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Molecular Characterization and Mitogenic Activity of a Lectin from Purse Crab *Philyra Pisum*

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A lectin from the hemolymph of purse crab, *Philyra pisum*, was found to have anti-proliferative activity on human lung cancer cells by our laboratory. In this study, *P. pisum* lectin (PPL) was molecularly characterized including molecular mass, amino acid sequences, amino acid composition, and the effects of metal ions, temperature, and pH on the activity. We found that PPL showed mitogenic activity on human lymphocytes and BALB/c mouse splenocytes. The mitogenic activity (maximum stimulation index, SI=9.57±0.59) of PPL on human lymphocytes was higher than that of a standard well-known plant mitogen, concanavalin A (maximum SI=8.80±0.59). The mitogenic activity mediated by PPL is required for optimum dosing, and higher or lower concentrations caused decreases in mitogenic response. PPL also induced mitogenic activity on mouse splenocytes, however, the maximum SI (1.77±0.09) on mouse splenocytes of PPL was lower than that (2.14±0.15) of concanavalin A. In conclusion, PPL is a metal ion-dependent monomer lectin with mitogenic activity, and could be used as a lymphocyte or splenocyte stimulator.

Key Words: Crab, Lectin, Molecular characterization, Mitogenic activity, Lymphocyte, Splenocyte

INTRODUCTION

Lectins are carbohydrate-binding proteins that agglutinate erythrocytes or cells [1]. Their ability to induce cell agglutination or the precipitation of glycoconjugates on cell surface receptors makes them potentially useful in various research fields for the targeting and delivery of drugs [2] as well as detection, localization, and isolation of glycoconjugates [3].

Moreover, one of the most important effects on the lectins with the cells is mitogenicity, as like triggering of nondividing lymphocytes into a state of growth and proliferation [4]. The mechanism of mitogenic activity is not fully understood, however, the process is initiated by the binding of lectin to cell surface sugars, followed by binding to the T-cell antigen or surface component involved in antigen-specific stimulation [5,6]. In contrast to some lectins exhibit mitogenic activity, including those described in our previous report [7], others exhibit nonmitogenic or antimitogenic activity [8].

Mitogenic lectins such as phytohemagglutinin, concanavalin A (Con A), and *Limulus polyphemus* lectin (horseshoe crab) are useful tools for the study of signal transmission into cells and for the analysis of biochemical events that occur during lymphocyte stimulation *in vitro*, and most of them stimulate mainly T-lymphocytes [9].

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The purse crab *Philyra pisum* is distributed throughout the muddy areas of the west coast of Korea. Our previous investigation indicated that a lectin, *P. pisum* lectin (PPL), from the hemolymph of *P. pisum* has hemagglutination activity and anti-proliferative activity onto human lung cancer cells [10]. The purification procedure, purification fold, and the first ten N-terminal amino acid sequences for PPL were also investigated.

In the present study, we investigated the more detailed molecular characterization of PPL including its molecular mass, *p*I value, amino acid sequences, amino acid composition, effects of pH, temperature, and metal ions on its activity. Mitogenic activities on human lymphocytes and mouse splenocytes were also investigated.

METHODS

Preparation of hemolymph and purification of a lectin

Hemolymph preparation from *P. pisum* and purification of a lectin from the hemolymph were performed as described previously [10]. The purified lectin concentration was determined by the Bradford method [11] using bovine serum albumin as the standard.

ABBREVIATIONS: PPL, *Philyra pisum* lectin; Con A, concanavalin A; SDS-PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis; IEF, isoelectric focusing; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; EDTA, ethylenediaminetetraacetic acid; BrdU, 5-bromo-2-deoxyuridine; SI, stimulation index.

Hemagglutination and hemagglutination inhibition assay

The hemagglutination activity was assayed using 2% native erythrocytes according to previously-described methods [12].

Electrophoretic analyses

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [13] using a 15% acrylamide slab gel in the presence and absence of 2-mercaptoethanol. The gel was stained with silver nitrate. Molecular mass markers (6.5～200 kDa) were purchased from Bio-Rad. Isoelectric focusing (IEF) on a gel was estimated using a 2% ampholyte IEF-ready gel (pH 3～10) according to the manufacturer's instructions (Bio-Rad).

