Biological and Chemical Properties of Slime Polysaccharide of *Xanthomonas campestris* pv. *oryzae*

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多糖質の生物学的および化学的性質

Abstract

The slime polysaccharide of *X. campestris* pv. *oryzae* caused wilting in rice cuttings. This substance was high molecular and was precipitated by ethanol. Wilt inducing potential of slime polysaccharides of virulent and avirulent clones could not be differentiated. The slime polysaccharide of all clones was composed of arabinol, mannose, glucuronolactone, glucose and glucuronic acid. The ratios of these sugars were quite different between large colony type and small colony type. In general, arabinol and glucose contents of slime polysaccharides were higher in small colony types (St and Sm) than in large colony types (Lw, Ld and Lh). Column chromatography on Sephadex G-200 revealed that the slime polysaccharides of each clone were heterogeneous in molecular weight. The wild type was composed of relatively greater amount of high molecular weight polysaccharide whereas the small colony type variants were of relatively low molecular ones. Intermediate types such as Ld and Lh were composed of both these types.

The hapten inhibition studies were carried out by using sugars of slime polysaccharide component and anti-Q7472-serum. In the case of the isolate Q7472 (serovar A), glucose, mannose and glucuronic acid inhibited precipitin reaction, while in the cases of Q7502 (serovar B-I) and N 5837 (serovar B-II), glucuronic acid and glucuronolactone were strong promoters of the precipitin reaction. These facts suggest that the component sugars of a major portion of the immunogenic regions are remarkably different with serovars.

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Key Words: *Xanthomonas campestris* pv. *oryzae*, virulence, bacteriological properties.

Introduction

The surface of gram-negative bacterial cells is covered with extracellular polysaccharide (EPS) and lipopolysaccharide, which have been implicated as determinants in pathogenesis to animals and human-beings²⁶⁾. The production of EPS in form of capsule and loose slime are also known to be associated with virulence in several plant pathogenic bacteria^{2,9,12,14,16)}. These polysaccharides are thought to be involved in symptom expression, *i. e.*, wilting due to decreased vascular flow^{3,10,12,14,31)}, and it has been suggested that the production of EPS may interfere with defense mechanism of host plants which involves recognition of alien bacterial cell-wall component such as lipopolysaccharide²⁵⁾.

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Cadmus *et al.*⁴⁾ reported that extracellular polysaccharide produced by the small colony type variants differed in solution properties from that of parent strain (large colony type) in *X. campestris* pv. *campestris*; it differed also in content of pyruvic acid and O-acetyl substituents. However, the polysaccharides of normal and variant strains were identical in molar ratios of sugars. Kuo *et al.*²⁰⁾ reported that *X. campestris* pv. *oryzae* produced polysaccharides which could induce wilting in plant cuttings. Fan and Kuo¹³⁾ and Tabei³⁰⁾ attributed this polysaccharide induced-wilting to plugging of xylem vessels.

According to the report of Misaki *et al.*²²⁾ extracellular polysaccharide of *X. campestris* pv. *oryzae* contains glucose, mannose and glucuronic acid, while others^{1,20)} reported that glucose, mannose, glucuronic acid and glucuronolactone may be the constituents.

In this experiment the relationships among colony types, serological properties, wilt inducing ability and chemical composition of slime polysaccharide of *X. campestris* pv. *oryzae* were investigated.

Materials and Methods

Isolates. Eight colony variants derived from 3 isolates of *X. campestris* pv. *oryzae* were used (Table 1).

Isolation of slime polysaccharide and Pronase treatment. The methods for isolation of slime polysaccharide and Pronase treatment were as follows⁸⁾. The bacteria cultured on PSA medium⁵⁾ in Roux Bottles at 25 C for 3 days were suspended in 40 ml of distilled water and shaken thoroughly. The bacterial cells were harvested by centrifugation at $26,000 \times g$ for 30 min. The supernatant was transferred to a flask and three volumes of 95 % cold ethanol was slowly added with stirring. The precipitate was collected by centrifugation at $6,000 \times g$ for 20 min, washed twice, first with 95 % ethanol and next with absolute ethanol, and finally dried at 50 C. The dried polysaccharide was dissolved in 0.1 M tris-buffer, pH 7.4, to obtain a final concentration at 5 mg/ml. Pronase (Kaken Chemical Co. Ltd.) was added at a concentration of 1 mg/ml and incubated at 37 C for 12 hr. The digest was then dialyzed for 24 hr against 40 volumes of distilled water. To the supernatant obtained by centrifugation at $12,000 \times g$ for 30 min was added three volumes of 95 % cold ethanol. The precipitate was washed with

Table 1. The relationships among serovar, colony type and virulence of the clones of *X. campestris* pv. *oryzae* used in this experiment

Clone	Serovar ^{a)}	Colony type ^{a)}	Virulence (cm) ^{b)}
Q7472-Lw-1	A	Lw	13.0
Q7472-Lh-5	B-I	Lh	3.5
Q7472-St-1	B-II	St	0
Q7447-Lw-1	A	Lw	16.0
Q7447-Sm-1	B-II	Sm	7.0
N2-Lw-1	A	Lw	10.7
N2-Ld-1	B-I	Ld	3.0
N2-St-1	B-II	St	0

a) Choi *et al.*⁷⁾

b) Choi *et al.*⁸⁾

95 % cold ethanol. The final precipitate was dried at 50 C.

