Characteristics of the Lipopolysaccharide from *Pasteurella piscicida**

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Chemical analysis and toxicity assay of *Pasteurella piscicida* lipopolysaccharide (LPS) preparations used for immunization of yellowtail (*Seriola quinqueradiata*) were performed. LPS fractions were obtained by the phenol-water (P-LPS) or phenol-chloroform-petroleum ether (PCP-LPS) methods followed by purification with Cetavlon or ultracentrifugation, respectively. P-LPS was composed of 0.87% protein, 24.0% sugar and 36.0% fatty acid. Whereas PCP-LPS of 0.34% protein, 18.0% sugar and 34.2% fatty acid. The monosaccharide of P-LPS and PCP-LPS was respectively: 30.6% and 16.5% hexose; 3.8% and 2.1% heptose; 2.2% and 3.6% pentose; 2.2% and 3.2% 6-deoxyhexose; 3.2% and 2.6% 2-keto-3-deoxyoctonate (KDO); and 2.8% and 2.4% hexosamine. The fatty acids were analyzed by gas-liquid chromatography (GLC) as their methyl esters. Their identities were confirmed by mass spectrometry. Those analyses showed that the principal constituents were: lauric acid, 3-hydroxy lauric acid, myristic acid and palmitic acid. The acute toxicity assay using mice injected i.p. showed that the P-LPS preparation was more toxic than PCP-LPS. The *Limulus* amoebocyte assay did not show differences between the two preparations.

Pseudotuberculosis is one of the most important diseases of cultured yellowtail (*Seriola quinqueradiata*) in Japan. *Pasteurella piscicida* is a fish pathogen which causes a systemic disease characterized by grayish white lesions particularly at spleen and kidney (SIMIDU and EGUSA, 1972; KUSUDA and YAMAOKA, 1972). Attempts have been made to prepare vaccines against pseudotuberculosis (FUKUDA and KUSUDA, 1981; KUSUDA and HAMAGUCHI, 1987, 1988; KUSUDA *et al.*, 1988), and a preparation of *P. piscicida* lipopolysaccharide (LPS) was shown to give good protection to an experimental infection (FUKUDA and KUSUDA, 1985).

Depending on the disease, discussed is the possibility of application of bacterial LPSs as useful vaccines. Moreover, bacterial LPSs play

an important role as antigen which could induce both humoral and cellular immune response of fish. Production of specific antibody to LPS was demonstrated in many fishes and also in yellowtail immunized with a *P. piscicida* LPS preparation (FUKUDA and KUSUDA, 1985). Activation of phagocytosis after immunization with bacterial LPS was demonstrated in other fish as eel (SALATI and KUSUDA, 1986a; SALATI *et al.*, 1987). And only recently activation of phagocytic cells was showed after immersion of yellowtail in a *P. piscicida* LPS (KAWAHARA and KUSUDA, 1988). In the present study, *P. piscicida* LPS was analyzed for chemical composition, lethal toxicity and *Limulus* amoebocyte-gelating activity.

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Materials and Methods

Microorganism

Pasteurella piscicida strain U-3 was used, which was originally isolated from the kidney of a diseased yellowtail from Tosa-Shi, Kochi Prefecture, Japan, in June 1979.

Preparation of LPSs and hydrolysates

Strain U-3 was cultured in Todd-Hewitt broth (Difco) containing 2% NaCl at 25°C for 48 hours, then centrifuged. The pellet was washed by centrifugation with distilled water. LPS was extracted from the pellet by the Westphal's phenol-water method (WESTPHAL and JANN, 1965), followed by purification with cetyltrimethylammonium bromide (Cetavlon) to afford P-LPS. Otherwise, it was extracted by the Galanos phenol-chloroform-petroleum ether method (GALANOS *et al.*, 1969), followed by ultracentrifugation at $100,000\times g$ for 4 hours to afford PCP-LPS. Both LPSs were lyophilized and stored at 5°C until used. For degradation of the P-LPS, mild acid hydrolysis was carried out and lipid A was separated as a sediment, with the polysaccharide (PS) fraction in the supernatant as previously described (SALATI *et al.*, 1984). Alkali-PS was obtained by hydrolysis of P-LPS with NaOH 0.25 N (50°C, 5 hours), followed by acidification with acetic acid and extraction with chloroform. These PS preparations were also lyophilized and stored at 5°C until used. *Salmonella minnesota* Re 595 LPS was obtained from List Biological Laboratories, INC. (Campbell, California, USA).