Gel-filtration chromatography

Gel-filtration chromatography was performed using a TSK-GEL G3000SW column (Tosoh) with a conventional high-performance liquid chromatography (HPLC) system (Shimadzu) equilibrated with 20 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl at a flow rate of 0.5 ml/min and monitored at 280 nm. Molecular mass markers (13.7～2,000 kDa) were used with a gel-filtration calibration kit (Amersham Biosciences).

Mass spectrometry analysis

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrum of intact PPL was obtained by nano-ESI on a Q-TOF2 mass spectrometer (Micromass) using sinnapinic acid as the matrix.

Amino acid sequences analysis

PPL (12 μg) was applied to a 15% SDS-PAGE gel, electroblotted onto a PVDF membrane (Bio-Rad), and analyzed using an Automatic Protein Sequencer (476A-01-120, Applied Biosystems). Sequence homologues were searched using EXPASy Proteomics Server (http://au.expasy.org/ tool/blast/). PPL was subjected to in-gel tryptic digestion according to the manufacturer's instructions (Sigma), and MS/MS of major internal peptides were obtained by nano-ESI on a Q-TOF2 mass spectrometer (Micromass) using α-cyano-4-hydroxycinnamic acid as the matrix. The data were processed using a Mass Lynx Windows NT PC system.

Amino acid composition analysis

PPL (100 μg) was hydrolyzed *in vacuo* with 6 N HCl for 24 h at 110°C. For the analysis of cysteic acid and Trp, oxidized PPL (50 μg) treated with performic acid and hydrolyzed PPL (50 μg) with methanesulfonic acid were prepared, respectively. Phenylisothiocyanate-amino acids were analyzed by HPLC (Waters) with a Nova-Pak C18 column (Waters).

Effect of pH, temperature, and metal ions on the activity

PPL dissolved in neutral buffer was substituted for various pH buffers (pH 3.2～10.1) containing 0.15 M NaCl through centrifugation at 600×g for 2 h at 4°C using Microcon YM-10 (Millipore), and incubated for 1 h at room temperature. The hemagglutination titer was then measured.

In the neutral condition, the hemagglutination titer against rabbit erythrocytes was measured after treatment of PPL at 4～70°C for 1 h.

PPL was dialyzed overnight against 100 mM ethylenediaminetetraacetic acid (EDTA) disodium salt, after which it was dialyzed against 0.15 M NaCl at 4°C. The hemagglutination titer was then measured in the absence or presence of 5 mM $CaCl_2$, 5 mM $MnCl_2$, or 5 mM $MgCl_2$ solution.

Mitogenic activity on human lymphocytes and mouse splenocytes

Human peripheral blood lymphocytes were isolated by density gradient method from healthy human blood using Ficoll-Hypaque (Pharmacia) at room temperature according to the manufacturer's instructions. The splenocytes were isolated from BALB/c mouse spleen and diluted with RPMI medium containing 10% fetal bovine serum. The lymphocytes or splenocytes (1.2×10^4 cells/0.1 ml/well) were diluted with RPMI medium containing 10% fetal bovine serum, respectively, and various concentrations of PPL (from 0.78 to 25.0 μg/ml) was added to a 96-well microplate. The same concentrations of Con A (Sigma) were used as a control. After incubation at 37°C in a humidified atmosphere of 5% CO_2 for 72 h, 5-bromo-2-deoxyuridine (BrdU)- incorporation assay (Roche Applied Science) was performed according to the manufacturer's instructions. All values were the means of duplicate samples. The stimulation index (SI) of assay was expressed as the ratio of the mean absorbance at 405 nm in the presence and absence of PPL. Data are shown as the mean±standard deviation of three experiments.

