Homeostasis as Regulated by Activated Macrophage. II. LPS of Plant Origin Other than Wheat Flour and Their Concomitant Bacteria

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In order to seek a macrophage-activating substance, lipopolysaccharide (LPS) of plant origin other than that of wheat flour was surveyed. A large amount of LPS (10—100 μg/g) was detected in *Laminaria japonica* (kelp, 昆布), *Curcuma longa* (turmeric, 鬱根), *Undaria pinnatifida* (若布) and other substaces. Since concomitant bacteria possibly existing in root of farm products can be considered to contribute to LPS of plant origin, a count was taken of bacterial cells both dead and alive. This count revealed that some LPS were derived from concomitant bacteria which had probably come from root. Few concomitant bacterial cells were found in seaweed, while stem-root contained enough bacterial cells. Three predominant bacteria have been isolated and identified; *Pantoea agglomerans*, *Enterobacter cloacae*, and *Serratia ficaria*. These LPSs were purified and their chemical compositions were examined. They are similar to that of *Escherichia coli* except that their molecular sizes are smaller. Since LPS is non-toxic when taken orally or percutaneously, these LPSs may also be advantageous in the cure of intractable diseases.

Keywords wheat flour; lipopolysaccharide; macrophage; *Pantoea agglomerans*; homeostasis; tumor necrosis factor

Introduction

In the preceding paper of this series we described that a new lipopolysaccharide (LPS) from wheat flour (LPSw) has possible therapeutic value for various intractable diseases either by the percutaneous or oral route.[1,2] The present report describes an extensive survey of the existence of such LPS in plants other than wheat.

During the survey we sometimes encountered LPS derived from concomitant bacteria persistent on samples of plant origin. We examined whether LPS of those bacteria are also beneficial for therapeutic use because of their small molecular size.

It may be explained that traditional therapies for various intractable diseases are often based on the oral or percutaneous uptake of plant water-extract by which an adequate amount of LPS for the cure of those diseases can be provided.

Materials and Methods

Materials of Plant Samples All plant samples were used commercially available products.

Dicotylkedoneae: *Sinomenium accutum* (漢防已), *Gynostemma pentaphylum*, *Saposhnikovia divaricata* (防風), *Panax ginseng* (人参), *Raphanus sativus* (radish), *Actinidia polygama*, *Bupleurum scorzoneraefloiurm*, *Bupleurum scorzoneraefloiurm* (紫胡), *Glycyrrhiza glabra* (甘草), *Atractylodes japonica* (白朮), *Pueraria lobata* (葛根), *Eugenia caryopyllata* (glove, 丁字), *Brassia oleracea* (cabbage), *Angelica sinensis* (当帰), *Phellodendron amurense* (黄柏), *Scutellaria baicalensis* (黄芩), *Lycopersicum esculentum* (tomato), *Carcurbita moschata* (pumpkin), *Citrus aurantium* (橙皮), *Uncaria rhynchophylla* (鈎藤鉤), *Illicium vern* (star anise, 八角茴香), *Pinellia ternata* (半夏), *Avena fatua* (oats), *Prunus persica* (peach, 桃仁), *Myristica fragrans* (nutmeg, 肉荳蔻), *Piper nigrum* (pepper), *Capsicum annuum* (red pepper), *Juglans regia* (walnut), *Cinnamomum cassia* (cinnamon, 桂皮), *Paeonia lactiflora* (芍薬), *Rehmannia glutinosa* (地黄), *Persea americana* (avocado), *Eriobotrya japonica* (Japanese medlar), *Vicia faba* (broad bean), *Phaselous angularis* (adzuki bean), *Glycine max* (soy bean).

Monocotyledoneae: *Curcuma longa* (turmeric, 鬱金), *Zingiber mioga*, *Zingiber officinale* (ginger), *Sasa albo-marginata*, *Avena fatus* (oats), *Asparasgus officinallis* (asparagus), *Ophiopogon japonicus* (麦門冬), *Triticum aestivum* (wheat), *Iris sanguinea* (iris), *Coix lachryma-Jobi* (薏苡仁), *Oryza sativa* (rice), *Zea mays* (maize), *Allinum sativum* (garlic).

