# Chemical and mass spectrometry characterization of the red alga *Pyropia yezoensis* chemoprotective protein (PYP): Protective activity of the N-terminal fragment of PYP1 against acetaminophen-induced cell death in Chang liver cells

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**Abstract.** In the present study, the chemical structure and chemoprotective activity of *Pyropia yezoensis* protein (PYP) were investigated using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, automated protein sequencing, matrix-assisted laser desorption/ionization-quadrupole ion trap-time-of-flight mass spectrometry and a chemoprotective assay using a synthetic peptide. The PYP fraction was demonstrated to contain two proteins: PYP1 (10 kDa, SDS-resistant dimer) and PYP2 (10 kDa). PYP1 is a novel protein showing sequence homology with the hypothetical function-unknown proteins of *Chondrus crispus* (Rhodophyta) and *Emiliania huxleyi* (Haptophyceae). PYP2 is a paralog of an extrinsic protein of photosystem II found in other Rhodophyta. The synthetic peptide PYP1 (1-20), corresponding to the N-terminal 20 residues of PYP1 (ALEGGKSSGGGEATRDPEPT), exhibits chemoprotective activity against acetaminophen-induced cell death in Chang liver cells, indicating that PYP1 is a chemoprotectant of the PYP fraction. A possible association between the structure of PYP and its chemoprotective activity is discussed.

## Introduction

Seaweed has been steadily utilized in food supplements and as human food, feed for fishery organisms and biofuel (1,2). The Bangiales (Rhodophyta), in particular *Pyropia* sp. and *Porphyra* sp., are cultured widely in East Asia, including Korea and Japan. Thus far, numerous studies have concentrated on the availability of seaweed with regard to the nutritional value of food and medical material. Previously, the effectiveness of seaweed has become known through biological activity experiments conducted using seaweed extract *in vivo* and *in vitro* (3-10). In particular, proteins isolated from *Pyropia yezoensis* (formerly known as *Porphyra yezoensis*) showed a positive effect on anti-inflammation (9) and protection from cell toxicity (7). However, the majority of these studies investigated the expression of physiological activity *in vivo* or *in vitro*. To the best of our knowledge, no study has reported the association between the molecular structure and function of seaweed with various bioactive substances.

*P. yezoensis* is a model marine plant for physiological and genetic studies in seaweed (11). In addition to expressed sequence tag (EST) analyses (12,13), the draft nuclear genome sequence (14) and the complete plastid genome sequence (15) of *P. yezoensis* have recently been published. Hwang *et al* (7) reported that the 14-kDa protein-containing fraction of *P. yezoensis* has chemoprotective functions. In the present study, the association between the chemical structure and chemoprotective activity of the *P. yezoensis* protein (PYP) was investigated. PYP components resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were chemically and mass spectrometrically identified and characterized. The synthetic N-terminal fragment of the major PYP protein was determined to be a chemoprotectant. The possible physiological role of PYP and the mode of chemoprotective action will be discussed.

## Materials and methods

*Preparation of PYP.* The PYP fraction was prepared from *P. yezoensis* farmed in Wando Gun (Jeolla Nam-Do, Korea), as described by Hwang *et al* (7). In brief, 40 g of powdered *P. yezoensis* was dissolved in 1l distilled water (DW) at room temperature for 3 h. The extract was filtered with a 5-$\mu$m Muller gauze and subsequently three volumes of ethanol were added.

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The ethanol extract was filtered (no. 3; Advantec, Tokyo, Japan), pelleted with ammonium sulfate, dissolved in DW, dialysed in DW overnight and concentrated at 40˚C in a rotary evaporator (Eyela/Tokyo Rikakikai, Tokyo, Japan). The concentrated solution was redissolved in double DW and freeze-dried (Eyela FDU-2100; Lab Corporation, Seoul, Korea). The protein content was determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

