B-Lymphocyte-Stimulating Polysaccharide from Mushroom *Phellinus linteus*

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Hot water extract prepared from the mycelial culture of mushroom *Phellinus linteus* stimulated polyclonal antibody production in an *in vitro* culture system. The active fraction PLP was purified from the extract *ca.* 1030-fold by ethanol precipitation followed by DEAE-cellulose and gel permeation chromatography. PLP contained 13.2% (w/w) peptide and 82.5% (w/w) carbohydrate. About 6.8% (w/w) of the total carbohydrate was uronic acid. The molecular weight distribution of PLP was found to be nearly homogeneous (153 kDa) in gel permeation HPLC analysis. Neutral sugar composition analysis revealed Ara (7.0%), Xyl (3.7%), Glc (21.1%), Gal (24.1%) and Man (44.2%). Uronic acid was identified as a glucuronic acid by gas chromatography. Ten amino acids were detected and Asp and Glu were the major components. In our assay system, the half-maximal concentration of PLP for B-lymphocyte stimulation was *ca.* 3 μg/ml. Partial acid hydrolysis as well as sodium periodate treatment of PLP decreased the activity significantly, suggesting that both the full molecular size and the sugar moiety were essential. However, proteinase K treatment for up to 48 h did not affect the activity.

Key words *Phellinus linteus*; polysaccharide–protein complex; immuno-stimulator; B-lymphocyte

Nakahara *et al.*[2] reported that some polysaccharides isolated from bamboo leaves, bagasse, were remarkably effective in inhibiting the growth and inducing regression of sarcoma-180 subcutaneously transplanted in mice. This tumor-inhibiting effect was considered to be indirect and host-mediated, and not due to their cytocidal action on tumor cells. Many polysaccharides extracted from Basidiomycetes have been reported to act as biological response modifiers (BRM).[3—5] Among 11 Basidiomycetes including *Ganoderma applanatum* and *Coriolus versicolor*, the hot water extract of *Phellinus linteus*, which belongs to Polyporaceae, was reported to be the most effective against mouse transplanted sarcoma-180 (96.7% inhibition ratio).[2a] This extract showed no cytocidal activity on sarcoma-180. Oh *et al.* have suggested that the immuno-stimulating activity of *P. linteus* might be associated with a functional stimulation of B-lymphocytes.[6] However, chemical studies on the active principle of *P. linteus* have hardly been carried out, partly due to the rarity of this mushroom in nature. Recently, Ko and his colleagues succeeded in establishing a mycelial culture system of *P. linteus*.[7] It was confirmed that the water extract of the cultured mycelium showed activity against sarcoma-180 and B-lymphocytes as potent as that of the natural mushroom.[6,8] These facts led us to purify the active principle from the mycelial culture of *P. linteus* guided by an antibody forming cell (AFC) assay system. This report describes the purification of the active polysaccharide and some of its physico-chemical properties.

Materials and Methods

Materials Mycelial culture of *P. linteus* was carried out according to the reported method.[7] Female (C57BL/6XC3H) F1 (B6C3F1) mice maintained in Korea Research Institute of Bioscience and Biotechnology (Taejon, Korea) were used in all experiments. The 17—20 g mice were used as the source of the spleen cells. Sheep red blood cells (sRBCs) were obtained from Korea Media Co., Ltd. (Seoul, Korea). Guinea pig

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complement and RPMI 1640 were purchased from Gibco BRL (Grand Island, NY, U.S.A.). Lipopolysaccharide (LPS) and Proteinase K (from *Tritirachium album*) were purchased from Sigma.

Colorimetric Assays The sugar and protein contents were determined by the method of Dubois *et al.*[9] and by Lowry's method[10] using glucose (Glc) and bovine serum albumin (BSA) as standards, respectively. Uronic acid content was analyzed by the *m*-hydroxybiphenyl method using glucuronic acid as a standard.[11]

Extraction, Fractionation, and Purification 1) Extraction: Filtered mycelia (1.2 kg) were extracted with 5 l of boiling water for 12 h and the extract was concentrated at 100 °C with a spray-type concentrator.

