Purification and Characterization of Three Mitogenic Lectins from the Roots of Pokeweed (Phytolacca americana)

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Three mitogenic lectins, designated PL-A, PL-B, and PL-C, were purified from the roots of pokeweed (Phytolacca americana) using Q-Sepharose column chromatography followed by gel filtration on Sephadex G-75, hydrophobic chromatography on Butyl-Toyopearl, and FPLC on a Mono-Q column. PL-A, PL-B, and PL-C are acidic proteins having isoelectric points of 4.35 and their apparent molecular masses were 22, 48, and 21 kDa by SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol, respectively. The three lectins have similar amino acid compositions rich in half-cystine and similar N-terminal sequences, indicating that they are homologous proteins. Identical sequences of N-terminal regions and six corresponding tryptic peptides in PL-A and PL-B suggested that PL-A may be an N-terminal half fragment of PL-B. Although all of three lectins have mitogenic activities, PL-B is a mitogenic lectin with the most potent hemagglutinating and mitogenic activities, and PL-C has almost no hemagglutinating activity.

Several plant lectins are endowed with a mitogenic property which allows them to stimulate mitosis in resting lymphocytes. Börjesson et al.1,2) have first shown that a lectin from the roots of pokeweed (Phytolacca americana) is mitogenic, and isolated a mitogen with a molecular mass of 32 kDa. Later, Wadada3) and Yokoyama et al.4) have isolated five mitogens (Pa-1-Pa-5) with molecular masses of 19-31 kDa from pokeweed roots, of which Pa-1 was mitogenic for both T- and B-cells unlike concanavalin A and phytohemagglutinin (PHA). These five mitogens differ from each other in molecular structure and biological properties such as cytoagglutination, mitogenicity, and carbohydrate-binding specificity. However, their primary structures and structure-function relationships have not been described and it was expected that an elucidation of the molecular structures of these mitogens will lead to new insights regarding the structure-function relationships in their biological properties.

Since color-free pure mitogenic lectins have not been obtained by the method of Wadada, we isolated three color-free pure mitogenic lectins using a new procedure not including hydroxyapatite chromatography, or trichloroacetic acid or ethanol-presentation, and found some differences in their properties from the other mitogens. Of these lectins, one seemed to be a novel mitogenic lectin with higher molecular mass than the mitogens isolated so far and another one had almost no carbohydrate in the molecule. Furthermore, the molecular masses of our three lectins greatly decreased on SDS-polyacrylamide gel electrophoresis (PAGE) in the presence of 2-mercaptoethanol (2-ME), unlike the mitogens isolated by Wadada, suggesting some differences in their configurations.

Here, we describe the purification and characterization of three mitogenic lectins from the young roots of pokeweed.

Materials and Methods

Materials. Pokeweed roots were harvested throughout the year except October from plants cultivated in the field of Kyushu University and frozen at −20 °C until use. Polyclar AT was purchased from Gokyo Sangyo Co., Q-Sepharose from Pharmacia Fine Chemicals, and Butyl-Toyopearl 650M from Toyo Sol Co. Other reagents were of analytical grade.

Preparation of crude pokeweed lectin. Young pokeweed roots (500 g) with outer bark removed were homogenized in 10 mM sodium phosphate buffer, pH 7.4, or in 1% acetic acid solution with 100 g of polyclar AT. The homogenate was squeezed through twice-folded gauze and centrifuged. The supernatant was saturated with ammonium sulfate and the resulting precipitate was collected by centrifugation as crude pokeweed lectin (PB) or crude pokeweed lectin (AcOH), respectively.

Purification of pokeweed lectin.

Step 1. The crude lectin was dialyzed against 10 mM sodium phosphate buffer, pH 6.0, and put on a Q-Sepharose column (1.0 × 33 cm) previously equilibrated with the same buffer. The adsorbed protein was eluted by a linear gradient of NaCl from 0 to 0.3 M in the same buffer.