*Protein sequencing*. SDS-PAGE was performed as described by Laemmli (16) using a 1.5-mm-thick 18% acrylamide gel. In brief, PYP (20 mg) was dissolved in 1 ml water, and dark-colored insoluble material was removed by centrifugation (15,000 x g for 10 min). The clear supernatant (5 $\mu$l) was mixed with an equal volume of Laemmli buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue], and incubated for 5 min at 95˚C. SDS-PAGE standard (Broad Range; Bio-Rad, Hercules, CA, USA) was used as the molecular weight marker. The molecular weight of each protein band was determined by the method of Weber and Osborn (17). Electroblotting was performed in 25 mM Tris-HCl (pH 8.3) and 192 mM glycine at 100 V for 30 min using a Criterion Transblot Cell (Bio-Rad). The polyvinylidene fluoride (PVDF) membrane Sequi-Blot (Bio-Rad) was used for transfer. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250 Staining solution (Bio-Rad) for 1 min and destaining with 50% methanol for 10 min. Protein bands were excised from the membrane and destained with 90% methanol prior to protein sequencing. The N-terminal amino acid sequence and initial yield of phenylthiohydantoin-amino acid (PTH-AA) of each protein band was analyzed using a gas phase protein sequencer PPSQ-33A (Shimadzu, Kyoto, Japan).

*Mass spectrometry*. PYP proteins separated by SDS-PAGE were visualized with Coomassie Brilliant Blue G-250 Staining solution in accordance with the manufacturer's instructions. The major band (corresponding to the 10-kDa section in Fig. 1) was excised using a utility knife. In-gel tryptic digestion and peptide extraction were performed as previously described (18), except that the destaining step (prior to in-gel digestion) was omitted to avoid loss of small proteins. Tandem mass spectrometry (MS/MS) spectra were obtained using a matrix-assisted laser desorption/ionization-quadrupole ion trap-time of flight (MALDI-QIT-TOF) mass spectrometer (AXIMA Resonance; Shimadzu) in the positive mode. All the spectra were externally calibrated using human angiotensin II (*m/z*: 1,046.54) and human adrenocorticotropic hormone (18-39) fragment (*m/z*: 2,465.20) in a ProteoMass® Peptide & Protein MALDI-MS Calibration kit (Sigma-Aldrich, St. Louis, MO, USA). Protein identification was carried out by MS/MS ion searches using MASCOT® version 2.3 (Matrix Science, London, UK) against EST_rhodophyta 2011_11 (2,715,402 sequences; 367,566,596 residues) and *Pyropia yezoensis* CDS (61,962 sequences; 17,483,926 residues), downloaded from the *Pyropia yezoensis* Genome Annotation ver. 1, Fisheries Research Agency, Japan (http://nrifs.fra.affrc.go.jp/ResearchCenter/5_AG/genomes/nori/), SwissProt 2012_09 (540,958 sequences; 192,206,270 residues, http://www.uniprot.org/downloads), and the non-redundant sequence database from the National Center for Biotechnology Information (NCBInr 20130721; 31,240,556 sequences; 10,789,480,161 residues, ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz) in our own MASCOT server. Search parameters used were as follows: Enzyme, trypsin; fixed modifications, none; variable modifications, oxidation (M); mass values, monoisotopic; peptide mass tolerance, ±0.3 Da; fragment mass tolerance, ±0.2 Da; and max missed cleavage, 2. Positive identification was assigned with MASCOT scores above the threshold level ($P<0.05$ was considered to indicate a statistically significant difference) and with at least two peptides (protein score >44).

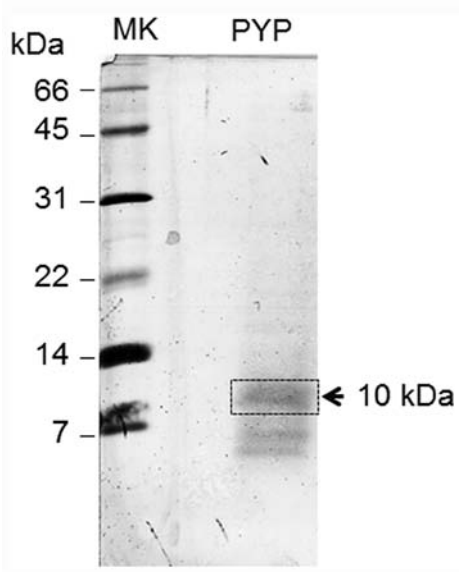


Figure 1. Separation of *Pyropia yezoensis* protein (PYP) components using 18% SDS-PAGE. Coomassie Brilliant Blue R-250-stained electroblot (PVDF membrane) of the water-soluble PYP proteins (5 $\mu$l of 20 mg/ml). Band section was excised for N-terminal protein sequencing and is indicated by the box.