Other Plants: *Undaria pinnatifida* (若布, 若布芽株), *Laminaria japonica* (昆布), *Cordyceps sinensis* (冬虫夏草), *Grifola frondosa*, *Hizikia fusiforme* (鹿尾菜), *Porphyra tenera* (海苔), *Pholiota nameko*, *Pleurotus ostreatus*, *Auricularia mesenterica*, *Agaricus bisporus*, *Pteridium aquilinum* (bracken), *Lentinus edodes*, *Gelidium amansii* (agar).

Preparation of Test Samples All samples were washed with distilled water, dried up to a constant weight by a heating dryer and vacuum desiccator. After mincing, one gram of powdered sample was suspended in 5 ml of distilled water and heated for 5 h at 60 °C for extraction of LPS. After extraction, the supernatant was obtained by centrifugation (at 2000 × *g* for 20 min).

Measurement of LPS Contents Toxicolor (a kit preparation of conventional Limulus test) and Endospecy (Limulus test-kit without G-factor), which reacts specifically with LPS, were purchased from Seikagaku Co. (Tokyo, Japan). Almost all tests were done with Toxicolor and the final check by Endospecy. LPS content in plant samples was expressed as the weight of *Escherichia coli* (*E. coli*) (μg) contained in 1 g of each sample by using a standard solution of *E. coli* LPS (42 pg/ml). LPS contaminated during the sample preparation was less than 0.05 μg/g.

Isolation and Identification of Concomitant Bacteria in Samples of Plant Origin From the water extract of samples concomitant bacteria were isolated on nutrient agar plates. Most of those bacteria were gram-negative form, and three kinds composed the majority of these colonies. Identification of these 3 species was carried out using an ID test kit, the details of which were described in the preceding report.[2]

Total Bacterial Count in Samples A new method was applied to those samples which involved possibly gram-negative bacteria. Principle of the method is based on the staining of nucleic acid with acridine orange in living or dead bacterial cells.[2]

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of LPS SDS-PAGE was carried out in 1.0 mm-thick 20% acrylamide concentration slab gel according to the method of Schägger and Jagow.[3] Details were described in the preceding paper.[2]

Chemical Analysis of LPS The LPS samples were analyzed for their hexosamine content by the Elson-Morgan method,[4] for 3-deoxy-D-mannoocutulosanic acid (KDO) by the diphenylamine method,[5] and for phosphorus by the method of Chen-Toribara.[6]

Acute Toxicity of LPS Inbred male C3H/He mice were used. Various doses of each LPS were administered intravenously. After 48 h, the number of dead and surviving mice were counted, and LD_{50} values were calculated by the method of Behren Käber.

Ulcer Protection Experimental gastric ulcers in mice were induced by 60 mg/kg of indomethacin (IM) or 50% ethanol. Preventive effect of the LPSs was compared with "percent inhibition" which gives the ratio of ulcer index of the test sample to that of negative control (A) as $(1-A)\times 100\%$. Details will be reported in a separate paper.[7]

