論文の内容の要旨

論文題目　PURIFICATION OF INNATE IMMUNITY STIMULANTS FROM GREEN TEA AND BROCCOLI BY USING SILKWORM MUSCLE CONTRACTION ASSAY

カイコの筋肉収縮を指標とした緑茶およびブロッコリーからの
自然免疫促進物質の精製

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Background:

Silkworm (*Bombyx mori*) muscle contraction assay is a newly established method in my laboratory for quantitative measurement of innate immunity stimulation (1). In this system, stimulants of innate immunity such as cell wall components (peptidoglycan or β-glucan) of pathogenic microorganisms triggers activation of hemocytes, immune cells in insect hemolymph. Activated hemocytes produce reactive oxygen species (ROS), resulting in the activation of a serine protease in hemolymph followed by conversion of the precursor of paralytic peptide to the active form. The active form of paralytic peptide induces not only stimulation of immune responses, but also muscle contraction. By using this system, I screened innate immunity stimulants from extracts of different plants. I found that hot water extracts of green tea (*Camelia sinensis)* and broccoli (*Brassica olereceae italica*) showed high activity. Here I describe purification of the active substances from those materials.

Experimental Procedures:

Assay of innate immunity stimulants

Muscle contraction values (C) were calculated by subtracting the final length (y) from the initial length (x) divided by initial length (figure 1). When an innate immunity stimulant is injected into hemolymph of silkworm muscle specimen, the immune cells are activated resulting in muscle contraction (1).

Sugar composition analysis

Monosachharide analysis was carried out by subjecting the polysaccharide to acid hydrolysis with 4M trifluoroacetic acid to convert polysaccharides to monosaccharides. The resulting monosaccharides were labeled by aminobenzoic acid ethyl ester-methanesulfonate and analyzed by Reverse Phase-HPLC (RP-HPLC). 1D NMR spectroscopic analysis was performed.

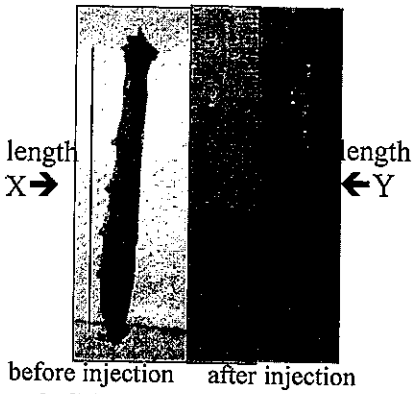

Figure 1: Silkworm muscle contraction by injection of an innate immunity stimulant.

Purification of an innate immunity stimulant from green tea

An innate immunity stimulant from green tea was purified with the following steps: hot water extraction, ethanol precipitation, 1st DEAE-cellulose chromatography at pH 7.9, 2nd DEAE-cellulose chromatography at pH 5.6, 1st MonoQ chromatography at pH 7.9, 2nd MonoQ chromatography at pH 5.6 and gel filtration with superdex 200.

Purification of an innate immunity stimulant from broccoli

A water-soluble and heat stable innate immunity stimulant was purified from broccoli. Purification was started with hot water extraction. Freeze-dried extract was treated with 67 % ethanol. Resulting precipitates were dissolved in a buffer and fractionated by gel filtration with Sepharose CL-4B and Superdex 200.

Interleukin-6 (IL-6) production by mouse macrophage

The peritoneal macrophages were harvested 3 days after injection of 4% thioglycollate medium from C57BL/6 male mice. The cells were incubated for 24 hours with different concentrations of purified fractions from green tea and broccoli. *E. coli* Lipopolysaccharide (LPS) was used as a positive control. The amount of IL-6 was determined by Enzyme-Linked Immunosorbent Assay (ELISA). Student's *t*-test was used as statistical tool.

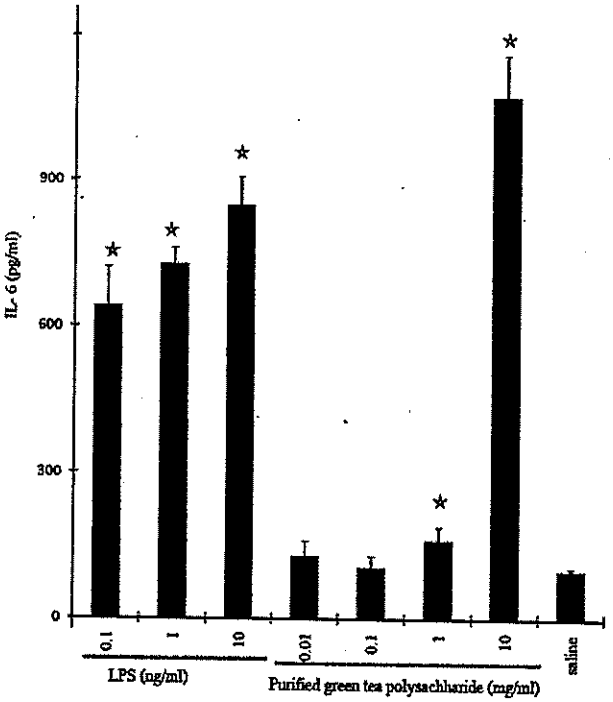

Figure 2: IL-6 production by mouse peritoneal macrophage cells stimulated with purified compounds from green tea