*Computational analysis*. Homology searches were performed using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were performed using CLUSTALW ver. 2 (19) and refined manually for representation. Sequence similarity (%) and identity (%) were calculated using a global pairwise alignment tool (Needle program) from EMBOSS (20). Theoretical mass, theoretical isoelectric point, grand average of hydropathy (GRAVY) and amino acid composition were calculated from the mature protein sequence using the ProtParm program in the ExPASy proteomic tools (http://web.expasy.org/protparam/). Secondary structure was predicted with the Jpred program (http://www.compbio.dundee.ac.uk/www-jpred/). Hydropathy plots and FoldIndex plots were created using the ProtScale program (http://web.expasy.org/protscale/) with a window size of 9, and FoldIndex (http://bioportal.weizmann.ac.il/fldbin/findex) with a window size of 12, respectively. Subcellular localization of the protein sequence was analyzed using WoLF PSORT (21). PYP sequence motifs were searched using MotifScan (http://myhits.isb-sib.ch/cgi-bin/motif_scan).

*Peptide synthesis*. The N-terminal 20 residues of PYP1 (ALEGGKSSGGGEATRDPEPT), designated PYP1 (1-20), were synthesized by Peptron (Daejeon, Korea). Purification

Table I. Tandem mass spectrometry (MS/MS) ion search of in-gel tryptic digest from the 10-kDa gel section.

| Protein ID | *m/z* | Delta[a], Da | Missed cleav.[b] | Score[c] | Sequence[d] | Accession no. | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Rhod_ EST[e] | Pye_ CDS[f] | Swiss Prot | NCBInr |
| PYP1 | 1687.96 | 0.08 | 0 | 40 | QQAIHVAPSFADYLK | AV429545 | 20922_g5127 | NA[g] | NA[g] |
| | 2197.09 | 0.09 | 1 | 20 | SSGGGEATRDPEPT AVDPNDPK | AV429545 | NA[g] | NA[g] | NA[g] |
| PYP2 | 1382.86 | 0.03 | 0 | 29 | IPGLTDLQIATLK | DR907360 | 19303_g4763 | NA[g] | NA[g] |
| | 1589.05 | 0.20 | 0 | 36 | LVALTPTPEYELDK | DR907360 | 19303_g4763 | NA[g] | NA[g] |
| | 2820.43 | 0.04 | 0 | 49 | YPGMYPTVAGLIAT NGPFETVSDLYK | DR907360 | 19303_g4763 | NA[g] | NA[g] |

[a]Mass difference between observed and theoretical mass of the matched peptide. [b]Number of missed cleavage sites in the tryptic fragment.[c]MASCOT peptide score. [d]Matched poptide sequence (Oxidized Met is underlined). [e]Rhodophyta expressed sequence tag (EST) encoding the longest mature protein sequence. [f]Abbreviated accession numbers of *P. yezoensis* coding DNA sequence (CDS) (14), omitting prefix 'contig_'(e.g., contig_20900_g5127). [g]Not available due to insignificant probability-based MASCOT score (P>0.05). PYP, *P. yezoensis* protein.

of PYP1 (1-20) was performed on a Shimadzu Prominence high-performance liquid chromatography apparatus and controlled using the software package Class-VP, 6.14 using a C18 column (Shiseido Capcell Pak; Shiseido, Tokyo, Japan) in 0.1% trifluoroacetic acid (TFA)/water and a gradient of 10-70% acetonitrile in 0.1% TFA, with a flow rate of 1 ml/min and ultraviolet detection at 220 nm. The molecular mass of PYP1 (1-20) was confirmed to be 1,916 Da (matched with the sequence mass) by mass analysis (HP 1100 Series LC/MSD; Agilent Technologies, Santa Clara, CA, USA).

*Cell culture*. The Chang liver cell line (CCL-13; American Type Culture Collection, Manassas, VA, USA) was maintained at 37˚C in a humidified atmosphere with 5% $CO_2$. Cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 mg/ml streptomycin.

*Cell proliferation and cytotoxicity*. Cell proliferation was measured using a CellTiter 96® aqueous non-radioactive cell proliferation assay (Promega, Madison, WI, USA), based on the cleavage of MTS into a formazan product soluble in tissue culture medium. Cells were seeded onto 96-well plates at $2x10^4$ cells/well and maintained for 24 h. Medium was replaced with serum-free medium (SFM). After 24 h, SFM was replaced with PYP1 (1-20; 250 or 500 ng/ml) containing 15 mM acetaminophen (APAP) for 24 h. Subsequently, cells were added to MTS/phenazine methosulfate solution for 30 min at 37˚C. Cell proliferation was measured at 490 nm absorbance using a microplate reader (Benchmark; Bio-Rad).