Step 2. The fraction having hemagglutinating activity was pooled and saturated with ammonium sulfate. The resulting precipitate was dissolved in a small amount of deionized water and dialyzed against 50 mM sodium borate-HCl buffer, pH 8.0. After removal of the insoluble materials by centrifugation, the supernatant was put on a Sephadex G-75 column (2.0 × 140 cm) and developed with the same buffer.

Step 3. The active fraction obtained by gel filtration was saturated with ammonium sulfate and the resulting precipitate was dialyzed against 4 M NaCl 10 mM sodium phosphate buffer, pH 6.0, and put on a Butyl-Toyopearl 650M column (1.5 × 10 cm). The undesorbed protein was washed out, the adsorbed protein was eluted by a linear gradient of NaCl from 4 to 0 M in 10 mM sodium phosphate buffer, pH 6.0.

Step 4. Final purification of pokeweed lectin was done by an FPLC apparatus (Pharmacia Fine Chemicals, Uppsala, Sweden) with a prepacked Mono-Q column (0.5 × 2.5 cm) in 10 mM sodium acetate buffer, pH 5.5. The protein was eluted by a linear gradient of NaCl from 0 to 0.5 M in the same buffer at a flow rate of 1 ml/min.

SDS-polyacrylamide gel electrophoresis. SDS PAGE was done by the method of Laemmli19) on a 15% acrylamide gel in 0.1 M Tris glycine buffer using a slab type apparatus. Proteins on the gel were stained with 0.25%
Coomassie brilliant blue R-250.

Molecular mass was estimated by SDS-PAGE in the presence of 2-ME according to the method of Weber and Osborn using bovine serum albumin (66kDa), egg white albumin (45kDa), z-chymotrypsinogen (24kDa), &-lactoglobulin (18.4kDa), and egg white lysozyme (14.3kDa) as standard proteins.

Isoelectric point. The isoelectric point of lectin was measured using electrofocusing on a 5% polyacrylamide gel column (0.5 x 10 cm) containing 2% carrier ampholine of pH 2.5-5.0 (Bio-Rad Ltd.) by the method of Svensson.80 The gel was run for 15 h at 200 V and subsequently at 400 V for 1 h at 6.0 C. The gel was sliced 3 mm thick, dipped in deionized water, and the pH value of gel-stein water was measured.

Amino acid composition and amino acid sequence. Lectin was hydrolyzed with constant-boiling HCl containing 0.05% 2-ME in an evacuated sealed tube at 110 C for 24, 48, and 72h. Amino acid analysis was done with a Hitachi 655-A amino acid analyzer. Half-cystine was measured as cysteic acid after performic acid oxidation and tryptophan by the method of Edelhoch.81 Amino acids were sequenced by the DABITC/PITC double coupling method of Change et al.82,83

Carbohydrate content. Neutral carbohydrate was measured by the phenol-sulfuric acid method of Dubois et al.110 and the content was expressed by weight percent as the amount of mannose.

Preparation of reduced and S-carboxymethylated (RCm) lectin. RCm-lectin was prepared principally according to the method of Crestfield et al. After reduction with 2-ME in the presence of 5 M urea at pH 8.6 for 3h, the lectin was carboxymethylated with monoiodoacetic acid for 15min. The reaction mixture was dialyzed against deionized water to remove the reagents, and lyophilized.

Tryptic digestion and separation of peptides. Lyophilized RCm-lectin (2 mg) was dissolved in a small amount of formic acid, dialyzed sufficiently against deionized water, and then digested with 1:50 (w/w) TPCK-trypsin (Sigma Co.) at 37 C and pH 8.0, adjusted with dilute ammonia solution, for 3h. The lyophilized digest was dissolved in 5 mm potassium phosphate buffer, pH 6.0. After centrifugation, the supernatant was subjected to RP-HPLC with a YMC-GEL C4 column (0.46 x 25 cm). The peptides were separated by a linear gradient of MeCN from 0 to 24% in 5 M potassium phosphate buffer, pH 6.0. Further purification of the peptides were done by RP-HPLC using a linear gradient of MeCN from 0 to 42% in 0.1% TFA solution.

