

カンキツ葉蛋白性成分によるカンキツかいよう病菌 (*Xanthomonas campestris* pv. *citri*) の凝集

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Agglutination of *Xanthomonas campestris* pv. *citri*, a Causal Pathogen of Citrus Canker, by Proteinaceous Components from Citrus Leaves

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高橋敏房*・道家紀志*：カンキツ葉蛋白性成分によるカンキツ
かいよう病菌 (*Xanthomonas campestris* pv. *citri*) の凝集

Abstract

Proteinaceous components which agglutinated unwashed bacterial cells of *Xanthomonas campestris* pv. *citri* and its extracellular polysaccharides (EPS), but not washed cells and unwashed EPS-nonproducing mutant cells, were isolated from citrus leaves of *Citrus unshiu*, *C. natsudaoidai* and *C. reticulata*, forming fibrous materials. The agglutinin was prepared by extraction with HCl-acidic water (pH 4.0) from homogenate of the leaves followed by precipitation with 50%-saturated ammonium sulfate. This agglutinin was heat and protease labile, insensitive to various salts except higher concentration than 100 mM, and was inhibited at pH higher than 6.0. The Similar agglutination of unwashed cells and EPS of other xanthomonads (*X. campestris* pv. *campestris* and *X. campestris* pv. *phaseoli*), but not that of pseudomonads and erwiniae, was also demonstrated. Of 18 sugars tested, only D-glucosamine (25 mM) showed a potential activity to inhibit the agglutination of *X. campestris* pv. *citri*, but not that of other xanthomonads.

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Key Words : *Xanthomonas campestris* pv. *citri*, agglutinin, citrus leaf.

Introduction

Xanthomonas campestris pv. *citri* has been known to be a pathogenic bacterium which causes citrus canker showing hypertrophic tissues in citrus leaves as an initial symptom. Although some elucidations of the disease mechanism were made from physiological point of view^{5,6)}, little knowledges on specific recognition mechanism for establishment of host-parasite interaction has been obtained.

There are many recent reports on the involvement of agglutinating factors of host-plants in recognition mechanisms of bacterial diseases^{2,4,7,12,13,14)}. Sequeira and Graham¹³⁾ have found that a lectin isolated from potato tubers agglutinates only strains of pseudomonads that were avirulent in potato. Slusrenko and Wood¹⁵⁾ reported that a fraction obtained from cotyledons of *Phaseolus vulgaris* aggregated cells of avirulent isolate of *Pseudomonas phaseolicola* more actively than those of virulent isolates. These phenomena brought out a potent assumption that agglutinin may be associated with establishing incompatible interactions.

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In the interactions of species of *Rhizobia* with their hosts, it has been also demonstrated that agglutinins (lectins) involve in regulating the host-bacterium specificity^{1,2,3}. However, attachment via the lectin of *Agrobacterium* to a specific wounded site on the host cells has been found to be prerequisite for infection¹¹. In these cases, the agglutinin of host plant involves in the process of establishing the symbiotic relationship.

In citrus canker, the relationship between the causal bacterium and host tissues seems to be rather symbiotic than necrobiosis because of the disease symptom showing a hypertrophic tissue in an early stage of infection. In this disease, it was also assumed that there may be an interaction between some components of bacteria and host tissues which involves in establishing host-parasite symbiosis.

In the present study, we describe a new agglutinin isolated from citrus leaves which agglutinates extracellular polysaccharides of *X. campestris* pv. *citri* *in vitro*, but not bacterial cells themselves. A brief report of this study has been published¹⁷.

Materials and Methods

Bacteria and culture. Virulent strains of *Xanthomonas campestris* pv. *citri* (U 9-1) were obtained from the collection at Plant Pathology Laboratory, Faculty of Agriculture, Shizuoka University and newly obtained from leaves with citrus canker of *C. natsudaidai* Hayata (Rutac.) (N1-1, N1-2, N1-4, N1-5, N1-6, N2-2, N2-4, and N5-2), *C. unshiu* Murc. (Rutac.) (U1, U2, U3 and U6), and *C. sinensis* Osbeck var. *brasiliensis* Tanaka (Na 2-1, Na 2-2 and Na 3-2). A mutant strain of *X. campestris* pv. *citri* (U9-1 CM), which did not produce extracellular polysaccharides and hardly grow in the host tissues, was isolated from slimeless colonies of the virulent strain (U9-1) naturally appeared on nutrient agar plates.