*Statistical analysis*. Significant differences in the effects of each factor on cell viability were examined using an analysis of variance (ANOVA), followed by the Duncan test.Differences with a probability value (P)<0.05 were considered significant.

## Results

*Protein separation and identification of the PYP fraction*. In our previous study, SDS-PAGE of the PYP fraction showed a single 14-kDa protein band on a 15% polyacrylamide gel (7). Since N-terminal protein sequencing analysis of the 14-kDa band yielded a composite and unreadable sequence, the PYP fraction was further resolved using an 18% polyacrylamide gel and electroblotted onto a PVDF membrane; protein bands were detected by Coomassie Brilliant Blue staining. As shown in Fig. 1, the PYP fraction showed a diffuse 10-kDa band and a ~7-kDa doublet band. These proteins were easily lost from the gel during destaining when the gel was stained with Coomassie Brilliant Blue R-250. The 10-kDa band of the blot was excised as a section (Fig. 1) and sequenced with a gas phase protein sequencer, yielding two unambiguous sequences: Ala-Leu-Glu-Gly-Gly-Lys-Ser-Ser-Gly-Gly-Gly -Glu-Ala-Thr-Arg-Asp-Pro-Glu-Pro-Thr (PTH-AA yield: 23.6 pmol/band) and Glu-Thr-Xaa-Tyr-Ala-Asn-Val-Pro-Phe -Leu (PTH-AA yield: 7.6 pmol/band). Thus, these two proteins were tentatively designated PYP1 (N-terminus: Ala-Leu-Glu-) and PYP2 (Glu-Thr-Xaa-), respectively. The protein sequence data reported will appear in the UniProt Knowledgebase (http://www.uniprot.org/uniprot) under the accession numbers C0HJG2 (PYP1) and C0HJG3 (PYP2).

To identify the genes encoding PYP1 and PYP2, the corresponding 10-kDa band was further analyzed by an MS/MS ions search following in-gel tryptic digestion. As summarized in Table I and shown in Fig. 2, two proteins encoded by EST clones (the nuclear-encoded genes of *P. yezoensis*) were identified. Availability of the gene sequences was evaluated with Rhodophyta EST, *Pyropia yezoensis* CDS, SwissProt, and NCBInr databases. Due to partial sequences of predicted gene models in *Pyropia yezoensis* CDS annotation ver. 1 (10,327 gene models) (14), the MASCOT protein score (sum of the peptide score) was less than that of the Rhodophyta EST (Table I). The cross-species MS/MS ions search against the SwissProt and NCBInr databases failed to identify genes for PYP1 and PYP2 (Table I). Therefore, precursor protein sequences for PYP1 and PYP2 were deduced from the Rhodophyta EST, and their homologs were searched from the NCBInr database to predict the structure and function. As shown in Fig. 2A, PYP1 showed significant sequence similarity with the hypothetical function-unknown proteins

