamine release from rat peritoneal mast cells have been reported.¹⁾ Kubo *et al.*^{1c)} also demonstrated that the immature fruit of *Citrus unshiu* MARKOVICH has strong anti-allergic effect against types I, II and IV allergic reactions. From these findings, it has been

gradually recognized that citrus fruits might have the potential to inhibit various allergies. To demonstrate this potential more clearly, it is necessary to identify the most effective among the citrus fruits and to isolate the effective component.

Kakegawa *et al.*^{2a)} demonstrated that compound 48/80, a non-specific histamine releaser from mast cells, strongly activated hyaluronidase, and, further, that hyaluronidase activated by compound 48/80 was inhibited by anti-allergic agents such as disodium cromoglycate (DSCG) or tranilast.

We have therefore been attempting the last few years to identify the anti-allergic principles from natural products using a hyaluronidase activation test described by Kakegawa *et al.*^{2a)} We previously reported that tea extracts inhibited the activation of inactive hyaluronidase induced by compound 48/80 (IC_{50} : 0.5–1.2 mg/ml), and that phenolic compounds such as tannins in the extracts were responsible for the effect.³⁾ We also isolated two pectins (IC_{50} : 45 μ g/ml) as the hyaluronidase inhibitors from *Gymnema sylvestre* R. Br.⁴⁾

In the present study, we investigated the inhibitory effects of the fruits of *C. reticulata* BLANCO, *C. tamurana* hort. ex TANAKA, *C. natsudaoidai* HAYATA, and *C. unshiu* MARKOVICH on the activation of inactive hyaluronidase induced by compound 48/80. We then tried to isolate the hyaluronidase inhibitors in the immature fruit of *C. reticulata* BLANCO, which has shown the strongest inhibitory effect on the activation step in this study.

Experimental

Materials — Fruits of *C. reticulata* BLANCO, *C. tamurana* hort. ex TANAKA, *C. natsudaoidai* HAYATA and *C. unshiu* MARKOVICH cultivated in Shizuoka Prefectural Citrus Experimental Station were harvested from June to December in 1988–1990. They were cut into round slices, dried and extracted twice with 50% ethanol. The extract was lyophilized.

Reagents for Measuring Inhibitory Effects of Hyaluronidase — Hyaluronidase (from bovine testis), hyaluronic acid potassium salt (from rooster comb) and compound 48/80 were purchased from Sigma Chemical Co., Wako Pure Chemical Co. and Sigma Chemical Co., respectively. DSCG was supplied by Fujisawa Pharmaceutical Co., Ltd.

Other Reagents — Pectin (from oranges, Sigma Chemical Co.) D-galacturonic acid (Wako Pure Chemical Co.), hesperidin (Kanto Chemical Co.), naringin (Bio Chemika Co.), pullulans (Shodex standard P-82, Showa Denko Co.) and a

trimethylsilyl ethers kit OT-3 (Shinwa Chemical Industries, Ltd.)) were used.

Assay of Inhibitory Effects of Hyaluronidase — Hyaluronidase activity was determined by the method of Davidson and Aronson⁵⁾ after incubation of 400 NF unit/ml of hyaluronidase with 0.4 mg/ml hyaluronic acid potassium salt at 37°C for 40 min in 0.1M acetate buffer of pH 4.0.^{2b)} Inhibitory effects on the activation of inactive hyaluronidase induced by compound 48/80 (0.1 mg/ml) were determined using the method described.^{2b)} Fifty percent inhibitory concentration (IC_{50}) was calculated using the mean of 3 observations at each point of 7 concentrations.

Isolation of Hyaluronidase Inhibitor from *C. reticulata* BLANCO — The hyaluronidase inhibitor in the fruit of *C. reticulata* BLANCO was purified by the following method. One hundred grams of the dried fruit was extracted with 1 l of methanol at 70°C for 3 h. After cooling, the mixture was filtered to give a methanol-extract (MeOH ext). The residue was further extracted with 1 l of water at 90°C for 3 h. After cooling, the mixture was filtered to give a water-extract (H_2O ext). Five liters of ethanol was added to the H_2O ext to form precipitates (EtOH ppt) and the EtOH ppt was collected by filtration. The EtOH ppt was dissolved in water, and separated by gel permeation chromatography (GPC) to give the hyaluronidase inhibitor. GPC was performed on a TSK-GEL G4000 SW column (21.5 mm i.d. $\times$ 300 mm) (Tosoh) in an oven at 25°C, with water as a mobile phase and at a flow rate of 3.0 ml/min. The eluted materials were monitored with a differential refractometer (RI-8000, Tosoh).