Results

LPS in the Plant Kingdom Test samples from many

TABLE I. LPS Content in the Various Plant Samples

No.	Scientific name	C.S.[a]	English name	Crude drug and seaweed name[b]	Part[c]	Limulus activity[d] (μg/g)
1	*Sinomenium accutum*	D		漢防已	Root and stem	600→ 400
2	*Undaria pinnatifida*	O		若布	Thallus	400→ 30
3	*Undaria pinnatifida*	O		若布	Sporophyte	400
4	*Laminaria japonica*	O	Kelp	昆布	Thallus	200
5	*Cordyceps sinensis*	O		冬虫夏草	Whole	200→ 60
6	*Grifola frondosa*	O			Basidiocarp	200→ 200
7	*Hizikia fusiforme*	O		鹿尾菜	Thallus	200→ 30
8	*Curcuma longa*	M	Turmeric	鬱金	Root	200→ 30
9	*Porphyra tenera*	O		海苔	Thallus	200→ 200
10	*Pholiota nameko*	O			Basidiocarp	100→ 8
11	*Pleurotus ostreatus*	O			Basidiocarp	80→ 60
12	*Auricularia mesenterica*	O			Basidiocarp	80
13	*Gynostemma pentaphylum*	D			Stem and leaf	70
14	*Saposhnikovia divaricata*	D		防風	Root	50
15	*Panax ginseng*	D		人参	Root	50→ 20
16	*Raphanus sativus*	D	Radish		Whole	50
17	*Actinidia polygama*	D			Fruit	40
18	*Bupleurum scorzoneraefloiurm*	D		柴胡	Root	40
19	*Zingiber mioga*	M			Flower	40
20	*Glycyrrhiza glabra*	D		甘草	Root	30→ 3
21	*Atractylodes japonica*	D		白朮	Rizome	30
22	*Pueraria lobata*	D		葛根	Root	20→ 5
23	*Eugenia caryopyllata*	D	Clove	丁子	Bud	20
24	*Zingiber officinale*	M	Ginger	生姜	Root	20
25	*Sasa albo-marginata*	M			Leaf	20
26	*Agaricus bisporus*	O	Mushroom		Basidiocarp	20→ 0.6
27	*Laminaria japonica*	O		昆布	Root	10→ 10
28	*Brassia oleracea*	D	Cabbage		Leaf	10
29	*Angelica sinensis*	D		当帰	Root	10
30	*Phellodendron amurense*	D		黄柏	Bark	10
31	*Scutellaria baicalensis*	D		黄芩	Root	10
32	*Lycopersicum esculentum*	D	Tomato		Fruit	10→ 0.8
33	*Carcurbita moschata*	D	Pumpkin		Seed	10→ 0.03
34	*Pteridium aquilinum*	O	Bracken		Thallus	10
35	*Citrus aurantium*	D		橙皮	Pericarp	8
36	*Uncaria rhynchophylla*	D		釣藤鉤	Stem	7
37	*Illicium verum*	D	Star anise	八角茴香	Fruit	6→ 0.9
38	*Pinellia ternata*	D		半夏	Solid bulb	6
39	*Avena fatua*	M	Oats		Seed	6→ 0.9
40	*Lentinus edodes*	O			Basidiocarp	6→ 0.6
41	*Prunus persica*	D	Peach	桃仁	Seed	5
42	*Asparagus officinallis*	M	Asparagus		Stem	5
43	*Ophiopogon japonicus*	M		麦門冬	Root	4
44	*Triticum aestivum*	M	Wheat		Seed	4→ 0.2
45	*Iris sanguinea*	M	Iris		Seed	3→ 0.02
46	*Gelidium amansii*	O	Agar	天草	Thallus	3
47	*Myristica fragrans*	D	Nutmeg	肉荳蔲	Seed	2
48	*Piper nigrum*	D	Pepper	胡椒	Fruit	2
49	*Capsicum annuum*	D	Red pepper		Fruit	2
50	*Juglans regia*	D	Walnut	胡桃仁	Seed	2
51	*Cinnamomum cassia*	D	Cinnammon	桂皮	Bark	2
52	*Paeonia lactiflora*	D		芍薬	Root	2
53	*Coix lachryma-Jobi*	M		薏苡仁	Seed	2
54	*Rehmannia glutinosa*	D		地黄	Root	1
55	*Persea americana*	D	Avocado		Seed	1→0.003
56	*Oryza sativa*	M	Rice		Seed	1
57	*Eriobotrya japonica*	D	Japanese medlar		Seed	0.8→0.001
58	*Vicia faba*	D	Broad bean		Seed	0.8
59	*Phaselous angularis*	D	Adzuki bean		Seed	0.5
60	*Glycine max*	D	Soy bean		Seed	0.2→ 0.02
61	*Zea mays*	M	Maize		Seed	0.2
62	*Allinum sativum*	M	Garlic		Bulb	0.07→0.004

a) Classification of samples: D, dicotyledoneae; M, monocotyledoneae; O, other plants (including algae and mushroom). *b*) Chinese character of each sample. *c*) Name of used parts. *d*) Underlined values indicate the Limulus activity of samples assayed by Endospecy kit.