X. campestris pv. *campestris* (NA-1), *X. campestris* pv. *phaseoli* (SH-1), *Pseudomonas syringae* pv. *syringae* (NEGE-1), *Ps. syringae* pv. *pisi* (SH-2), and *Erwinia herbicola* (V2) were also obtained from the collections at the laboratory stated above.

The pathogenesis of these bacteria was periodically checked by observing typical disease symptom appeared in their host plant leaves following inoculation. Each culture was maintained at 28°C by at least weekly transfer of cells on a PSA slant consisting of hot water extracts of 200 g potato tuber tissues, 10 g of sucrose and 15 g of agar in 1,000 ml.

The bacterial cells used agglutination assay were grown on the PSA slant for 2 days at 28°C. The cells were harvested from the typical colonies and gently suspended in sterile distilled water. These cells are henceforth referred to as unwashed bacteria. The unwashed bacterial suspension was stirred with a Vortex mixer, followed by being centrifuged at $20,000 \times g$ for 30 min. Precipitated cells were resuspended in distilled water and the suspension was subjected to centrifugation. This washing procedure was repeated more than once. These cells obtained by centrifugation are henceforth referred to as washed bacteria. Concentration of cells was adjusted to about 5×10^8 cells/ml by dilution after measuring optical density of the suspension at 540 nm. Each extinction coefficient was obtained from the relationship between the O. D. and number of colonies formed.

Preparation of agglutinin. Leaves of *C. unshiu* var. *miyagawa-wase* (100 g)

were rinsed in distilled water and then homogenized with 500 ml of distilled water containing HCl (pH 4.0) for 5 min in a blender. After the homogenate was allowed to stand overnight with a constant agitation, pH was again adjusted to 4.0 with HCl, and filtrated through two layers of cheesecloth. The filtrate was centrifuged at $20,000 \times g$ for 30 min. The resultant supernatant was added with ammonium sulfate to 50 % saturation, stirred for 30 min and then centrifuged at $20,000 \times g$ for 30 min. Suspension of the pellet was dialyzed against distilled water overnight. Nondialyzable fraction was subjected to ultrafiltration using Amicon P-30 filter. Sample retained on the filter was used for agglutinin activity assay. All procedures for extraction were carried out at 4°C.

Agglutinin preparations were also extracted from leaves of other *Citrus* species, *C. natsudaoidai* and *C. reticulata* Blanco (Rutac.)

Preparation of extracellular polysaccharides. Supernatant obtained by centrifugation from unwashed bacterial suspension as described above was filtrated through a membrane filter (pore size: $0.22 \mu\text{m}$). Filtrate was added with 3 volumes of ethanol, yielding precipitate. The precipitate collected by centrifugation at $10,000 \times g$ was dissolved in distilled water followed by treatment with phenol-water according to the method of Sequeira and Graham¹³⁾. Soluble components in water was dialyzed against distilled water and used as extracellular polysaccharides (EPS).

Assay for agglutinin activity. Agglutinin activity of the preparation (ca. 7 mg/ml) was checked in a test tube by pipetting 0.5 ml of the preparation dissolved in various solution to be tested into 0.5 ml of bacterial cell suspension or EPS solution at various concentrations. As a control, distilled water was used for the suspension medium. The mixture was vigorously mixed with a Vortex mixer at room temperature, and then examined the appearance of aggregates by naked eyes or under a light microscope (Nikon optiphot) equipped with a Normalski interference apparatus (Nikon NT).

To determine effect of pH on agglutinating activity, pH of both agglutinin preparation and bacterial suspension was adjusted by 10 mM citrate buffer from pH 3 to 4 and by 10 mM phosphate buffer from pH 5 to 8.

