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IMMUNOCHEMICAL STUDIES ON O-ANTIGENS OF *VIBRIO PARAHAEMOLYTICUS*. III. STUDIES ON INHIBITION OF THE PRECIPITIN REACTION BY KNOWN SUGARS

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SUMMARY The inhibitory effects of sugars and sugar derivatives with known molecular structures on the precipitation reactions of *Vibrio parahaemolyticus* O-antigens (O1 to O10) were studied. From the results the immunodominant sugars seemed to be α-linked-N-acetyl-D-galactosamine for the O1 antigen, D-galactose for O2, α-linked-D-glucose and D-glucosamine for O3, D-glucose and N-acetyl-D-glucosamine for O4, D-galactose for O6, N-acetyl-D-galactosamine and D-galactose for O7, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine for O9, and β-liked-N-acetyl-D-galactosamine for the O10 antigen. The immunodominant sugars of the O5 and O8 antigens are still unknown.

INTRODUCTION

O-Antigens were extracted from boiled cells of *Vibrio parahaemolyticus* by the phenol-water method (Torii et al., 1969). The serological specificities of the antigens from 32 pilot strains shown by the agar diffusion technique coincided well with those shown by O-agglutination. Chemical studies on purified O-antigens from 10 pilot strains, O1 to O10, showed that these antigens were mainly composed of glucose, galactose, glucosamine, heptose, phosphorus, fatty acid ester and nitrogen compounds. In addition galactosamine was found in 5 of the 10 samples (Torii et al., 1969).

The purified O-antigens were also treated with alkali and acid (Torii and Igarashi, 1969). On treatment with 0.2 N sodium hydroxide at 50 C for 24 hr all the O-antigens tested lost their original precipitin lines in agar and produced new lines, which still had the respective O-group specificities. Treatment with 0.2 N hydrochloric acid at 50 C for 48 hr had complicated effects. Namely, after this treatment one group of antigens (O4, O5 and O8) completely lost their ability to precipitate with homologous antisera. Another group (O2 and O9) produced new precipitin lines in agar instead of the original lines. A third group (O3, O6 and O10) gave new lines besides the original ones and a fourth group (O1 and O7) was not significantly affected by the treatment.

It was interesting to see how the major sugars found in the O-antigens contributed to the precipitin reaction as antigenic determinants. This paper reports the determination of the

immunodominant sugars in ten purified O-antigens, O1 to O10, by examination of the specific inhibition of homologous precipitation systems with sugars and sugar derivatives with known molecular structures.

MATERIALS AND METHODS

1. *Antigens*

Ten O-antigens (O1 to O10) were prepared by the phenol-water method from boiled cells of 10 pilot strains (O1 to O10) and purified by the method of Westphal et al. (1952 and 1965) as described in the previous report (Torii et al., 1969). Specimens of the dextrans NRRL B1299 and N4 were kindly prepared for us by Meito Sangyo Co., Ltd., Nagoya, Japan, from *Leuconostoc mesenteroides*, strains NRRL B1299 and N4, respectively.

2. *Antisera*

Anti O-sera were prepared by immunizing rabbits with boiled cells by the method of Burrows (1946) (cf. Miwatani et al., 1969) as described in the previous paper (Torii et al., 1969). Rabbit anti-dextran sera were prepared by intravenous injection of formalin-killed cells of *Leuconostoc mesenteroides* NRRL B1397 as described by Torii and Sakakibara (1970).

3. *Serological test*

The quantitative preciptin reaction and quantitative precipitation inhibition were performed in the usual ways (Kabat, 1961). Precipitated protein was determined by the method of Goodman et al. (1968) with the slight modification that the final volume was 2.0 ml.

4. *Sugars and sugar derivatives*

D-Glucose, D-galactose, N-acetyl-D-glucosamine and lactose were obtained from Wako Pure Chemical Industries, Ltd., Osaka. D-Galactosamine hydrochloride, β-phenyl-D-galactoside, α-phenyl-N-acetyl-D-galactosaminide, β-phenyl-N-acetyl-D-galactosaminide and melibiose were obtained from Nakarai Chemicals, Ltd., Kyoto. D-Glucosamine hydrochloride was a product of Sigma Chemical Co., St. Louis, Mo. N-Acetyl-D-galactosamine was from Seikagaku Kogyo Co., Ltd., Tokyo. α-Methyl-D-glucoside and β-methyl-D-glucoside were from Pierce Chemical Co., Rockford, Ill. α-Methyl-N-acetyl-D-glucosaminide and β-methyl-N-acetyl-D-glucosaminide were generous gifts from Prof. Y. Matsushima, Department of Chemistry, School of Science, Osaka University, Osaka.