plant origins have been surveyed by Limulus reaction and the content of Limulus positive substances in each sample are expressed as μg/g of sample weight. Primary screening was done with a Toxicolor kit which contains G-factor, indicating the content of LPS plus β-1,3 glucan in the samples. Some samples showing more than 10 μg/g were

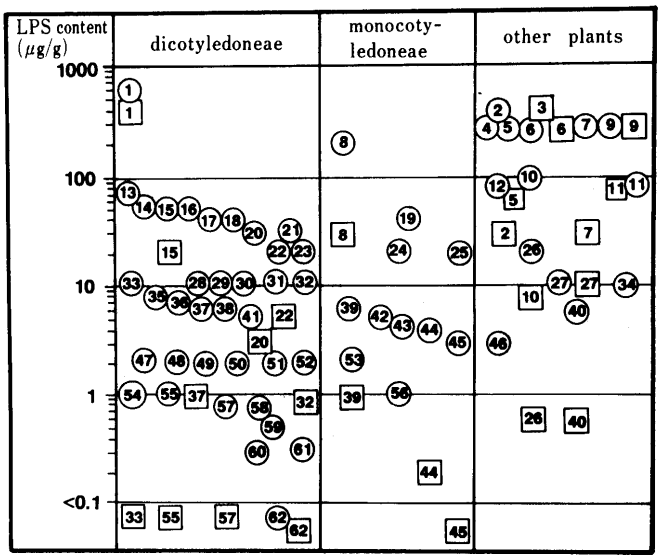

Fig. 1. LPS Contents in Plants

Each number in ○ and □ correspond to the sample number in Table I. ○, limulus activity of samples were assayed by Toxicolor kit; □, limulus activity of samples were assayed by Endospecy kit.

TABLE II. Number of Bacterial Cells Dead and Alive in Seaweed and Root which Contained a High Amount of LPS

Type	Sample	LPS content (μg/g)	No. of bacterial cell ($\times 10^8$ cells/g)	Calculated[a] LPS (μg/g)	Origin[b] of LPS
Seaweed					
	Laminaria japonica	14	0.2	0.6	P
	Hizikia fusiforme	32	0.8	2.4	P
	Undaria pinnatifida	370	0.07	0.2	P
	Porphyra tenera	150	59	180	B
Plant sample in soil					
	Sinomenium accutum	400	180	540	B
	Curcuma longa	20	5	15	B
	Panax ginseng	5.3	25	75	B
	Pueraria lobata	3	0.5	1.5	B
	Cordyceps sinensis	20	3	9	B

a) We calculated the maximum LPS contents from the number of bacterial cells living and dead in plant samples by the ratio: 3 μg of LPS/10^8 bacterial cell. *b*) If the calculated LPS was under 10% the detected LPS weight, LPS in the plant was considered to have derived from the plant itself (P). If over 10%, LPS was considered to have derived from bacterial origin (B).

retested with the Endospecy kit which contains no G-factor, indicating the real content of LPS in the samples.

The results are shown in Table I and Fig. 1. A large amount of LPS was detected in *Laminaria japonica* (kelp, 昆布) and other plants. The difference in values obtained between the Toxicolor and Endospecy kit indicated the content of β-1,3 glucan in samples.

Concomitant Bacteria in Samples of Plant Origin As shown in Table I, samples showing a high content of LPS are stem-root, seeds and others, except seaweed, which have a close connection with the soil. Actually, quite a large number of bacteria, both living and dead were isolated and counted from stem-root in *Sinomenium accutum* (漢防已), *Panax ginseng* (人参), *Curcuma longa* (Turmeric, 鬱金), *etc.* (Table II). There is no bacteria in seaweed except *Porphyra teneta* (海苔). These results suggest that seaweed has its own LPS which is not derived from concomitant bacterial cells, while major LPSs in stem-root are derived from bacterial cells in the soil.