To determine effect of salts and metal ions on the agglutinating activity, bacteria were suspended in each salt solution at concentrations from 25 to 125 mM. Salts tested were as follows; sodium chloride, sodium sulfate, potassium chloride, potassium sulfate, magnesium chloride, barium chloride, and calcium chloride.

To determine effect of sugars on agglutinating activity, the following sugars were tested; D-glucose, D-galactose, D-xylose, D-ribose, 2-deoxy-D-glucose, myo-inositol, D-cellobiose, maltose, α -lactose, D-raffinose, D-mannosamine, D-glucosamine, D-galactosamine, α -methyl-D-galactoside, α -methyl-D-mannoside, α -methyl-D-glucoside, N-acetyl-D-glucosamine. Each sugar was dissolved at a concentration of 25 mM in the agglutinin preparation and the mixture was incubated for 30 min at 27°C before it was mixed with bacterial suspension or EPS solution.

Heat- and protease-stability. Protease (Sigma Chem. Co.) was added to 0.5 ml of agglutinin preparation in 10 mM phosphate buffer (pH 7.0) at a final concentration of 500 $\mu\text{g/ml}$. The mixture was incubated at 37°C for 2 hr, dialyzed against 10 mM phosphate buffer (pH 5.0), and used for the assay. Agglutinin preparations dissolved in

distilled water was incubated at 50 to 100 C for 10 min. After precipitate was removed by centrifugation at $2,000 \times g$ for 10 min, the supernatant was used for the assay.

Results

Agglutination of xanthomonad cells

When agglutinin preparation from citrus leaves (*Citrus unshiu*) was mixed with unwashed bacteria of *X. campestris* pv. *citri* in distilled water, an amorphous aggregate appeared just after mixing (Table 1, Plate I-1 and -2). Microscopic observations showed that the aggregate was composed of many fibrous materials in which most of bacterial cells were embedded (Plate I-3). Thus, turbidity derived from bacterial cells

Table 1. Agglutination of bacterial cells and extracellular polysaccharides (EPS) of xanthomonads, pseudomonads and erwineae by proteinaceous components isolated from citrus leaves (*Citrus unshiu*)

Bacteria		Agglutination ^{a)}		
		Bacterial cells		EPS ^{b)}
		Unwashed	Washed	
<i>Xanthomonas campestris</i>				
pv. citri	(U 9-1)	+	—	+
pv. citri	(N 1-1)	+	—	nt
pv. citri	(N 1-2)	+	—	nt
pv. citri	(N 1-4)	+	—	nt
pv. citri	(N 1-5)	+	—	nt
pv. citri	(N 1-6)	+	—	nt
pv. citri	(N 2-2)	+	—	nt
pv. citri	(N 2-4)	+	—	nt
pv. citri	(N 5-2)	+	—	nt
pv. citri	(U 1)	+	—	nt
pv. citri	(U 2)	+	—	nt
pv. citri	(U 3)	+	—	nt
pv. citri	(U 6)	+	—	nt
pv. citri	(Na 2-1)	+	—	nt
pv. citri	(Na 2-2)	+	—	nt
pv. citri	(Na 3-2)	+	—	nt
pv. citri	(U 9-1 CM) ^{c)}	—	—	nt
pv. campestris	(NA-1)	+	—	+
pv. phaseoli	(SH-1)	+	—	+
<i>Pseudomonas syringae</i>				
pv. syringae	(NEGI-1)	—	nt	nt
pv. pisi	(SH-2)	—	nt	nt
<i>Erwinea herbicola</i>	(V 2)	—	nt	nt

a) Assay was conducted as described in Materials and Methods. + : fibrous aggregate appeared, — : absence of aggregate, nt: not tested.

b) Isolated from the supernatant of bacterial suspension by phenol-water method¹³⁾.

c) A mutant lacking extracellular slime of polysaccharide derived from isolate U 9-1. Little EPS was isolated.