The error bars show the standard deviations (n=3).(★ p,<0.05 compared with saline)

Results:

An innate immunity stimulant purified from green tea

The purified active substance was eluted earlier than the elution of β-lactoglobulin (35 kDa) in gel filtration, suggesting that its molecular mass was greater than 35 kDa. Green tea polyphenols [catechin and polyphenon], which have been reported to modulate immunity (2), did not induce muscle contraction in the system of silkworms. Neither plant cellulose nor chitin did induce the contraction. The final fraction purified from green tea induced the production of IL-6 from mouse proinflammatory macrophage cells (figure 2). The amount of green tea polysaccharide needed for IL-6 production by mouse macrophage cells was much greater than that of LPS, that means the specific activity of immune stimulating polysaccharide purified from green tea is much less than that of LPS.

The activity of the purified fraction was lost by acid hydrolysis.

Sugar composition analysis of acid hydrolyzed samples by RP–HPLC and NMR analysis of purified fractions show that the purified substances were polygalactouronic acids. The NMR analysis data also showed that some of the carboxylic residues were methyl estered.

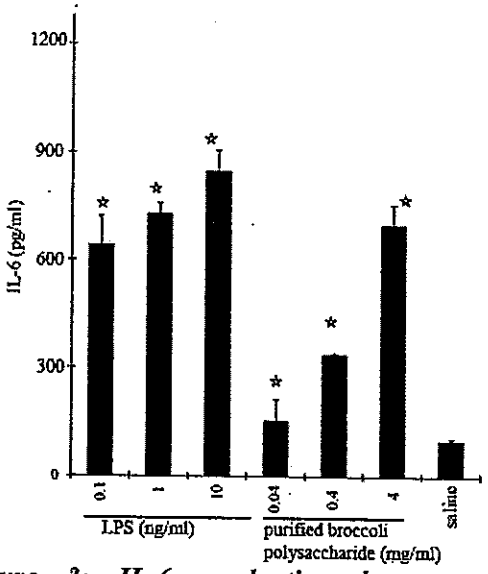

Figure 3: IL-6 production by mouse peritoneal macrophage cells stimulated with purified compound from broccoli

The error bars show the standard deviations (n=3).(★p,<0.05 compared with saline)

An innate immunity stimulant purified from broccoli

The active substance of broccoli was eluted earlier than the elution of Aprotinin (6511 Da) in gel filtration suggesting that the molecular mass of the active substance was greater than 6.5 kDa. The acid hydrolysis of final fraction lost the activity, suggesting that the purified materials were polysaccharides. The broccoli final fractions induced the production of significant amount of IL-6 from murine macrophage cells (figure 3). This result suggests that the broccoli innate immunity stimulant purified by silkworm muscle contraction assay induces innate immunity in mammals.

Discucussion:

I have established purification of novel innate immunity stimulants from green tea and broccoli. This is the first report for purification of immune stimulants from plants by using silkworm muscle contraction assay. These substances induced IL-6 production by mouse macrophage cells. Since specific activity of the fractions for IL-6 production is much less than

that of LPS, I propose that the mechanism of stimulation of innate immunity by those polysaccharides might be different from that of LPS.

References:

1) Ishii K, Hamamoto H, Kamimura M, Sekimizu K. Activation of the silkworm cytokine by bacterial and fungal cell wall components via a reactive oxygen species-triggered mechanism. *J. Biol. Chem.* 2008: **283**: 2185-2191.

2) Stephen D. Hsu, D. P. D., Haiyan Qin, James Borke, Kalu U. E. Ogbureke, Julia N. Winger, Amy M. Camba, Wendy B. Bollag, Hubert J. Stöppler, Mohamed M. Sharawy and George S. Schuster. Green tea polyphenols reduce autoimmune symptoms in a murine model for human Sjogren's syndrome and protect human salivary acinar cells from TNF-α-induced cytotoxicity. *Autoimmunity*. 2007: **40**:138-147.