RESULTS

1. *Quantitative precipitin reactions of ten anti O-sera (anti O1 to O10)*

Quantitative precipitin reactions were performed with 50 μl of ten anti O-sera (anti O1 to O10) and ten homologous O-antigens (O1 to O10). The quantitative precipitin curves are given in Fig. 1. The arrows in the figure indicate the equivalent points at which subsequent inhibition analyses were carried out.

2. *Quantitative inhibition of the precipitin reaction by known sugars*

Since the major constituent sugars were found to be glucose, galactose, glucosamine and galactosamine, as described in the previous paper (Torii et al., 1969), these sugars and their derivatives were used as inhibitors. The effects of two disaccharides, melibiose and lactose were also tested.

Individual antisera contained different amounts of antibodies, so the quantities of antisera used in inhibition experiments were adjusted to give almost the same level of antibodies (38 μl to 100 μl). The total volume of the reaction mixture was 500 μl. The results of the inhibition test are shown in Tables 1 and 2.

None of the sugars or sugar derivatives tested caused significant inhibition of the O5-anti O5 and O8-anti O8 systems. However, in the other eight systems some of the samples caused various degrees of inhibition of precipitin reactions.

In the O1-anti O1 system N-acetyl-D-galactosamine (20 μmoles) and α-phenyl-N-acetyl-D-galactosaminide (10 μmoles) caused 23% and 25% inhibition, respectively. So, the antigenic determinant of this system may include the α-N-acetyl-D-galactosaminyl group. In the O2-anti O2 system melibiose (10 μmoles) and lactose (20 μmoles) caused 23% and 35%

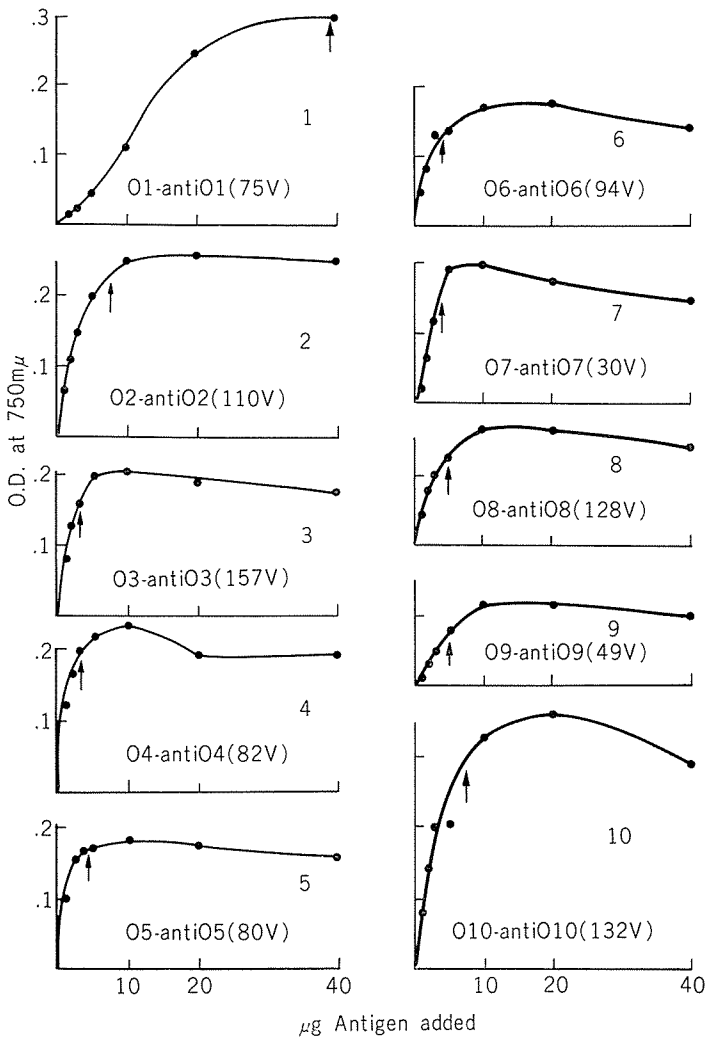

FIGURE 1. *Quantitative precipitin curves of O-antigens and O-antisera. 1 to 10 are the precipitin reaction systems of O-antigens, O1 to O10, respectively.*