2) Ethanol Precipitation: The extract (154 g, designated as fr.-1) was suspended in 1 l of distilled water and 4 volumes of ethanol were added. This solution was allowed to settle for 3 d at 4 °C. The clear yellowish supernatant was decanted off from the dark gray residue and the precipitate was centrifuged for 30 min at 4 °C and 5000 × *g* to remove remaining soluble materials. The precipitated pellet was dissolved in water and 80% ethanolic precipitation was repeated three times in the same manner. The supernatants were combined and lyophilized (132 g, fr.-2). The final pellet was dissolved in 400 ml deionized water and dialyzed at 4 °C against water for three days with three exchanges of water. The insoluble material which formed inside the dialysis bag was filtered off through a Whatman GF/D filter and the filtrate was lyophilized (16.5 g, fr.-3). Fraction-3 (15 g) was dissolved in 1 l of water and 1.5 l of ethanol was added to make a 60% ethanolic solution. The solution was centrifuged for 30 min at 4 °C and 10000 × *g*. After removal of ethanol, the supernatant (fr.-4, 7.9 g) and pellet (fr.-5, 5.3 g) were lyophilized.

3) DEAE-Cellulose Chromatography: Two grams of fr.-4 was applied to a DEAE-cellulose column (Merck Art. 3201, 4 × 35 cm) which had been equilibrated with 5 mM sodium phosphate buffer (pH 7.7). The column was initially washed with the equilibration buffer (total *ca.* 400 ml) and every 10 ml fractions was collected. The non-binding fraction which was positive to both phenol-sulfuric acid method and Lowry method was combined and dialyzed (4-I, 450 mg). The bound material was eluted with a linear gradient from 0.1 to 1.0 M NaCl in the same buffer (total *ca.* 800 ml). It was finally washed with 400 ml 1.0 M NaCl in the same buffer. The eluates were combined appropriately and fractionated into 4-II to 4-V (49, 430, 475 and 120 mg, respectively) (Fig. 1).

4) Gel Permeation Chromatography on HW65F: Fraction 4-III (150 mg) was dissolved in 10 ml of water then applied to a gel permeation column [Toyopearl HW65F, Tosoh Co., Ltd., Japan (2.5 × 51 cm); mobile phase, distilled water]. Fractions of 4 ml were collected and aliquots were employed to monitor sugar and peptide contents. The tubes were combined to give fractions 4-III-1 (designated as PLP, 6 mg) to 4-III-4 according to the results of bioassay and elution pattern

(Fig. 2).

Characterization of PLP 1) Gel Permeation HPLC: HPLC conditions were as follows: column, Tosoh GMPW (7.8 × 300 mm); column temp., 40 °C; detection, UV 215 nm and refractive index; mobile phase, 0.1 M NaCl; flow rate, 0.8 ml/min.

2) Complete Acid Hydrolysis and Reduction of Monosaccharides[12,13]: PLP [2 mg in 2 ml of 2 M trifluoroacetic acid (TFA)] was hydrolyzed at 121 °C for 1 h in a sealed tube. The hydrolysate was filtered through a Whatman GF/D filter and the filtrate was evaporated to dryness. Two ml of 1.0 M NH₄OH containing 10 mg of sodium borohydride was added to the residue and the reaction was permitted to continue for 12 h in a refrigerator. After the reaction was completed, remaining sodium borohydride was removed according to the reported method.

3) Separation of Alditols and Aldonic Acid[13]: The dried residue was taken up in 5 ml of distilled water, and this solution was transferred to a test tube containing 10 g of IRA-68 anion exchange resin (Sigma). The suspension was stirred for 30 min at room temperature, then resin was filtered through filter paper. The resin was washed twice with distilled water and the filtrate and washings were combined to be evaporated. The aldonic acids were eluted from the resin by suspending it in 10 ml of 1.0 M HCl and stirring for 1 h at room temperature. The resin was then removed by filtration on a Whatman GF/D filter.

4) Lactonization and Reduction of the Aldonic Acid: The filtrate from the 1.0 M HCl washings was evaporated to dryness. The dried residue was then stored overnight in a vacuum desiccator in the presence of KOH pellets to remove residual HCl. The dried sample was dissolved in 1 ml of 10 mM sodium borate, pH 7.5, and 20 mg of sodium borohydride in 1 ml of the borate buffer was added to the sample. The reduction was permitted to proceed for 12 h in a refrigerator, after which the remaining sodium borohydride was removed as before. The acidified solutions were then evaporated to dryness.