Table 2. Effect of various salts on agglutination of unwashed bacterial cells of *Xanthomonas campestris* pv. *citri* by agglutinin preparation from citrus leaves (*Citrus unshiu*)

Salts	Agglutination ^{a)} in salt solution at ^{b)}				
	25mM	50mM	75mM	100mM	125mM
NaCl	++	++	+	—	—
Na ₂ SO ₄	++	++	+	—	—
KCl	++	++	+	—	—
K ₂ SO ₄	++	++	+	—	—
MgCl ₂	++	++	+	—	—
BaCl ₂	++	++	+	—	—
CaCl ₂	++	++	+	—	—

a) ++: most of bacterial cells aggregated with fibrous material, +: a little aggregate appeared, —: absence of aggregate.

b) Final concentration in reaction mixture.

disappeared in the space of reaction mixture without the aggregate. However, washed bacteria and unwashed colony mutant of *X. campestris* pv. *citri* produced no agglutinate with the agglutinin preparation (Table 1, Plate I-4 and -5).

The agglutinin preparation also agglutinated unwashed bacterial cells of *X. campestris* pv. *campestris* and *X. campestris* pv. *phaseoli* (Table 1, Plate I-2, -6, and -7). Washed bacteria of these xanthomonads were not agglutinated with the agglutinin preparation. Unwashed bacterial cells of *Ps. syringae* pv. *syringae*, *Ps. syringae* pv. *pisi* and *E. herbicola* were not agglutinated with the agglutinin preparation.

Leaf preparations isolated from other species of *Citrus* (*C. natsudaidai* and *C. reticulata*) also showed the activity to agglutinate unwashed xanthomonad cells similarly to that from *C. unshiu*.

Agglutination of EPS of xanthomonads

In the supernatant obtained by centrifugation from suspension of unwashed bacterial cells of xanthomonads, some materials which produced the similar fibrous aggregate with agglutinin preparation from *C. unshiu* leaves to that appeared in the case of unwashed bacterial cells, were found to exist (Plate I-8). EPS isolated from the supernatant was also found to be agglutinated with the agglutinin preparation (Table 1, Plate I-9). The agglutinated materials were fibrous and similar to those appeared with unwashed bacterial cells except no appearance of bacterial cells embeded in the fibrous materials.

Effect of salts and pH on the agglutination

In the reaction mixtures containing salts in distilled water (NaCl, Na₂SO₄, KCl, K₂SO₄, MgCl₂, BaCl₂ and CaCl₂) at concentrations lower than 75 mM, the agglutination of unwashed bacterial cells of *X. campestris* pv. *citri* occurred without detectable difference in the agglutination aspect as compared with that in the solution containing no salts (Table 2). However, all of salts tested inhibited the agglutination at concentrations higher than 100 mM. When each of salts was added to the mixture

Table 3. Effect of pH values on agglutination of unwashed bacterial cells by agglutinin preparation from citrus leaves (*Citrus unshiu*)

Bacteria	Agglutination ^{a)} in buffer solution at pH of					
	3 ^{b)}	4 ^{b)}	5 ^{c)}	6 ^{c)}	7 ^{c)}	8 ^{c)}
<i>Xanthomonas campestris</i>						
pv. <i>citri</i> (U 9-1)	+	+	+	—	—	—
pv. <i>campestris</i> (NA-1)	+	+	+	—	—	—
pv. <i>phaseoli</i> (SH-1)	+	+	+	—	—	—
<i>Pseudomonas syringae</i>						
pv. <i>syringae</i> (NEGI-1)	—	—	—	—	—	—
pv. <i>pisi</i> (SH-2)	—	—	—	—	—	—
<i>Erwinia herbicola</i> (V 2)	—	—	—	—	—	—

a) Assay was conducted as described in Materials and Methods. + : fibrous aggregate appeared, — : absence of aggregate.

b) Adjusted by 10 mM phosphate buffer.

c) Adjusted by 10 mM citrate buffer.

forming the aggregate with unwashed bacterial cells of *X. campestris* pv. *citri* in distilled water, the visible aggregate disappeared just after addition with the salt at concentrations higher than 100 mM (final concentration).

The agglutinating activity was found to depend on pH in the reaction mixture (Table 3). The agglutination of unwashed bacterial cells of xanthomonads was strongest at pH 3.0 and gradually weakened with increase in pH value to 6.0. At higher pH over 6.0 no agglutination occurred. At every pH tested, no agglutination of unwashed bacterial cells of pseudomonads and erwiniae were detectable.