Wilt induction by slime polysaccharide. Cuttings of 3-week old rice seedlings were placed in tap water for an hour before use. Four concentrations of aqueous solution ranging from one to 1,000 ppm of the slime polysaccharide were prepared. Five ml of each solution was poured into micro-tubes, and cuttings of rice seedling were inserted. The test tubes were held at temperatures ranging from 20 to 25 C for 24 hr in shadow.

Fractionation of slime polysaccharide. Slime polysaccharide of *X. campestris* pv. *oryzae* was chromatographed on a column (1.7×47 cm) of Sephadex G-200 (Pharmacia Fine Chem. Co.) and eluted with distilled water²⁷⁾ at a flow rate of 10 ml/hr. The amount of polysaccharides eluate in each fraction (2 ml) was determined by phenol-sulfuric acid method¹¹⁾.

Acid hydrolysis. Slime polysaccharides (10 mg) were hydrolyzed in sealed tubes containing 3 ml of 1 N H₂SO₄ at 100 C for 5 hr, and after cooling, the hydrolysates were neutralized with a saturated solution of Ba(OH)₂. After centrifugation at 8,000× g for 20 min, the supernatants were collected and freeze-dried.

Analysis of sugar residue by gas-liquid chromatography. Sugar components of slime polysaccharide were analyzed by the method of Sweeley *et al.*²⁹⁾ Hydrolyzed slime polysaccharide was treated with 1 ml of anhydrous pyridine, 0.2 ml of hexamethyldisilazane, and 0.1 ml of trimethylchlorosilane. The mixture was shaken vigorously for about 30 sec and then warmed at 75 to 85 C for 2-3 min. A Shimazu Model GC-7AG gas chromatograph with hydrogen flame ionization detector was used. The temperature of injection port was 270 C. Column temperatures were changed from 140 to 240 C at 4 C/min. One microliter of the sample was injected. Identification of sugars, glucuronolactone and glucuronic acid was based on co-chromatography with known standards. Sugar ratio was automatically shown on "Chromatopac" (Shimazu Model C-RIA) as peak area.

Quantitative precipitin inhibition assay. One ml of sugar solution (100 mg/ml) was incubated with 1 ml of diluted (1 : 10 in saline) antiserum at 37 C for 1 hr. One ml of the polysaccharide solution (2 mg/ml) in saline was then added to the reaction mixture, further incubated at 37 C for 1 hr and kept overnight at 4 C. The mixtures were centrifuged at 6,000× g for 20 min and washed three times with 0.85 % NaCl solution. The pellet was dissolved in 1 ml of 0.1 N NaOH and optical density of the solution was determined at 287 nm¹⁸⁾.

Results

Wilting effect of slime polysaccharide on rice cuttings

The cuttings of rice seedlings placed in colloidal solution of slime polysaccharide of *X. campestris* pv. *oryzae* exhibited wilting symptom within 2 hr and were completely wilted by the end of 12 hr incubation. The rapidity and severity of the wilting were correlated with concentration of polysaccharide but not with colony type or virulence (Table 2). Rice cuttings wilted after 2 hr at 1,000 ppm, 12 hr at 10 to 100 ppm and weak or no symptom was observed at 1 ppm.

Table 2. The effect of slime polysaccharide of *X. campestris* pv. *oryzae* on the induction of wilting in rice cuttings^{a)}

Clone and isolate	Serovar	Concentration (ppm)			
		1,000	100	10	1
Q7472-Lw-1	A	+++ ^{b)}	++	+	±
Q7472-St-1	B-II	+++	++	+	±
Q7447-Ld-1	B-I	+++	++	+	±
Q7447-Sm-1	B-II	+++	++	+	±
Q7502	B-I	+++	++	+	±
N5837	B-II	+++	++	+	±

a) Twelve hr after dipping in slime polysaccharide solution.

b) +++~±: Severity of wilting.