TABLE III. Phenotypic Characteristics of Gram-Negative Bacteria in Wheat Flour

Characteristics	*Pantoea agglomerans*	*Serratia ficaria*	*Enterobacter cloacae*
Production of yellow pigment	+	−	−
Growth at 37 °C	+	+	+
Motility	−	+	+
Indole production	−	−	−
Voges-Proskauer reaction	+	+	+
Nitrate reduction			
H_2S production	−	−	−
Hydrolysis of esculin	+	+	+
Gas from D-glucose	−	+	+
Lysine decarboxylase	−	−	−
Arginine dihydrolase	−	−	+
Ornithine decarboxylase	−	−	+
Phenylalanine deaminase	−	−	−
Deoxyribonuclease	−	−	−
Oxidase (Kovacs')	−	−	−
β-Galactosidase	+	+	+
Urease	−	−	−
Utilization of:			
Citrate (Simmons')	+	+	+
Malonate	+	−	+
Acid production from:			
Inositol	−	−	−
Sucrose	+	+	+
Adonitol	−	−	−
Raffinose	−	+	+
L-Arabinose	+	+	+
D-Mannitol	+	+	+
L-Rhamnose	+	+	+
D-Sorbitol	−	+	+

+, reaction present in at least 90% of the strains within 24 to 48 h. −, reaction absent in at least 90% of the strains after 2 d.

TABLE IV. Quantitative Analysis, Acute Toxicity and Anti-ulcer Activity of Purified LPS from Gram-Negative Bacteria Found in Wheat Flour

LPS source		*E. coli*	*P. aggl.*	*S. fic.*	*E. cloac.*
Chemical analysis[a]	P[b]	2	2	2.5	1.5
	KDO[c]	0.8	2	2	1
	Hexosamine[d]	8	5.5	9	7
LD_{50}[e] mg/kg		8.4	5.0	4.2	5.0
Percent ulcer protection[f]	i.v.	44	100	84	36
	p.o.	15	27	ND	ND

a) Molecular weight of these samples was assumed to be 5 kDa. *b*) Chen–Toribara method. *c*) 3-Deoxy-D-manno-octulosonic acid; diphenylamine method. *d*) Elson–Morgan method. *e*) LD_{50} was calculated by the method of Behrens Käber. *f*) Mice ($n=5$) were fasted for 24 h, then LPSs were administered intravenously (1 μg/mouse) or orally (200 μg/mouse), and after 1 h, 200 μl of 50% ethanol was administered orally. One hour later, gastrectomized and formalin fixed stomach samples were photographed and the length of the ulcer was measured. Preventive effect of samples was compared with inhibition rate of the ulcer length. ND, not done.

Three species of bacteria were isolated from wheat flour, wheat bran, and oats: *Pantoea agglomerans* (*P. aggl.*), *Enterobacter cloacae* (*E. cloac.*) and *Serratia ficaria* (*S. fic.*). *P. aggl.* is the most remarkable, since it acounts for 40—70% of all living bacteria in wheat bran and wheat flour and is also persistantly isolated from all kinds of wheat flour produced in districts as different as, Canada, U.S.A. (three areas), Australia and Japan.[2)]

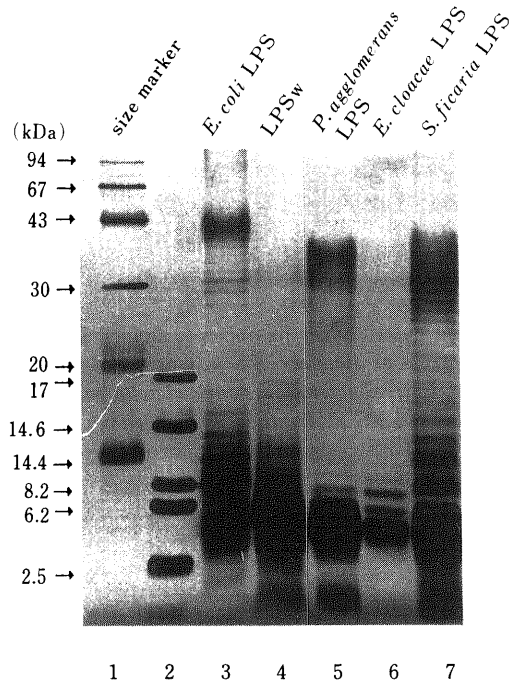

Fig. 2. Profile of SDS–PAGE of Purified LPS of Gram-Negative Bacteria from Wheat Flour