Heat and protease stability

The agglutinin preparation incubated at temperatures lower than 70 C for 10 min retained the agglutinating activity, but that incubated at higher temperatures than 85 C lost the activity completely with irreversible denaturation (Table 4). The agglutinin preparation treated with protease lost its agglutinating activity.

Table 4. Effect of thermal treatment of agglutinin preparation from citrus leaves (*Citrus unshiu*) on the activity to agglutinate unwashed bacterial cells of *Xanthomonas campestris* pv. *citri*

Temperature treated (C) ^{a)}	Agglutination ^{b)}
50	++
60	+
70	+
85	—
100	—

a) The agglutinin preparation was incubated in distilled water at designated temperatures for 10 min.

b) ++: most of bacterial cells aggregated with fibrous materials, +: a little aggregate appeared, —: absence of aggregate.

Effect of sugars on the agglutination

An attempt to prevent the agglutinating activity was made by using various sugars. In the reaction mixture containing each sugar tested at a final concentration of 25 mM, only D-glucosamine prevented the unwashed bacterial cells or EPS of *X. campestris* pv. *citri* from the agglutination caused by agglutinin preparation. However, D-glucosamine as well as other sugars tested caused no inhibition of the agglutination of unwashed bacterial cells and EPS of other xanthomonad than *X. campestris* pv. *citri*. Addition with D-glucosamine into solution containing aggregate of unwashed bacteria or EPS of *X. campestris* pv. *citri*, but not of other xanthomonads, dissociated the aggregate.

Discussion

Citrus leaves, which are host tissues of *X. campestris* pv. *citri*, was found to contain an agglutinin which caused agglutination with unwashed bacterial cells and the extracellular polysaccharides, but not with washed bacterial cells or unwashed colony mutant cells lacking extra-polysaccharidal slime. This agglutinin also showed the activity to agglutinate red blood cells of rabbit and human (unpublished data). Therefore, it may be called a kind of lectin with a unique nature which interacts with extracellular polysaccharides of xanthomonads. The agglutinin seems to be a proteinaceous components with molecular weight more than 3×10^4 daltons since the agglutinin preparation was precipitated with 50 % saturated ammonium sulfate, heat and protease labile and nonpermeable through PM-30 membrane filter.

The agglutinin exhibited the activity to agglutinate unwashed bacterial cells or EPS of xanthomonads nonpathogenic to citrus plants. It seems that the agglutination may be nonspecific among xanthomonads as far as we are concerned. However, a finding that D-glucosamine specifically inhibited the agglutination of only *X. campestris* pv. *citri* suggested that there may be difference in the mode of agglutination between *X. campestris* pv. *citri* and other xanthomonads nonpathogenic to citrus plants.

It is also marked that the agglutinates formed after mixing the agglutinin with EPS or unwashed bacterial cells shows fibrous products. This is quite different from the agglutinate which have been reported in other bacteria-host agglutinin systems^{12, 13, 14}. Although the details of the mechanism of agglutination in the present system have to wait for future investigations, the fibrous materials may be assumingly formed through end to end association of EPS molecules mediated by the agglutinin molecules.

The agglutinin was isolated from the supernatant of leaf homogenate at acidic condition (pH 4.0). Preliminary experiments showed that the agglutinin was not extracted in the supernatant in the case of tissue-homogenate at pH 7.0, retaining in insoluble materials at $20,000 \times g$ centrifugation. The isolated agglutinin was very soluble at pH 7.0 without agglutinating activity. Therefore, it is assumed that the agglutinin might bind to insoluble materials of citrus tissues and that conformational changes of the agglutinin may be caused depending on pH, resulting in change in agglutinating activity. The inhibition of agglutinin activity by high ionic strength also suggested that ionic factor may involve in the agglutinating activity.