RESULTS

Molecular characterization of PPL

The lectin, PPL, was purified from the hemolymph of *P.*

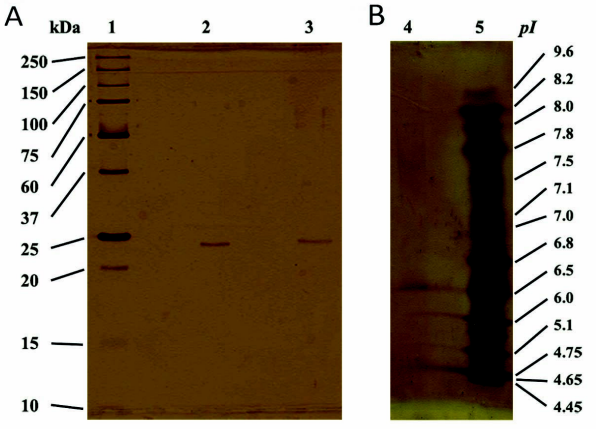

Fig. 1. Electrophoretic analyses of PPL. (A) Lane 1, molecular standard marker; lane 2, PPL; lane 3, PPL with 2-mercaptoethanol; (B) lane 4, PPL; lane 5, *p*I standard markers.

pisum according to the previous investigation [10], and exhibited the same hemagglutination activity to on mouse, rat, and rabbit erythrocytes at a titer of 8, 64, and 256, respectively, whereas it did not agglutinate human ABO and chicken erythrocytes (data not shown). To investigate its purification efficiency, the molecular mass and *p*I value of PPL were assessed, and the electrophoretic analysis analyses using SDS-PAGE and IEF gels, were performed. A single protein band corresponding to a molecular mass of approximately 24 kDa (Fig. 1A) was observed with and without 2-mercaptoethanol in SDS-PAGE stained with silver nitrate. PPL was observed as a single peak when applied to an anion exchange column (Resource Q, Pharmacia) (data not shown), and was observed as one band with an estimated *p*I of 6.5 in the IEF gel (Fig. 1B). The gel-filtration chromatography results indicated that PPL has a Kav value of 0.68, corresponding to a molecular mass of 24.0 kDa for the native molecule (data no shown). The MALDI-TOF mass spectrum showed that the exact molecular mass of PPL was 24.060 kDa (data not shown).

Table 1. Amino acid composition of PPL

Amino acid	Molar ratio (%)
Asp/Asn	8.90
Glu/Gln	7.66
Ser	7.62
Gly	13.55
His	2.09
Arg	3.02
Thr	6.97
Ala	6.65
Pro	5.49
Tyr	1.04
Val	5.02
Met	1.74
Ile	4.20
Leu	5.96
Phe	3.46
Trp	6.04
Lys	4.42
Cys	6.17
Total	100

Amino acid sequences of PPL

The single N-terminal amino acid sequence of PPL was determined to be Ile-Val-Gly-Gly-Thr-Glu-Ala-Thr-Pro-His-Xaa-Trp-Xaa-Tyr-Gln-Val-Xaa-Leu-Phe-Ile (Xaa indicates that the residue could not be identified). The internal amino acid sequences of two major tryptic-digested peptides from PPL was determined by MS/MS analysis as 1,386.66 m/z (Ser-Trp-Leu-Leu-Thr-Asn-Asp-Leu-Ala-Leu-Leu-Lys) and 1,331.87 m/z (Leu-Pro-Gln-Ser-Val-Phe-Thr-Asn-Ala-Asn-Leu-Lys) (data not shown).

Amino acid composition of PPL

As shown in Table 1, the amino acid composition of PPL showed high contents of Gly (13.55%), Asp/Asn (8.90%), Thr (6.97%), Ala (6.65%), Cys (6.17%), and Trp (6.04%), and lower contents of Met (1.74%) and Tyr (1.04%).

Effects of pH, temperature, and metal ions of PPL on the activity

The hemagglutination activity of PPL was stable at pH values from 4.0 to 8.0 and at temperatures below 40°C; the activity decreased at pH 3.2 or 9.1 and was completely inactivated at pH value 10.1 and at temperatures above 70°C (Table 2). PPL lost its hemagglutination activity following dialysis with EDTA and was recovered by the addition of 2.5 mM $CaCl_2$ or 2.5 mM $MnCl_2$, but not by 2.5 mM $MgCl_2$ (data not shown).