5) Acetylation and Gas Chromatography (GC): Acetylation was carried out in a routine manner. The resulting alditol acetates were analyzed by Varian 3400 GC. GC conditions were as follows: column, fused silica capillary column (Supelco, SP-2330, 0.32 mm i.d. × 30 m); detection, flame ionization detector (FID); detector temperature, 260 °C; injection temperature; 250 °C; column temperature, after 2 min of initial heating at 200 °C, increased at a rate of 4 °C/min to 250 °C and maintained for 10 min; carrier gas, helium at a flow rate of 30 ml/min.

6) Amino Acid Analysis: PLP (1 mg) was dissolved in 200 μl of 5.7 mM HCl and 50 μl solution was transferred to a mini-vial, then evaporated to dryness. The residue was hydrolysed with a Waters Pico-Tag hydrolyzing kit using 5.7 M HCl for 2 h. The hydrolysate was dried again and dissolved in 2 ml of elution buffer, followed by centrifugation to precipitate insoluble materials. After 50-fold dilution, the supernatant (250 μl) was subjected to HPLC analysis. HPLC conditions were as follows: column, Hitachi No. 2617 ion exchange column (4 × 250 mm); elution buffer, sodium citrate buffer (pH 3.2 to 10.5); flow rate, 0.3 ml/min; detection, UV 440 and 570 nm. The contents of amino acids were determined by comparing the retention times and peak heights with those of standard materials.

Modification of PLP 1) Partial Acid Hydrolysis of PLP[14]: PLP (3 mg) in 3 ml of 2 M TFA was divided equally into three sealed tubes then hydrolyzed at 85 °C for 2.5, 5 and 7.5 h, respectively. The reaction mixture was allowed to cool to room temperature, then filtered on a Whatman GF/D filter and evaporated to dryness. One ml of H₂O was added to the residue and the solution was filtered through cotton. Aliquots of the solution (10 μl) were submitted to gel permeation HPLC. The remainder was lyophilized and used for bioassay at a concentration of 10 μg/ml.

2) Proteinase K Hydrolysis of PLP[15]: Two mg of proteinase (ca. 40 unit) in 100 μl of 0.25 M potassium phosphate buffer (pH 7.5) was added to 900 μl of buffer containing 2 mg of PLP. Three tubes were prepared and the mixtures were incubated at 37 °C for 6, 24 and 48 h, respectively. After boiling, the precipitated enzyme was removed from the mixture by centrifugation. The supernatant was filtered through a membrane filter (cut-off range was 10 kDa). The non-filtrate was recovered and lyophilized. Since less than 1% (w/w) of Lowry-positive material was contained in the non-filtrate, the enzyme reaction was thought to have been successful. Ten μg/ml of each enzymatic hydrolysate was bioassayed. Boiled enzyme plus PLP and boiled enzyme were used as controls.

3) Sodium Periodate Treatment of PLP[16]: The treatments used were (i) 2 mg of PLP plus 1 ml of 20 mM sodium periodate (NaIO₄),

incubated for 12 or 24 h, (ii) 2 mg of PLP plus 1 ml of 20 mM NaIO₄, previously exposed to 200 μl of ethylene glycol for 48 h to inactivate the periodate, and (iii) 2 mg of intact PLP. After incubation in sealed vials in an incubator in the dark at 25 °C, 200 μl of ethylene glycol was added to the first and the third treatment groups to inactivate the periodate. After a further 48 h of incubation, the solutions were filtered through a membrane filter and each 10 μg/ml pellet was bioassayed.

In Vitro **Activation and AFC Assay in Lymphocyte** These procedures were performed as described in the previous report.[7,17] Briefly, the spleen cells were suspended in RPMI 1640 with 10% fetal calf serum and adjusted to 5 × 10⁶ cells/ml. The cultures (0.5 ml aliquot/well; 4 replicate wells/treatment group) were located in individual wells of a 48-well cluster plate (Costar) and samples or LPS (25 μg/ml, purchased from Sigma) were added to them. For *in vitro* stimulation, the plates were incubated with rocking (8—10 rocks/min) at 37 °C in Bellco (Bellco Biotech., Vineland, NJ, U.S.A.) stainless-steel tissue culture boxes in an atmosphere of 10% CO₂, 7% O₂ and 83% N₂ at 4—5 psi. The polyclonal antibody response was measured on day two after inoculation. AFCs against TNP (trinitrophenyl)-haptenated sRBC were enumerated using a modified Jerne plaque assay and the cell number was counted by using a hemacytometer.[7] The number of antibody-forming cells was considered as an index of activity. The specific activity of each fraction was calculated as follows. The amounts of samples required to give the same activity as 25 μg/ml LPS were calculated from the dose-activity curves and then each activity was normalized to that of fr.-1 taken as 1. This can be summarized by the quation: specific activity = the amount (mg) of fr.-1 required to produce as many AFCs as 25 μg/ml LPS (10.3 mg of fr.-1 is needed by calculation)/the amount (mg) of samples required to produce as many AFCs as LPS. Since the activity of LPS was almost saturated at 25 μg/ml, this concentration was used for all experiments as a positive control.