inhibition, respectively, so the antigenic determinant must be D-galactose. In the O3-anti O3 system D-glucose (20 μmoles), α-methyl-D-glucoside (20 μmoles), β-methyl-D-glucoside (20 μmoles) and D-glucosamine (20 μmoles) caused 47%, 70%, 27%, and 33% inhibition, respectively. Thus α-methyl-D-glucoside was much more inhibitory than β-methyl-D-glucoside. These results show that the antigenic determinant group (or groups) must include α-D-glucosyl and D-glucosaminyl groups. In the O4-anti O4 system D-glucose (20 μmoles), α-methyl-D-glucoside (20 μmoles) and β-methyl-D-glucoside (20 μmoles) caused 33%, 62% and 48% inhibition, respectively and D-glucosamine (20 μmoles), α-methyl-N-acetyl-D-glucosaminide (20 μmoles) and β-methyl-N-acetyl-D-glucosaminide (20 μmoles) caused 23%, 43% and 35% inhibition, respectively. So, D-glucose and N-acetyl-D-glucosamine must be present in the antigenic determinant. In the O6-anti O6 system only melibiose (20 μmoles) was inhibitory, causing 22% inhibition, so the D-galactosyl group may be part of the antigenic determinant. In the O7-anti O7 system β-phenyl-D-galactoside (20 μmoles), D-galactosamine (20 μmoles), α-phenyl-N-acetyl-D-galactosaminide (10 μmoles) and β-phenyl-N-acetyl-D-galactosaminide (10 μmoles) showed 23%, 32%, 26% and 24% inhibition, respectively. So, the N-acetyl-D-galactosaminyl and D-galactosyl groups are probably present in the antigenic determinants. In the O9-anti O9 system N-acetyl-D-glucosamine (20 μmoles), N-acetyl-D-galactosamine (20 μmoles), α-methyl-N-acetyl-D-glucosaminide (20 μmoles), and β-methyl-N-acetyl-D-glucosaminide (20 μmoles) showed 36%, 38%, 23%, and 40% inhibition, respectively. So, the N-acetyl-D-glucosaminyl and N-acetyl-D-galactosaminyl groups are probably present in the antigenic determinant. Finally in the O10 system β-phenyl-N-acetyl-D-galactosaminide (20 μmoles) caused significant inhibition of precipitation (32%), so, β-

TABLE 1. *Percentage inhibition of quantitative precipitation by sugars and their derivatives*

System	01			02			03			04			05		
μmoles of compound	5	10	20	5	10	20	5	10	20	5	10	20	5	10	20
compound															
D-Glucose	0		3	0		14	17	26	47	20		33	4		4
D-Galactose	0		6	5		6	8	16	16	13		16	0		1
D-Glucosamine	7		11	3		13	21		33	25		23	0		0
D-Galactosamine	5		9	2		16	12		19	0		0	0		0
N-Acetyl-D-glucosamine	8		5	4		1	0	7	5	17		23	3		7
N-Acetyl-D-galactosamine	9		23	0		1	0	0	0	0	13	0	0		0
α-Methyl-D-glucoside	0		2	5		4	45	58	70	29	38	62	9		3
β-Methyl-D-glucoside	0		9	0		12	11	19	27	11	39	48	6		2
β-Phenyl-D-galactoside	4		4	5		5							1		2
α-Methyl-N-acetyl-D-glucosaminide	2	2		0	0		0		11	11	25	43	11	4	
β-Methyl-N-acetyl-D-glucosaminide	0	8		0	4		4	0	0	15	23	35	4	6	
α-Phenyl-N-acetyl-D-galactosaminide	10	25		4	7		0	0		0	0		8	9	
β-Phenyl-N-acetyl-D-galactosaminide	0	0		6	7		15	0		0	3		3	4	
Melibiose	0		0	9	23	22[a]							0		4
Lactose	0		0	19	33	35									

[a] 15 μmoles were used.