Citrus canker is characterized by formation of hypertrophic tissues with the multiplication of *X. campestris* pv. *citri* in the early stages of infection^{8, 9, 10}. Necrotrophic

tissues typical in canker disease appeared at late stages of infection. This pathogen seems to have symbiotic nature rather than necrotrophic one. The colony mutant (U 9-1 CM) of *X. campestris* pv. *citri*, which lacked extracellular polysaccharidal slime, failed to grow in the host tissues (unpublished data). However, growth of the mutant was recovered to an extent in the case of inoculation with EPS of a virulent strain, causing early disease symptom (unpublished data). It was also found that attachment of the bacterial cells to the wounded tissue of citrus leaves were specifically inhibited by D-glucosamine which was found to dissociate the agglutination of unwashed bacterial cells or the EPS¹⁶⁾. These findings suggested that interaction between EPS and the agglutinin in citrus leaves may possibly involve in establishing the host-parasite relationship in citrus canker showing hypertrophic tissues. Further characterization of this agglutinin and the significance of interaction between EPS and the agglutinin in citrus canker will be reported elsewhere.

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和 文 摘 要

カンキツ葉蛋白質性成分によるカンキツかいよう病菌
(*Xanthomonas campestris* pv. *citri*) の凝集

高橋敏房・道家紀志

カンキツかいよう病菌の非洗浄菌体および菌体外多糖質 (EPS) を凝集するが、洗浄菌体や非洗浄 EPS 非生産菌体を凝集しない蛋白質性の成分が、ウンシュウ、ナツダイダイおよびボンカンの葉より得られた。同成分は、葉の磨砕液から HCl 酸性溶媒 (pH 4.0) で抽出され、50%飽和硫酸で沈殿回収された。この凝集素は、熱および蛋白質分解酵素に不安定で、100 mM より低濃度の各種の塩類には影響を受けず、pH 6 以上で活性が阻害された。同様な凝集は、他の *Xanthomonas* 菌であるカンラン黒腐病菌およびインゲン葉焼病菌の非洗浄菌体、およびそれらの EPS でもみられた。しかし、検定した18種の糖のうち、D-glucosamine のみがカンキツかいよう病菌の凝集を阻害したが、他の *Xanthomonas* 菌の凝集は阻害しなかった。

Explanation of Plate

Plate I. Agglutination of xanthomonads by agglutinin preparation isolated from citrus leaves (*Citrus unshiu*).

1. Appearance of the mixtures containing the agglutinin and unwashed bacterial cells of *Xanthomonas campestris* pv. *citri* (A), washed bacterial cells (B), cell free supernatant obtained from unwashed bacterial cell suspension (C), extracellular polysaccharides isolated from the supernatant (D), or unwashed bacterial cells of colony mutant which produce little extracellular polysaccharidal slime (E).
2. Appearance of the mixtures containing the agglutinin and unwashed bacterial cells of *X. campestris* pv. *citri* (A), *X. campestris* pv. *campestris* (B) or *X. campestris* pv. *phaseoli* (C).
3. A light micrograph of the aggregate formed in the mixture of unwashed bacterial cells of *X. campestris* pv. *citri* and the agglutinin. Bacterial cells were embedded in fibrous materials.
4. A light micrograph of the mixture of washed bacterial cells of *X. campestris* pv. *citri* and the agglutinin. No aggregate appeared.
5. A light micrograph of the mixture of unwashed bacterial cells of colony mutant and the agglutinin. The mutant lacking extracellular polysaccharidal slime was derived from *X. campestris* pv. *citri*. No aggregate appeared.
6. A light micrograph of the aggregate formed in the mixture of unwashed bacterial cells of *X. campestris* pv. *campestris* and the agglutinin.
7. A light micrograph of the aggregate formed in the mixture of unwashed bacterial cells of *X. campestris* pv. *phaseoli* and the agglutinin.
8. A light micrograph of the aggregate formed in the mixture of supernatant obtained from unwashed bacterial cell suspension of *X. campestris* pv. *citri* and the agglutinin. Fibrous materials appeared.
9. A light micrograph of the aggregate formed in the mixture of partially purified extracellular polysaccharides of *X. campestris* pv. *citri* and the agglutinin. Fibrous materials appeared.

Plate I