Results and Discussion

On the basis of their solubility in aqueous EtOH, four fractions (fr.-2 to 5) were obtained from hot water extract of *P. linteus* (fr.-1). Samples containing 0.1 to 1.0 mg/ml of each fraction were bioassayed to compare their immuno-stimulating activities. The relative activity of fr.-4 was 91% of the positive control LPS, taken as 100%, while the others showed values of 22 to 68% (data not shown), so fr.-4 was considered as the active fraction to be employed for further purification. DEAE-cellulose chromatography of fr.-4 afforded a non-binding fraction (4-I, *ca.* 30% of fr.-4 by weight) and four NaCl-eluted fractions (4-II to 4-V) (Fig. 1). The B-lymphocyte-stimulating activity of each DEAE-cellulose fraction (0.01 to 1.00 mg/ml) was measured. The main activity was found in 4-III, which contained 32.4% (w/w) of carbohydrates and 45.9% (w/w) of Lowry-positive materials. The uronic acid content was 5.2% (w/w) of total detected carbohydrate (Table 1). Further purification of 4-III was attempted by gel permeation chromatography using Toyopearl HW65F. Fifty tubes were collected and 10 μg/ml of each was submitted to bioassay. Near void fraction (tube number 3 to 10) showed the highest B-cell-stimulating activity. On the basis of this result and the elution pattern (Fig. 2), the eluates were separated into four fractions, namely 4-III-1 to 4-III-4. The most active 4-III-1 was designated as PLP. The half-maximal concentration of PLP for B-lymphocyte-stimulating activity was *ca.* 3 μg/ml (Fig. 3). The specific activity of the active fractions is presented in Table 1.

PLP showed a relatively homogeneous molecular weight distribution on gel permeation HPLC (Fig. 4). Its molecular weight was estimated to be 153 kDa from the retention time on HPLC compared with those of standard
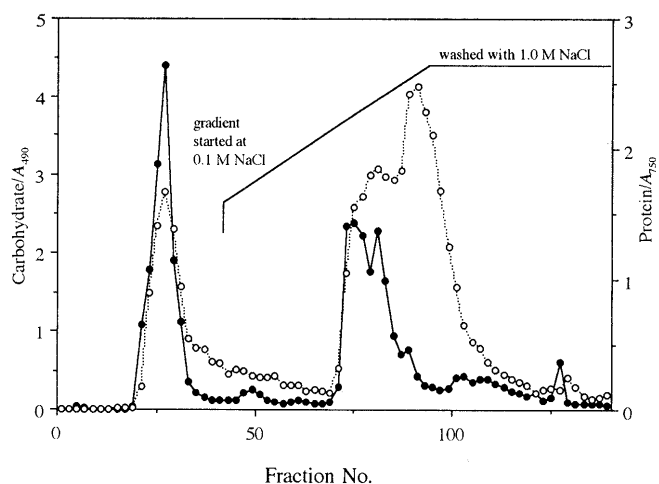
Fig. 1. Chromatogram of Fraction-4 on a DEAE Cellulose Column

Each fraction was monitored by the phenol-sulfuric acid method at A_{490} (—●—) and Lowry's method at A_{750} (---○---). The gradient was started after washing with 400 ml of equilibration buffer. Fraction numbers 21—33, 34—69, 70—87, 88—111 and 112—137 were collected, dialyzed, lyophilized and designated as 4-I, 4-II, 4-III, 4-IV and 4-V, respectively. For details see Materials and Methods.
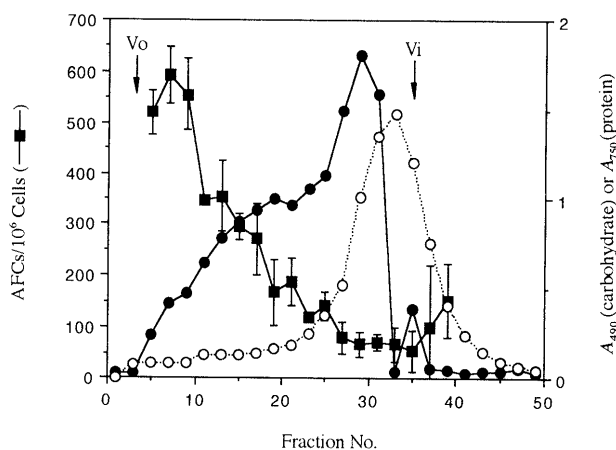