Fractionation of slime polysaccharide

The soluble slime polysaccharide was precipitated in 95% ethanol, treated with Pronase, dialyzed against water, precipitated with ethanol and dried at 50°C. The crude slime polysaccharide from each clone was fractionated on a gel permeation column, and the amount of polysaccharide in eluate was measured with phenol-sulfuric acid method⁽¹¹⁾. The molecular size of polysaccharides of each clone was distributed in wide range, and

the ratio of high- and low-molecular weight polysaccharide in each clone was also different.

The clone Q7472-Lw-1 (large colony type and virulent) excreted relatively high-molecular polysaccharides whereas Q7472-St-1 (small colony type and avirulent) excreted relatively low-molecular polysaccharides. The clone Q7472-Lh-5 (large colony with halo and weakly virulent), however, showed intermediate pattern between Lw-1 and St-1 (Fig. 1).

The fractions containing antigen "a" were identified by the ordinary agar gel diffusion test against anti-Q7472-serum. Most of the fractions from Q7472-Lw-1 formed precipitin band "a" while all fractions of Q7472-Lh-5 and Q7472-St-1 did not.

Fig. 1. Sephadex G-200 chromatography of the slime polysaccharides obtained from the clones of *X. campestris* pv. *oryzae* different in virulence.

Chemical composition of slime polysaccharide

Gas-liquid chromatograms of the sugars obtained from the hydrolysis

of slime polysaccharide of *X. campestris* pv. *oryzae* showed five peaks (Fig. 2). The peaks were arabinol, mannose, glucuronolactone, glucose and glucuronic acid. The slime polysaccharide from all colony types had the same monomeric components. Glucose, mannose and glucuronic acid were the major monomers detected in the slime polysaccharide of all types with small amounts of glucuronolactone and arabinol (Table 3). Significant difference was not observed among Lw, Lh and Ld types in the contents of these major monomers. In St type, however, the major sugar of arabinol was particularly higher than in Lw, Ld and Lh types.

The molar ratios of arabinol, glucose, mannose, glucuronic acid and glucuronolactone in the slime polysaccharide of Q7472-Lw-1 (serovar A) were 0.8, 37.5, 30.2, 30.7 and 0.8, while in that of Q7472-St-1 (serovar B-II) they were 7.6, 41.0, 20.4, 26.5 and 4.5, respectively. In general, the ratio of mannose and glucuronic acid were greater and those of arabinol and glucuronolactone were much smaller in the slime polysaccharide from large colony types as compared to small colony types.

Quantitative precipitin inhibition assay

Serological identity of antigens is an expression of similarity in serological determinants. To characterize specificity of the slime polysaccharide antigen, quantitative precipitin inhibition studies were conducted. Component sugars of slime polysaccharides were used

Fig. 2. Chromatogram of TMS-derivatives of acid-hydrolyzed slime polysaccharide of *X. campestris* pv. *oryzae*.

Table 3. Component sugars of slime polysaccharide of *X. campestris* pv. *oryzae*

Clone	Arabinol	Mannose	Glucuronolactone	Glucose	Glucuronic acid
Q7472-Lw-1	0.8%	30.2%	0.8%	37.5%	30.7%
Q7472-Lh-5	1.1	30.0	1.1	37.7	30.1
Q7472-St-1	7.6	20.4	4.5	41.0	26.5
N2-Lw-1	0.2	28.5	0.6	36.8	33.9
N2-Ld-1	0.7	26.6	0.7	38.8	33.2
N2-St-1	15.6	24.3	3.0	41.1	16.0
Q7447-Lw-1	0.2	31.4	1.0	33.3	34.1
Q7447-Sm-1	0.7	21.9	1.1	43.1	33.2

Table 4. Precipitin inhibition by component sugars of *X. campestris* pv. *oryzae* polysaccharide

Inhibitor (100 mg/ml)	Q7472 (serovar A)		Q7502 (serovar B-I)		N5837 (serovar B-II)	
Arabitol	0.66 ^{a)}	0 ^{b)}	0.26	7.2	0.27	3.5
Glucose	0.39	40.9	0.27	0.1	0.28	0
Mannose	0.51	22.7	0.30	+ 7.1	0.29	+ 3.5
Glucuronic acid	0.56	15.2	0.90	+121.4	1.28	+357.1
Glucuronolactone	0.70	+ 6.1	1.33	+327.0	1.11	+326.8
NaCl	0.66	0	0.28	0	0.28	0

a) Determined at 287nm.

b) Inhibition or promotion (%), + : Promotion.

to inhibit the precipitation with anti-Q7472-serum. The results are shown in Table 4.

In the cases of Q7502 and N5837 which belong to serovar B, 121.4-375.0 % promotion of precipitin reaction was observed with glucuronolactone and glucuronic acid, while promotion effect was not so remarkable with glucose, arabitol and mannose. In the case of Q7472 belonging to serovar A, the results were remarkably different showing high inhibition with glucose, mannose and glucuronic acid.