Hemagglutination assay. The hemagglutination assay was done using human O type erythrocytes. Trypsin-treated erythrocytes were prepared by incubation of the cells in 0.01% trypsin solution at 37 C for 1h. To 50 μl of two-fold serial dilutions of the lectin in PBS was added 20 μl of a 4% suspension of human erythrocytes, or trypsin-treated erythrocytes in PBS, and mixed well. After allowing this to settle at 37 C for 1h, the agglutination was observed by eye. Hemagglutinating activity was expressed by a minimum concentration (optical density at 280 nm) of lectin necessary to agglutinate the cells.

Cell culture and monitoring of [3H]thymidine incorporation. Human blood lymphocytes from a normal volunteer were isolated by Lymphoprep (Nycomed Pharma AS) density gradient and cultured at a concentration of 5 x 106/ml in RPMI 1640 medium with 15% fetal calf serum, 100 μg/ml streptomycin, and 100 U/ml penicillin in the presence of the indicated concentrations of lectins at 37 C in an atmosphere of air containing 5% CO2 (v/v), and viability was measured by Trypan Blue exclusion.

[Methyl-3H]Thymidtine (0.2μCi) was added to 0.2 ml of 5 x 106/ml human lymphocyte cells during 4h before harvesting the cells at the indicated times. The cells were harvested on the filter paper and washed with PBS. Then, they were fixed with 7% TCA and washed with 99% ethanol. The filter paper containing DNA was dried and the radioactivity was measured.

Results

Purification of pokeweed lectins

Elution profile of the crude lectin (PB) in Q-Sepharose column chromatography at pH 6.0 is shown in Fig. 1.
one band on SDS-PAGE, these lectins are designated pokeweed lectin (PL)-A, PL-B, and PL-C, respectively, from the elution order in Q-Sepharose column chromatography (Fig. 1).

Final purifications of PL-A, PL-B, and PL-C were done by FPLC with a Mono Q-column in 10 mm sodium acetate buffer, pH 5.5 (Fig. 4). The purified PL-A, PL-B, and PL-C gave a single band corresponding to molecular masses of approximately 30, 60, and 42 kDa, respectively, on SDS-PAGE. Yields of the purified PL-A, PL-B, and PL-C from 500 g of the roots were 15, 22, and 115 as total optical density at 280 nm, respectively.

Physico-chemical properties of purified pokeweed lectins
The SDS-PAGE patterns of the purified lectins are shown in Fig. 5. In the absence of 2-ME, the approximate molecular masses were estimated to be 30, 60, and 42 kDa for PL-A, PL-B, and PL-C, respectively, and these values were unchanged on SDS-PAGE of the lectins treated with formic acid or 8 M urea. However, on the SDS-PAGE in the presence of 2-ME, the bands for PL-A, PL-B, and PL-C changed to the positions corresponding to molecular masses of 22, 48, and 21 kDa, respectively.

The isoelectric points of purified PL-A, PL-B, and PL-C were found to be approximately 4.35. Although a difference in their pl values was expected from their varied chromatographic behaviors, our analysis was unable to detect any such deviations.

The amino acid compositions, N-terminal amino acid sequences, and carbohydrate contents of three lectins are given in Table 1. The amino acid compositions of PL-A, PL-B, and PL-C were similar to each other. The extraordinarily large number of half-cystine residues is unique and the contents of Asx, Glx, and glycine are also high; these four amino acids account for 60% of the residues in each lectin. Isoleucine was absent in PL-A and PL-C.

In addition to the amino acid compositions, the similarities in their N-terminal sequences indicate that three lectins are homologous proteins, and PL-A and PL-B are more related to each other than to PL-C. The carbohydrate contents of three lectins differ somewhat; PL-A and PL-B are glycoproteins, but PL-C is not likely to be a glycoprotein.