Analytical methods

Protein was determined by the method of LOWRY *et al.* (1951). Sugar was determined using the phenol-sulfuric acid method (DUBOIS *et al.*, 1956). For determination of 2-keto-3-deoxyoctonate (KDO), the periodate-thiobarbituric acid method was used (DRÖGE *et al.*, 1970). Pentose was determined with the cysteine-sulfuric acid method (DISCHE, 1949); hexose with the cysteine-sulfuric acid method (DISCHE *et al.*, 1949); heptose with a modification of the Osborn method (OSBORN, 1963) and 6-deoxyhexose with the cysteine sulfuric acid method

(DISCHE and SHETTLES, 1948). Hexosamine was determined by the Elson-Morgan method (BLIX, 1948). The quantity of fatty acids was determined enzymically using a Non-Esterified Fatty Acid (NEFA)-C Test (Wako Pure Chemical Ind., Osaka, Japan). The components of the fatty acids were identified using gas chromatography. Alkali hydrolysis of the LPSs was made as previously described (SALATI and KUSUDA, 1985). Gas-liquid chromatography/mass spectrometry (GLC-MS) of the fatty acid methyl esters was performed on a Shimadzu GCMS-QP 1000 instrument, using a 1% SE-30 column, source temperature at 250°C, ionizing voltage at 70 eV and helium as carrier gas.

Lethality test

Male 5-week-old ICR mice were injected intraperitoneally with 10 mg of each preparation per kg. Mortality was recorded after 7 days and LD₅₀ was determined by the method of Litchfield and Wilcoxon (1949).

Limulus assay

Limulus Amoebocyte Lysate (LAL) assay was performed using a *Limulus* HS-Single Test (Wako Pure Chemical Ind., Osaka, Japan). The preparations examined were: P-LPS, PCP-LPS, and PSs of *P. piscicida* strain U-3 and LPS of *S. minnesota* strain Re 595 as positive control.

Results

The chemical analysis of the two LPSs from *P. piscicida* strain U-3 is summarized in Table 1. P-LPS and PCP-LPS contained respectively: 0.87 and 0.34% protein, 24.0 and 18.0% sugar, 36.0 and 34.2% fatty acid, 3.2 and 2.6% KDO, 2.2 and 3.6% pentose, 30.6 and 16.5% hexose, 3.8 and 2.1% heptose, 2.2 and 3.2% 6-deoxyhexose, 2.8 and 2.4% hexosamine.

As much as one third of total weight was accounted for fatty acids (Table 1). In gas chromatography, the fatty acid methyl esters from P-LPS elicited 6 peaks (Fig. 1). Peaks 1, 4, and 6 were identified as 12:0, 14:0, and 16:0, respectively. Peaks 2, and 3 were respectively identified as 12:1, and a 3-hydroxy fatty acid (3-OH 12:0). Peak 5 was judged to

Table 1. Chemical analysis of two types of lipopolysaccharide obtained from *Pasteurella piscicida* strain U-3

Constituents	P-LPS (%)	PCP-LPS (%)
Protein	0.87	0.34
Sugar	24.0	18.0
Fatty acid	36.0	34.2
Monosaccharide:		
KDO	3.2	2.6
Pentose	2.2	3.6
Hexose	30.6	16.5
Heptose	3.8	2.2
6-Deoxyhexose	2.2	3.2
Hexosamine	2.8	2.4

Fig. 1. Gas-chromatogram of fatty acids from *Pasteurella piscicida* P-LPS.

be an unsaturated fatty acid of 16°C (mass spectra not showed).

The acute toxicity of the LPSs and hydrolysates preparations is shown in Table 2. P-LPS was more toxic than PCP-LPS. PS preparations from P-LPS were found to be less

Table 2. Acute toxicity in mice injected i.p. with P-LPS, PCP-LPS, PS, and alkali PS obtained from *Pasteurella piscicida* U-3

Preparations	LD ₅₀ (mg/kg)
P-LPS	25.6–34.1 (30.5) *
PS	>100
alkali PS	>100
PCP-LPS	74.1–89.1 (81.6)

* Figures in parentheses indicate a mean of values from a group of five mice.