Lane 1, 2: size marker, indicates molecular size (kDa). Lane 3: 20 μg of *E. coli* LPS. Lane 4: 20 μg of LPSw. Lane 5: 20 μg of *P. agglomerans* LPS. Lane 6: 20 μg of *E. cloacae* LPS. Lane 7: 20 μg of *S. ficaria* LPS.

P. aggl. is a species of gram-negative soil bacteria, quite recently assigned as such,[8] ubiquitously distributed, especially in cotton-seed[9] and wheat,[9] and contributes to the growth of plant by nitrogen fixation[10] and also by release of phosphorus.[11] Results of the ID-kit of this bacterium are shown in Table III.

LPS from Isolated Bacteria LPS of *P. aggl.*, *E. cloac.*, and *S. fic*, were extracted by Westphal's method.[12] These LPSs were purified to more than 95% by conventional methods.[2] As a control, LPS from *E. coli* was compared. Table IV shows the chemical composition, the LD_{50} and the anti-ulcer activity of those LPSs. Figure 2 shows their pattern of gel electrophoresis. In general, the pattern of LPS on SDS–PAGE is multi-band because of its heterogenity of sugar chain such as *E. coli* LPS in Fig. 2. It is to be noted that all the LPSs have molecular size of *ca.* 5 kilodaltons (Da), smaller than that of *E. coli* LPS.

Discussion

Since Coley's findings in 1946,[13] bacterial LPS has been recognized as a potentially good drug, however, its benefits in therapeutic use were unclear. As described in our previous report,[1,2] LPSw with its small molecular size (5 kDa) is available for clinical use, especially for the cure of various diseases by possibly inducing the precursor of tumor necrosis factor (TNF) in macrophage of experimental animals as well as in human.[14] In order to seek LPSs of other plant origin, we made extensive survey in many species of plants with the Limulus reaction kit. As shown in Table I, *Laminaria japonica* (kelp) and other seaweed themselves contain a large amount of LPS, since few concomitant bacteria were found. However, those LPSs cannot be easily isolated because of its attendant highly viscous polysaccharide. Other troubles may occur in the case of mushroom and other plants, and cost of those materials is another problem.

A large amount of LPS is commonly found in samples of stem-root or root, as well as of seeds. As stated, we isolated 3 species of gram-negative bacteria, *Pantoea agglomerans*, *Enterobacter cloacae* and *Serratia ficaria* from wheat. LPS of all these bacteria belong to a group with a small molecular size of *ca.* 5 kDa, whereas LPS of *E. coli* is of a large molecular size, 5—50 kDa (Fig. 2). In the test for anti-ulcer activity (Table VI), LPS of *Pantoea agglomerans* showed activity even by the oral route. Details of the correlation of molecular weight and anti-ulcer activity will be described later.[12]

Recently Swain *et al.* reported the merked effect of oat bran against hyperlipidemia.[15] As will be described in a later report of this series,[16] LPSw shows a marked antihyperlipidemic effect in rabbit (WHHL). We are not sure whether oats bran has its own LPS or not, but the bran which Swain and his colleagues used contains a large quantity of LPS of small molecular size enough to activate macrophage, regardless of whether its origin is oats or concomitant bacteria.

Similarly, LPS contained in preparations of Chinese drugs derived primarily from stem-root such as *Sinomenium accutum* (漢防已), *Cordyceps sinensis* (冬虫夏草), *Curcuma longa* (鬱金), *Saposhnikovia divaricata* (防風), *Panax ginseng* (人参), *et al.* may be rich enough to induce activated macrophage (Tables I, II), regardless of its origin, from plant or concomitant bacteria. They may also be effective in activating macrophage. Details will be discussed elsewhere.

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