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**Fig. 3.** Hydrophobic Chromatographies of Fractions F-1a (A), F-2a (B), and F-3a (C) Obtained in Fig. 2 on Butyl-Toyopearl 650M Columns.

Fractions F-1a, F-2a, or F-3a obtained by gel filtration on Sephadex G-75 column in Fig. 2 were put on a Butyl-Toyopearl 650M column (1.5 x 10 cm) pre-equilibrated with 4 mM NaCl 10 mM sodium phosphate buffer, pH 6.0, and eluted with a linear gradient of NaCl from 0 to 0.6 in 10 mM sodium phosphate buffer, pH 6.0.

**Fig. 4.** FPLC Elution Profiles of PL-A (A), PL-B (B), and PL-C (C) Obtained in Fig. 3.

Fraction PL-A, PL-B, or PL-C obtained by Butyl-Toyopearl column chromatography in Fig. 3 was put on a Mono Q-column (0.5 x 5 cm) equilibrated with 10 mM sodium acetate buffer, pH 5.5, and eluted by a linear gradient of NaCl from 0 to 0.5 M in the same buffer at a flow rate of 1 ml/min.

**Fig. 5.** SDS-Polyacrylamide Gel Electrophoresis of Purified Three Pokeweed Lectins.

Purified PL-A, PL-B, and PL-C were analyzed by electrophoresis on a SDS polyacrylamide gel in the absence (1 3) and presence (4 6) of 2-ME. 1 and 4. PL-A: 2 and 5. PL-B: 3 and 6. PL-C: Std. standard proteins as described in Materials and Methods.
Comparison of the amino acid sequences of tryptic peptides from PL-A and PL-B

To examine whether or not PL-A is derived from PL-B by post-translational processing, the peptides were isolated from the tryptic digests of two lectins and their amino acid sequences were compared. Elution profiles of the pH 6 soluble tryptic peptides in RP-HPLC using a phosphate-MeCN elution system are shown in Fig. 6. Two peptides (T4a and T4b) in the peak 4 from PL-A were separated by RP-HPLC using a TFA-MeCN elution system. Nine peptides thus obtained from both lectins were sequenced directly by the manual DABITC/PITC double coupling method. The amino acid sequences of these peptides are shown in Table II. Of the isolated 9 peptides, the corresponding 6 peptides had identical sequences. This suggests the possibility that PL-A is an N-terminal fragment of PL-B or a protein with extremely high homology to the N-terminal half of PL-B.

Hemagglutinating activities of pokeweed lectins

Hemagglutinating activities of these three lectins were measured using human O type erythrocytes. Toward native erythrocytes, as shown in Table III, PL-B had very high activity, while the agglutinating activity of PL-A was about 1.35% of that of PL-B, and PL-C did not agglutinate the cells even at 170-fold the concentration of PL-B. Toward trypsin-treated erythrocytes, on the other hand, PL-A and PL-B had much higher activities than toward native erythrocytes; especially the activity of PL-A increased about 260-fold. However, the activity of PL-C was extremely low, indicating that PL-C is not a hemagglutinin.

Mitogenic activities of pokeweed lectins

The mitogenic activities of three lectins were examined by incorporation of [3H]thymidine into human peripheral lymphocyte cells at various doses using PHA-L₄ as the reference lectin. As shown in Fig. 7, PL-B had the highest mitogenic activity, and PL-A and PL-C had very low activities. The maximal activity was obtained with PL-B at the concentration of 10 μg/ml-culture, whereas it was achieved by 1 μg/ml-culture by PHA-L₄. The dose-response
**Table III.** Hemagglutinating Activities of Three Pokeweed Lectins.