TABLE 2. *Percentage inhibition of quantitative precipitation by sugars and their derivatives*

System	06			07			08			09			010		
μmoles of compound	5	10	20	5	10	20	5	10	20	5	10	20	5	10	20
compound															
D-Glucose	5		1	0		3	2		3	1		0	0		1
D-Galactose	7		16	0		12	3		2	0		4	0		0
D-Glucosamine	0		8	4	0	18	0		0	11		18	9		4
D-Galactosamine	18		5	10		32	0		0	0		21	1		4
N-Acetyl-D-glucosamine	3		10	0		0	0		4	30		36	8		5
N-Acetyl-D-galactosamine	13		13	4		18	7		12	31		38	3		12
α-Methyl-D-glucoside	5		8	0		0	5		5	1		7	2		0
β-Methyl-D-glucoside	1		2	1		8	9		0	4		5	0		0
β-Phenyl-D-galactoside	19		14	11		23	7		5				16		9
α-Methyl-N-acetyl-D-glucosaminide	0	3		3	2		6	2		6		23	3	7	
β-Methyl-N-acetyl-D-glucosaminide	5	8		0	0		1	3		26		40	11	12	
α-Phenyl-N-acetyl-D-galactosaminide	0	0		19	26		8	9		12	19		5	3	
β-Phenyl-N-acetyl-D-galactosaminide	13	12		30	24		7	11		16	9		9	22	32
Melibiose	17		22	0		17	2		3				0		16
Lactose	14		13	7		13	2		4				4		10

TABLE 3. *Possible sugar residues in antigenic determinants of O-antigens*

O-antigen	Sugar residues
01	α-N-acetyl-D-galactosaminyl
02	D-galactosyl
03	α-D-glucosyl, D-glucosaminyl
04	D-glucosyl, N-acetyl-D-glucosaminyl
05	unknown
06	D-galactosyl
07	N-acetyl-D-galactosaminyl, D-galactosyl
08	unknown
09	N-acetyl-D-glucosaminyl, N-acetyl-D-galactosaminyl
010	β-N-acetyl-D-galactosaminyl

N-acety-D-galactosaminyl may be present in the determinant.

The sugar residues which may be present in the individual antigenic determinants of O-antigens (O1 to O10) are summarized in Table 3.

3. *Examination of the cross reaction between dextran and O-antigens*

α-Methyl-D-glucoside strongly inhibited the O3-anti O3 and O4-anti O4 systems, so cross reactions between these O-antigens and dextrans, which are solely composed of α-glucosyl linkages, were examined by the ring test. The reactions examined were that between O3 or O4 antigen and anti dextrans and that between dextrans and anti O3 or anti O4 serum.

TABLE 4. *Examination of cross reactions between dextrans and O-antigens*

Antiserum	Antigen			
	03	04	Dextran B 1299	Dextran N 4
153 D (anti dextran)	−	trace	+	+
154 D (anti dextran)	−	−	+	+
155 D (anti dextran)	−	trace	+	+
156 D (anti dextran)	−	−	+	+
157 V (anti 03)	+	−	−	−
82 V (anti 04)	−	+	−	−

As shown in Table 4, there was no significant cross reaction between dextrans and O3 or O4 antigen. Dextrans contain α-linked glucoses as the immunodominant group (Kabat, 1968). Antigens O3 and O4 also have immunodominant α-linked glucosyl groups. The lack of a cross reaction may be because the α-glucosyl group is linked to a different position of glucose or to different constituents attached to the dominant groups.

DISCUSSION

This study was a screening experiment on the inhibitory effects on the precipitin reaction of O-antigens of various sugars and their derivatives of known structure. Generally an inhibition of about 20% was considred significant, since experimental errors may be about 10 percent in this kind of experiment. When a small amount of a compound caused about 20% inhibition while a larger amount caused much less inhibition the compound was not regarded as inhibitory.

The inhibitory effects of the inhibitors tested were rather low except for that of α-methyl-D-glucoside in the O3 and O4 systems. This may be because the structures of inhibitory sugars have only a weak effect on binding with antibodies, or because there may be some other unrelated determinant and its antibodies present in large quantities in the antisera tested. As yet unidentified minor constituents may also be important immunodominants.

It would be interesting to see whether α-phenyl-D-galactoside inhibits the O2 system, in which two galactosyl oligosaccharides caused some inhibition, but unfortunately this could not be tested because this compound was poorly soluble in water.

α-Methyl-glucoside and α-methyl-N-acetyl-D-glucosaminide separately caused 62% and 43% inhibition of the O4 system. It will be interesting to determine their effect in combination.

In this study the inhibitory effects of sugars and their derivatives of known structure were

tested. Cross-reactions were also examined, to obtain information on some partial structures of the antigenic determinants of the O-antigens of *Vibrio parahaemolyticus*. However, it is obviously important to obtain antigenic determinants by partial degradations of the antigens and also to see the contributions of minor components to the determinants.

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