Determination of Molecular Weight — The molecular weight of the hyaluronidase inhibitor in the extract from *C. reticulata* BLANCO was determined by GPC using pullulans (Shodex standard P-82) as a standard for calibration curve. GPC was conducted on a TSK-GEL G4000 SW column (7.0 mm i.d. $\times$ 300 mm) (Tosoh) in an oven at 25°C, with water as a mobile phase at a flow rate of 0.5 ml/min. The eluted materials were monitored with a differential refractometer.

Hydrolysis of the Hyaluronidase Inhibitor

— The purified hyaluronidase inhibitor (1 mg) was dissolved in 10% HCl (5 ml), and the solution was heated at 150°C in an oil bath for 20 min. The reaction mixture was trimethylsilylated (TMS) by the OT-3 kit, and the derivative was subjected to gas chromatography-mass spectrometry (GC-MS). GC-MS was performed under the following conditions. Column, 2% OV-17 on Uniport HP (80/100 mesh) 3 mm i.d.×2 m glass column; carrier gas, He 40 ml/min; temp., col. 160°C, inj. 200°C, ion source 190°C; ionization voltage, 70 eV; instrument, M-80A (Hitachi).

Results and Discussion

1. Inhibitory Effects of Citrus Fruits on the Activation of Inactive Hyaluronidase

The inhibitory effects of 50% ethanolic extracts from immature and mature citrus fruits on the activation of inactive hyaluronidase are shown in Table I. All of the fruits inhibited the activation step, with the effects

of the immature fruits being stronger than those of the mature fruits. Especially, in *C. reticulata* BLANCO, IC₅₀ of the immature fruit decreased to about one thirty-third that of the mature fruit, and was the lowest of the values among the fruits tested.

Immature fruit of *C. unshiu* MARKOVICH has been reported to exhibit a strong anti-allergic effect against types I, II and IV allergic reactions.¹⁰⁾ In the present study, it was found that the inhibitory effect of immature fruit of *C. reticulata* BLANCO was about six times stronger than that of immature fruit of *C. unshiu* MARKOVICH, suggesting that the former fruit may have a strong anti-allergic effect.

Table II shows the changes in inhibitory effects of 50% ethanolic extracts from the fruit of *C. reticulata* BLANCO during maturation. These effects weakened with maturation of the fruit, and the immature fruit, harvested in July, showed the strongest inhibitory effect

TABLE I. Inhibitory Effects of 50% Ethanolic Extract from Citrus Fruits on Activation of Inactive Hyaluronidase by Compound 48/80

Material	IC ₅₀ (mg/ml) ^{a)}	
	Immature ^{b)}	Mature ^{c)}
<i>Citrus reticulata</i> BLANCO	0.5	17.4
<i>Citrus tamurana</i> hort.ex TANAKA	3.0	64.0
<i>Citrus natsudaidai</i> HAYATA	6.2	40.0
<i>Citrus unshiu</i> MARKOVICH	3.0	N.T. ^{d)}

a) Fifty % inhibitory concentration.

b) Harvested in July.

c) Harvested in December.

d) Not tested.

TABLE II. Changes in Inhibitory Effect of 50% Ethanolic Extract from *Citrus reticulata* BLANCO on Activation of Inactive Hyaluronidase during Maturation

Material	Date of harvest	Weight (g)	Diameter (cm)	Yield ^{a)} (%)	IC ₅₀ ^{b)} (mg/ml)
<i>Citrus reticulata</i> BLANCO	1	1989. 7.24	7.2— 9.2	2.4—2.5	7.2±0.1
	2	9.21	41.4— 53.8	4.4—4.7	5.5±0.2
	3	10.26	80.0— 96.7	5.7—6.0	6.1±0.2
	4	12. 8	115.2— 144.4	6.2—7.0	8.0±0.2

a) Yield of 50% ethanolic extract from citrus fruits. Mean±S.D.

b) Fifty % inhibitory concentration.

($IC_{50}=0.5$ mg/ml).