Mitogenic activity of PPL on human lymphocytes and mouse splenocytes

PPL showed mitogenic activity on human lymphocytes and BALB/c mouse splenocytes (Table 3). In particular, the relative maximum mitogen SI (9.57±0.59) on human lymphocytes of PPL (1.56 μg/ml) was higher than well-known mitogenic lectin, Con A (25.0 μg/ml, SI=8.80±0.59). PPL also induced mitogenic activity on mouse splenocytes as the relative maximum mitogen SI (1.77±0.09) of PPL (12.5 μg/ml), even though this SI was lower than Con A (SI=2.15±0.15)

Table 2. Effects of pH and temperature on the activity of PPL

	pH								Temperature (°C)					
	3.2	4.4	5.6	6.0	7.3	8.0	9.1	10.1	4	25	40	50	60	70
Activity (%)	20	100	100	100	100	100	40	0	100	100	100	50	20	0

Table 3. Stimulation index (SI) of BrdU-incorporation assay of PPL on human lymphocytes and mouse splenocytes. Con A was used as a control

	SI	Dose (μg/ml)						
		25.0	12.5	6.25	3.13	1.56	0.78	0
Lymphocytes	PPL	1.28±0.33	5.17±0.66	7.13±0.59	8.94±0.39	9.57±0.59	7.13±0.53	1.00±0.02
	Con A	8.80±0.59	7.97±0.66	8.30±0.53	7.07±0.39	7.18±0.20	6.19±0.66	1.00±0.02
Splenocytes	PPL	1.19±0.08	1.77±0.09	1.47±0.08	1.48±0.07	1.37±0.08	0.83±0.08	1.00±0.03
	Con A	2.00±0.09	2.15±0.15	2.04±0.08	0.96±0.10	1.12±0.05	0.89±0.08	1.00±0.03

at the same concentration. The mitogenic activity of PPL was required for optimum dose and the higher or lower concentrations of PPL caused a decrease in mitogenic response to lymphocytes or splenocytes.

DISCUSSION

The results of electrophoretic analyses including SDS-PAGE, IEF, gel-filtration and ion-exchange column chromatography, and mass analysis indicate that PPL is a single acidic protein and exists as a monomer of 24.060 kDa, also confirming that no isolectin contamination occurred during the purification procedure. The molecular mass of reported crab lectin was from 24 to 700 kDa and the molecular mass of PPL was similar to that of tachylectin from the Japanese horseshoe crab *Tachypleus tridentatus* [14,15]. The *p*I value was similar with that of lectin from the portunid crab *Liocarcinus depurator* [16].

The single N-terminal amino acid sequence of PPL was determined and was highly heterogeneous compared to those of lectins obtained from other sources. Interestingly, even though the internal amino acid sequences are quite different from the reported proteins, the N-terminal amino acid sequences of PPL was quite similar (identities 75%) with amino acid sequences of crustacean proteases such as collagenolytic protease from the red king crab *Paralithodes camtschaticus* (45～64; Ile-Val-Gly-Gly-Gln-Glu-Ala-Thr-Pro-His-Thr-Trp-Val-His-Gln-Val-Ala-Leu-Phe-Ile), and chymotrypsin from the whiteleg shrimp *Litopenaeus vannamei* (46～65; Ile-Val-Gly-Gly-Val-Glu-Ala-Thr-Pro-His-Ser-Trp-Pro-His-Gln-Ala-Ala-Leu-Phe-Ile).

PPL contained a high proportion of hydrophobic (45.77%), aromatic (12.63%), and acidic (16.56%) amino acids. The amino acid composition of PPL accounts for a relatively high proportion of hydrophobic (45.77%) and acidic (16.56%) amino acids.

The BrdU (an analogue of thymidine)-incorporation assay is a one-step non-radioactive assay used to determine the proliferation of lymphocytes or splenocytes, and is easily detectable by immunochemical methods [17,18]. The results showed that PPL has mitogenic activities on both human lymphocytes and mouse splenocytes. The mitogenic activity was stronger than those of commonly used mitogens such as Con A, even when using 1/16-dose of Con A in human lymphocytes. The mitogenic activity mediated by PPL is required for optimum dose, as observed in previous reports including Con A [19]. Higher concentrations of Con A have been reported to cause decreases in mitogenic response due to toxic action [4] and PPL is probably similar in action.

In conclusion, based on the present results and those of previous investigations, PPL is a metal ion-dependent 24 kDa monomer C-type lectin from *P. pisum* with hemagglutination activities on mouse, rat, and rabbit erythrocytes, anti-proliferative activity on human lung cancer cells, and mitogenic activities on human lymphocytes or mouse splenocytes. The results of mitogenic acivity indicate that PPL could be used as a lymphocyte or splenocyte stimulator.

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