Fig. 2. Elution Profile of 4-III on Gel Permeation Chromatography

Each fraction was monitored by the phenol-sulfuric acid method (—●—) and by Lowry's method (---○---). Closed squares (—■—) indicate the number of antibody-forming cells/10^6 cells produced by treatment with 10 μg/ml of each fraction. According to the results of bioassay and elution pattern, tubes 3—10, 11—23, 24—33 and 34—43 were combined to give fractions 4-III-1 (PLP) to 4-III-4, respectively.

Fig. 3. Dose-Dependent B-Lymphocyte-Stimulating Activity of PLP

Relative Intensity

Fig. 4. Gel Permeation HPLC Chromatogram of PLP

The left trace shows a chromatogram under UV 215 nm and the right one is detected under RI. For experimental conditions, see Materials and Methods.

Table 1. Specific Activity and Contents of Carbohydrate, Protein and Uronic Acid of Active Fractions Purified from *P. linteus*

Sample	Specific activity[a]	Carbohydrate[b]	Protein[b]	Uronic acid[b]
Fr.-1	1	100.8	250.1	—
Fr.-4	8	331.8	747.4	—
4-III	34	323.5	458.9	52.3
PLP	1030	824.6	131.5	68.0

a) For the evaluation of specific activity, see Materials and Methods. b) Presented as μg/mg and determined by the phenol-sulfuric acid, Lowry's, and the m-hydroxybiphenyl methods, respectively.

pullulan molecular markers (Showa Denko, Japan). PLP contained 13.2% (w/w) protein and 82.5% (w/w) carbohydrate when determined by Lowry's method and the phenol-sulfuric acid method, respectively. About 6.8% (w/w) of the carbohydrate was found to be uronic acid. This was identified as a glucuronic acid by GC analysis of the corresponding alditol acetate. The IR spectrum showed hydroxyl absorptions at 3200—3400 cm^{-1} and a carbonyl at 1645 cm^{-1} which was supposed to be due to intermolecular hydrogen-bonded carboxylic acid (the spectrum was recorded on a JEOL JIR-RFX-3001 instrument in a KBr disk). After hydrolysis of PLP, liberated monosaccharides were derivatized to their corresponding alditol acetates. The presence of galactose (Gal), glucose (Glc), mannose (Man), arabinose (Ara), and xylose (Xyl) were confirmed by GC as neutral sugars. Man was predominant (Table 2). Due to the poor solubility in NMR solvents (D_2O and aqueous NaOD
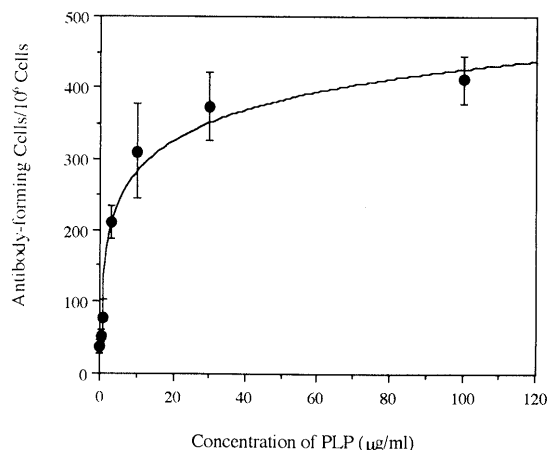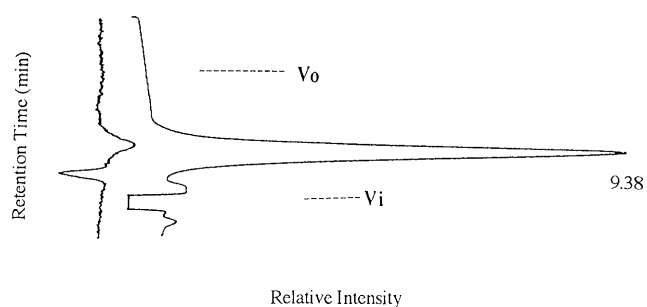
solution) and diversity of component monosaccharides no informative resonance signals were detected in an NMR experiment. To determine the amino acid composition and contents, the HCl-hydrolysate of PLP was analyzed by HPLC. Ten kinds of amino acids were detected and Glu and Asp comprised 37 molar % of the total detected amino acids (Table 3). From these observations, B-lymphocyte-stimulating PLP was characterized as a heteroglycan-protein complex containing glucuronic acid. There was no direct evidence to show whether PLP was a glyco-peptide or a mixture of protein and carbohydrate although gel permeation HPLC showed a nearly homogeneous molecular weight distribution under simultaneous detection with RI and UV_{215}. However, increase in A_{241} during alkaline β-elimination reaction indicated the presence of O-glycosidic linkage[18] in the structure of PLP. The type of glycopeptide linkage of PLP is under