Table 3. *Limulus* Amoebocyte Lysate (LAL) activity of P-LPS, PCP-LPS, PS, and alkali PS from *Pasteurella piscicida* U-3 and LPS from *Salmonella minnesota* Re 595

Preparations	LAL activity (μg/ml saline)
U-3 P-LPS	10 ⁻⁴
U-3 PS	10 ⁻¹
U-3 alkali PS	10 ⁻²
U-3 PCP-LPS	10 ⁻⁴
Re 595 LPS	10 ⁻⁵

toxic. Results of the *Limulus* amoebocyte gelation assay are shown in Table 3. *S. minnesota* LPS, which was used as a reference, was positive with a dose of 10⁻⁵ μg/ml; while both *P. piscicida* LPS preparations were less active: one tenth of the *S. minnesota* LPS activity. The PS preparations were the less active.

Discussion

The damages caused by *P. piscicida* infection of cultured yellowtail in Japan stress the necessity of protective vaccine. One of the substances which seems to activate the phagocytic cells (KAWAHARA and KUSUDA, 1988) and which showed protection to experimental infection (FUKUDA and KUSUDA, 1985) was LPS extracted from *P. piscicida* cells. In this study, the composition and some characteristics of LPS preparations from *P. piscicida* strain U-3 were examined.

The monosaccharide constituents of LPSs from *P. piscicida* U-3 were similar to those from other *Pasteurella*, confirming a bacteriological relationship with *Enterobacteriaceae* (LÜDERITZ *et al.* 1968). It was indicated that *Pasteurella* LPS is the major antigen responsible

for typing based on gel diffusion precipitin tests (MANNING, 1984). In the sugar composition of LPSs, *P. piscicida* showed similarities to several strains of *P. multocida*, *P. pestis*, *P. septica* and *P. pseudotuberculosis* groups (LÜDERITZ *et al.*, 1968). However, the content of KDO of *P. piscicida* LPSs was more than that of *P. multocida* LPS, while the protein content was less (MANNING, 1984). The difference in hexose contents between P-LPS and PCP-LPS of *P. piscicida* were explained by the different methods employed for extraction (GALANOS *et al.*, 1977).

The principal components of *P. piscicida* lipid A were, as for many other bacteria, lauric acid, myristic acid and palmitic acid. Also 3-hydroxy lauric acid was found in the fatty acids of *P. piscicida*, as for *Pseudomonas aeruginosa*, *P. rubescens*, *Bordetella bronchiseptica* and *Vibrio cholerae* (GALANOS *et al.*, 1977). While, different from *E. coli*, *S. minnesota*, *P. aeruginosa*, *P. putrefaciens* and *Edwardsiella tarda*, 3-hydroxy myristic acid was not detected (TAYLOR *et al.*, 1966; KASAI and NOWOTNY, 1967; FENSOM and GRAY, 1969; WILKINSON and CAUDWELL, 1980; SALATI and KUSUDA, 1985); and different from *V. ordalii*, arachidic acid was not found in *P. piscicida* lipid A (SALATI and KUSUDA, 1986b). The quantity of fatty acids present in P-LPS and PCP-LPS of *P. piscicida* was very high (36.0% and 34.2%, respectively). This quantity was more than that found using the same analysis method for *E. tarda* (8.3%) (SALATI and KUSUDA, 1985) and for *V. ordalii* and *V. anguillarum* (1.4% and 11.3%) (SALATI and KUSUDA, 1986b). However, for *P. pestis* LPS, a quantity of 50% of fatty acids has been reported (LÜDERITZ *et al.*, 1968). This high quantity of fatty acids explains and justifies the practical difficulties to obtain a good *P. piscicida* LPS preparation for subadministration.

The results of the acute toxicity test on mice demonstrated that P-LPS was most toxic, while, the PS preparations did not show remarkable toxicity. This is obvious that the preparations without 3-hydroxy fatty acids were inactive in *in vivo* tests (TANAMOTO *et al.*, 1984). The results of the *Limulus* amoebocyte gelation assay did not show differences between LPS preparations. Both LPSs were,

however, less active than *S. minnesota* LPS used as positive control. Even if routinely used as a general toxicological tests, the validity of the *Limulus* Amoebocyte Lysate (LAL) assay for fish will be discussed, since *P. piscicida* LPS was not toxic in yellowtail (FUKUDA and KUSUDA, 1985).

Finally, from the point of view of vaccinal preparation, the results of this study supported by the above mentioned induction of cell mediated immunity and the absence of toxicity for yellowtail of the LPS from *P. piscicida*, indicate the possibility of practical applications, provided that difficulties on expenses for production are overcome.

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