2. Isolation of Hyaluronidase Inhibitor from *C. reticulata* BLANCO

In our preliminary experiment, the well-known constituents in citrus fruits, such as hesperidin, naringin and synephrine, little inhibited the activation of inactive hyaluronidase at a concentration of 2.0 mg/ml. Hyaluronidase inhibitor was therefore isolated from the immature fruit of *C. reticulata* BLANCO harvested in July (Chart 1). Since the H_2O ext showed stronger effect than the MeOH ext, the H_2O ext was further fractionated by the addition of ethanol. The inhibitory effect of the H_2O ext was recovered mainly in the EtOH ppt. The active constituent purified from the EtOH ppt by GPC, which was detected as a single peak on the chromatogram (Fig. 1), showed strong inhibitory effect at $91 \mu\text{g/ml}$ of IC_{50} . On the basis of the yield and IC_{50} value, it appears that the active constituent makes a large contribution

Chart 1 Isolation Procedure of Hyaluronidase Inhibitor from *Citrus reticulata* BLANCO

Each yield (g) and each 50% inhibitory concentration (IC_{50} : $\mu\text{g/ml}$) of fractions from 100 g of dried *Citrus reticulata* BLANCO is indicated in parenthesis.

Each total activity (yield/ $IC_{50} \times 10^3$) is indicated in brackets.

to the production of the inhibitory effect of the fruit.

3. Properties of the Hyaluronidase Inhibitor of *C. reticulata* BLANCO

The molecular weight of the active constituent was estimated to be 1.1×10^5 using GPC (Fig. 2). The constituent showed a pur-

Fig. 1. Gel Permeation Chromatogram of the Hyaluronidase Inhibitor in the Extract from *Citrus reticulata* BLANCO

Fig. 2. Molecular Weight Determination of the Hyaluronidase Inhibitor in the Extract from *Citrus reticulata* BLANCO by Gel Permeation Chromatography on a TSK-Gel G4000 SW

Sample-A: hyaluronidase inhibitor in the extract from *Citrus reticulata* BLANCO; P-800 (MW: 8.53×10^5), P-400 (MW: 3.8×10^5), P-200 (MW: 1.86×10^5), P-100 (MW: 1.0×10^5), P-50 (MW: 4.8×10^4) and P-20 (MW: 2.37×10^4): pullulans (Shodex Standard P-82).

ple color by Molisch reaction⁶⁾ known as a qualitative reaction of the saccharide, and an absorption maximum near 480 nm by the phenol-H₂SO₄ method.⁷⁾ The acid hydrolysis product of the constituent reduced the Fehling solution, although the constituent by itself did not.

From the above results, the hyaluronidase inhibitor was suggested to be a substance containing saccharides with a molecular weight of 1.1×10^5 .

The acid hydrolyzed product of the active constituent was trimethylsilylated, and the TMS derivative was analyzed by GC-MS to identify constituting monosaccharides. Though several peaks were detected on GC-MS chromatograms, which were suggested to be TMS-saccharides by their mass spectra, only the retention time (9.7 min) of a peak agreed with that of a TMS derivative of D-galacturonic acid as a standard; the mass spectrum of the peak also agreed with that of the standard. Furthermore, the content of D-galacturonic acid in the active constituent was determined as 58% by the carbazole method.⁸⁾

Pectin is well-known to be a polysaccharide in cell walls of all plant tissues, and occurs naturally as the partial methyl ester of α -(1 $\rightarrow$ 4) linked D-galacturonate sequences. Its molecular weight has been reported to be from 1.0×10^5 to 1.5×10^6 .⁹⁾ The TMS derivatives of the acid hydrolyzed active constituent showed the same retention time and mass spectra as those of the TMS derivatives of the

acid hydrolyzed commercial orange pectin.

Based on the above results, the hyaluronidase inhibitor of the fruit of *C. reticulata* BLANCO was suggested to be a pectin containing 58% D-galacturonic acid with molecular weight of 1.1×10^5 . The hyaluronidase inhibitor from *C. reticulata* BLANCO showed almost the same inhibitory effect as that of the commercial orange pectin (IC₅₀: 47 μ g/ml) and a current anti-allergic drug, DSCG (IC₅₀: 110 μ g/ml) on the activation of inactive hyaluronidase.

We recently isolated two pectins with molecular weights of 4.0×10^4 and 2.0×10^4 , as the hyaluronidase inhibitor (IC₅₀: 45 μ g/ml) from *Gymnema sylvestre* R. Br.⁴⁾ The pectin with a molecular weight of 1.1×10^5 from *C. reticulata* BLANCO showed about half the inhibitory effect as that of the pectin from *Gymnema sylvestre* R. Br. We further found that the effect of *C. reticulata* BLANCO decreased with the maturation of the fruit. Thus, the inhibitory effect of pectins might be related to their structure. It is interesting to examine this effect of various kinds of pectins on the activation of inactive hyaluronidase and to learn the relation of the structures to the effect. Isolation of pectins from natural products is now in progress in our laboratory.

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