Table 2. Neutral Sugar Composition of PLP

	T value	Content (molar %)
Ara	1.000	7.0
Xyl	1.135	3.7
Man	1.412	44.2
Gal	1.490	24.1
Glc	1.566	21.1

Table 3. Amino Acid Contents of PLP

Amino acid	Content (nmol/mg)
Asp	220.8
Thr	112.0
Ser	166.4
Glu	238.4
Gly	164.8
Ala	120.0
Val	102.4
Ile	16.0
Leu	35.2
Lys	64.0

Table 4. Changes in Immuno-Stimulating Activity of PLP after Treatment with 2 M TFA, NaIO$_4$ and Proteinase K

	Treatment										
	2 M TFA[a]				NaIO$_4$[b]				Proteinase K[c]			
Time (h)	0	2.5	5	7.5	0	12	24	Ctrl	0	6	24	48
Relative activity (%)	100	36	32	32	79	42	47	100	100	129	120	100

a) Zero time means TFA-untreated 10 μg/ml PLP. Activity is presented as relative % to this control. The peak (R_t 9.38) of intact PLP in gel permeation HPLC disappeared completely after 2.5 h of hydrolysis and a new main peak appeared at R_t 11.84 (ca. 10 kDa). This peak did not move significantly on further hydrolysis up to 7.5 h. b) Zero time means deactivated NaIO$_4$ plus 10 μg/ml PLP. Ctrl indicates 10 μg/ml intact PLP. Activity of each treatment is expressed as relative % to that of Ctrl as 100. c) Zero time indicates the boiled enzyme plus 10 μg/ml PLP. Activity is shown as relative % to that of 0 h as 100. The boiled enzyme showed no significant activity.

investigation.

In order to investigate which moieties are essential for the activity, PLP was converted to polyol by NaIO$_4$ treatment or hydrolyzed with proteinase K or 2 M TFA. Partial hydrolysis with 2 M TFA was carried out for 2.5, 5.0 and 7.5 h, respectively. Each hydrolysate showed a shift of the molecular weight distribution to the lower side in gel filtration HPLC. The changes in molecular weight decreased the activity significantly, suggesting that the molecular size is important for the activity (Table 4). Also, chemical modification of the sugar moiety using NaIO$_4$ dramatically decreased the activity (Table 4). More than 50% of the activity was lost after 12 or 24 h of the treatment, indicating that the sugar moiety was necessary for B-cell stimulation. In contrast, although proteinase K treatment liberated more than 99% (w/w) of the peptide (calculated by Lowry's method), the activity of PLP was

almost remained unchanged (or even slightly increased) up to 48 h of hydrolysis (Table 4). Therefore, the peptide moiety seems not to be essential for the activity.

Hirase et al. reported that the sugar structure of an anti-tumor active polysaccharide of Coriolus versicolor was β-D-glucan.[19] Sasaki and Takasuka confirmed the antitumor active polysaccharide as β-1,6-D-glucosyl branched β-1,3-D-glucan.[20] Many polysaccharides from Basidiomycetes have been found to be active against cancer and most of them are β-D-glucans. In plants, acidic polysaccharides (AIP1 and AIP2) of ca. 25 kDa containing mainly Glc (88.3 and 97.9%, respectively) and small amounts of uronic acid (ca. 2% in both cases) have been purified from the leaves of Artemisia species.[21] These active fractions were found to activate the growth of mouse splenocytes in vitro. PLP is a rare example of an immuno-stimulating acidic heteroglycan-peptide complex produced by Basidiomycetes.

References and Notes

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