<table>
<thead>
<tr>
<th>Lectins</th>
<th>Hemagglutinating activities* toward native erythrocytes</th>
<th>Hemagglutinating activities* toward trypsin-treated erythrocytes</th>
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<tbody>
<tr>
<td>PL-A</td>
<td>0.696 (1.35%)</td>
<td>0.0027 (21.85%)</td>
</tr>
<tr>
<td>PL-B</td>
<td>0.0094 (100%)</td>
<td>0.00059 (100%)</td>
</tr>
<tr>
<td>PL-C</td>
<td>&gt;1.6 (&lt;0.6%)</td>
<td>0.2 (0.3%)</td>
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</table>

* Hemagglutinating activity was expressed by the optical density at 280 nm of the lectin solution required to agglutinate the erythrocytes.

Purification of Pokeweed Mitogenic Lectins

We purified three mitogenic lectins (PL-A, PL-B, and PL-C) in this experiment, but another lectin with very low hemagglutinating activity was found in the fractions F-3b and F-3c obtained by gel filtration on a Sephacryl G-75 column in Fig. 2. Recently we found this to be a chitin-binding protein and designated it PL-D.

In the case of purification from the crude pokeweed lectin (AcOH) prepared from the AcOH-extract, the yields of three lectins were lower than those from the crude pokeweed lectins (PB) prepared from the phosphate buffer-extract, but the amount of contaminating proteins was small and fairly pure lectins were obtained only by Q-Sepharose column chromatography in some cases. This AcOH-extraction from the roots was effective especially for protection against colorization of proteins in the purification process of lectins from the aged pokeweed roots.

PL-A and PL-B were adsorbed on the Butyl-Toyopearl column and eluted at 2.5 and 1.4 m NaCl, respectively, while PL-C was not adsorbed, indicating the difference in their surface hydrophobicities of the molecules: the surface of the PL-C molecule was fairly hydrophilic.

The behaviors of three lectins on SDS–PAGE were characteristic. Although each of five mitogens (Pa-1–Pa-5) isolated by Wadxl showed the same molecular masses by SDS–PAGE whether or not the disulfide bonds had been reduced with 2-ME, the molecular masses of our three lectins greatly decreased in the presence of 2-ME. Since such a decrease of molecular mass in the presence of 2-ME was observed in many cystine-rich proteins, they may differ in the susceptibilities of intra-disulfide bonds to 2-ME, probably due to the differences in their configurations. With regard to this phenomenon, we found in our early work that the molecular masses of PL-A and PL-B isolated from the aged roots were unchanged even in the presence of 2-ME, but that of PL-C (42 kDa) changed to 21 kDa in the presence of 2-ME, and speculated that PL-C forms a dimeric structure.

The values of molecular masses of PL-A (30 kDa) and PL-C (21 kDa) obtained by SDS–PAGE in the absence and presence of 2-ME were very similar to those of Pa-2 (31 kDa) and Pa-4 (21 kDa), which are the major mitogens isolated by Wadxl, respectively. However, the high value of molecular mass of PL-B (60 or 48 kDa) was not found in any pokeweek mitogens (19–31 kDa) isolated so far, suggesting a novel mitogenic lectin. Our preliminary experiment using the method of Wadxl showed that PL-B was contained in an unadsorbed fraction in his hydroxyapatite column chromatography, and some of PL-B was adsorbed on the hydroxyapatite column and eluted near the elution position of Pa-2 in the gel filtration, and moreover that the elution positions of PL-C and PL-D in the gel filtration correspond to those of Pa-4 and Pa-5, respectively.

The amino acid compositions of PL-A and PL-C were similar to those of Pa-2 and Pa-4, respectively, though the sugar content of PL-C was somewhat lower than Pa-4 (Table I). The amino acid compositions also showed that a lectin corresponding to Pa-1 was not isolated in this.
experiment.

In conclusion, our study overall suggests that PL-A and PL-C correspond to Pa-2 and Pa-4, respectively, and PL-B may be a novel mitogenic lectin with a higher molecular mass and much higher mitogenic activity toward human peripheral lymphocytes than the others.

The amino acid sequence of PL-C will be presented in our following paper.

References