Discussion

The slime polysaccharides of *X. campestris* pv. *oryzae* induced wilting in rice seedling. However, there was no difference in wilt inducing potential between the slime polysaccharides of virulent and avirulent isolates. These results indicate that wilting is not specific and could be caused by the plugging with polysaccharides produced by the parasite which is compatible with the host. Hogdson *et al.*¹⁶⁾ observed two types of wilting in tomato plant cuttings: one affecting the leaflet margins which was caused by relatively small molecular weight substances like inulin, soluble starch, glucosan from crown gall bacteria and carbowax (1,500 and 3,000) and the other type affecting stems and petioles which was caused by macro-molecular substances as xylan, pectin and carbowax 6,000 and 9,000.

Since the virulence of *X. campestris* pv. *oryzae* isolates are closely related to the amount of polysaccharides production, the wilting of rice seedlings can partially be attributed to a similar molecular plugging. However, the differences in molecular size and molar ratio of component sugars seem to be related to virulence in the case of *X. campestris* pv. *oryzae*. Kuo *et al.*²⁰⁾ and Angadi¹⁾ already reported that *X. campestris* pv. *oryzae* produced polysaccharides which were capable of inducing wilting in plant cuttings. The molecular weights of these substances were reported to be 200,000 or bigger with glucose and mannose making up most of their weight²⁰⁾.

The chemical composition of slime polysaccharide described in this paper mainly agreed with that in earlier reports^{1,20,22,28)} with some minor differences. Slime polysaccharide of clones showing different colony types or serovars was the same in chemical components. Each contains five sugars such as arabitol, mannose, glucuronolactone, glucose and glucuronic acid. The molar ratios of these sugars were remarkably different

depending upon serovars or colony types.

Misaki *et al.*²²⁾ and Angadi¹⁾ isolated slime polysaccharide from *X. campestris* pv. *oryzae* with Fehling solution. However, we found out that this method was unsuitable for serological test, because the polysaccharide obtained by this method lost its serological properties,⁸⁾ indicating that slime polysaccharide might have been denatured.

The ratio of component sugars may reflect the structure of slime polysaccharide. Arabitol and glucose contents were higher in slime polysaccharide of small colony types (avirulent) than in those of large colony types. These differences could directly or indirectly reflect on the structural, serological, morphological and pathogenic differences of *X. campestris* pv. *oryzae*.

Iacono *et al.*¹⁸⁾ and others^{21,23)} analyzed immunodominant region of the antigen by hapten inhibition studies in *Streptococcus*. Similar experiments were also conducted by Jann *et al.*¹⁹⁾ and Nhan *et al.*²⁴⁾ According to the report by Jann *et al.*, partial structures of an antigen which inhibit the homologous serological system also fulfil the requirements for inhibition in a cross reacting system.

The hapten inhibition studies were also carried out in our experiment by using sugars of slime polysaccharide component and anti-Q7472-serum. In the case of Q7472, glucose, mannose and glucuronic acid inhibited precipitin reaction. These sugars, therefore, seem to constitute a major portion of the immunogenic region of serovar-specific antigen. In the cases of Q7502 (serovar B-I) and N5837 (serovar B-II), remarkable promotion effect was observed with glucuronic acid and glucuronolactone. Supposedly, the promotion effect also suggests some correlations with component sugars, but its mechanism is unknown.

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和 文 摘 要

イネ白葉枯病菌粘液多糖質の生物学のおよび化学的性質

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イネ白葉枯病菌の培養ろ液からエタノール沈殿によって得られた粘液多糖質はイネ幼苗を萎ちょうさせるが、濃度を同一にした場合には血清型の異なるクローン間にも活性の差は認められなかった。各クローンの粘液多糖質は血清型に関係なくいずれもアラビトール、マンノース、グルクロノラクトン、グルコースおよびグルクロン酸から成っているが、これらの含量比は血清型によって異なり、野生型（コロニー型Lw, 血清型A）の粘液多糖質では変異型（コロニー型St, 血清型B-II）のそれよりもアラビトール、グルコースおよびグルクロノラクトンの含量比が小であった。また血清型Aの産生する粘液多糖質は血清型B-II変異株の産生するそれに比べて高分子量のものの含量が相対的に大であった。粘液多糖質の各構成糖を用いて粘液多糖質の凝集反応抑制実験を行った結果、血清型Aの凝集反応はグルコース、マンノース、およびグルクロン酸によって著しく抑制されたが、血清型B-IおよびB-IIの場合にはグルクロン酸とグルクロノラクトンによって反応が著しく促進された。これらの結果は血清型によって主要免疫部分の構成糖に顕著な差があることを示唆するものと考えられる。