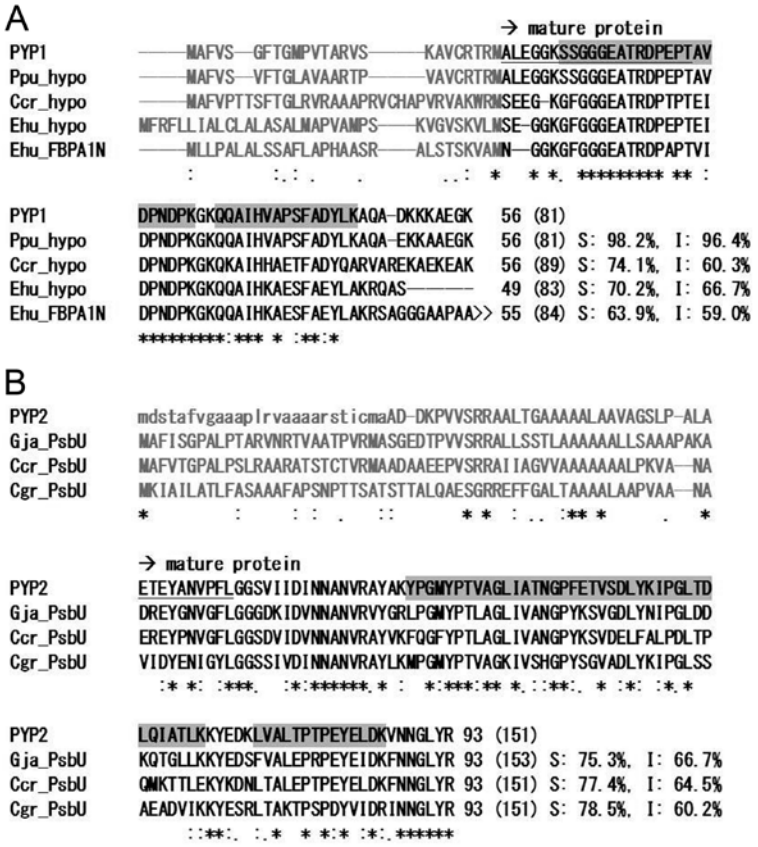


Figure 2. Multiple sequence alignment of protein sequences of (A) *Pyropia yezoensis* protein (PYP)1 and (B) PYP2 with homologous sequences from Rhodophyta, Haptophyceae and Stramenopiles. Gray letters indicate putative chloroplast targeting signal sequences (transit peptides). Dashes denote blanks or gaps. Underlined sequences are N-terminal protein sequences of mature proteins determined experimentally. Sequences highlighted in gray represent tryptic peptides assigned by tandem mass spectrometry (MS/MS) ions searches. Asterisks, colons and dots represent identical amino acids, conserved substitutions, and semiconserved substitutions, respectively. Chain length of the mature protein (that of the precursor in parenthesis), and percentage identity (I) and similarity (S) are shown after the C-terminus. Abbreviations and accession numbers: Ppu_hypo, hypothetical protein from *Porphyra purpurea* (esContig7275); Ccr_hypo, unnamed protein product from *Chondrus crispus* (CDF35169); Hhu_hypo, hypothetical protein from *Emiliania huxleyi* (AFE02913); Hhu_FBPA1N, N-terminal domain of putative fructose 1,6-bisphosphate aldolase class I from *Emiliania huxleyi* (AFE02906); Gja_PsbU, Photosystem II 12-kDa extrinsic protein (PsbU) from *Griffithsia japonica* (AAP80721); Ccr_PsbU, PsbU from *C. crispus* (CDF33931); Cgr_PsbU, PsbU from *Chaetoceros gracilis* (BAG85212). Precursor protein sequences of PYP1 and PYP2 were deduced from expressed sequence tags (ESTs) (AV429800 and DR907360, respectively). Due to the lack of an N-terminal transit peptide-coding region in the PYP2 EST clone (DR907360), a putative corresponding sequence (small letters) is supplemented by another EST clone (AV438439). '>>' indicates that the N-terminal domain homologous to PYP1 is followed by a putative fructose 1,6-bisphosphate aldolase class I.

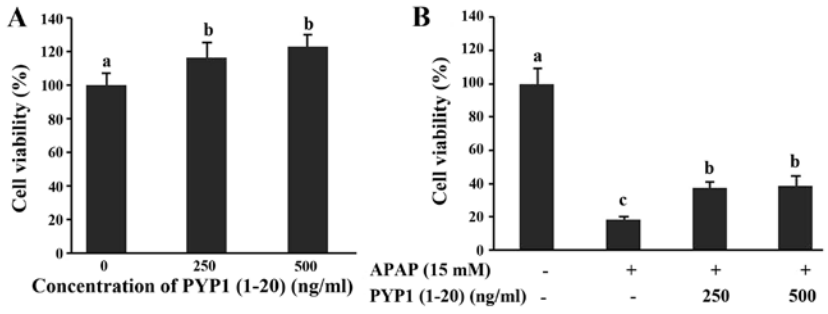


Figure 3. (A) Effect of *Pyropia yezoensis* protein (PYP)1 (1-20) on cell proliferation and (B) protective effect of PYP1 (1-20) against APAP-induced hepatotoxicity. Cells were cultured with acetaminophen (APAP) or APAP + PYP1 (1-20). Cell viability was measured by the MTS assay. Values represent the means ± standard deviation; $n$=3 ($P<0.05$). Letters next to the values indicate significant differences among groups by Duncan's multiple-range test.

of Rhodophyta (*Porphyra purpurea* and *Chondrus crispus*) and a Haptophyceae (*Emiliania huxleyi*), and the N-terminal domain of a putative fructose 1,6-bisphosphate aldolase class I from *E. huxleyi*. Thus, PYP1 is a novel protein conserved in Rhodophyta and Haptophyceae.

*Cell proliferation and cytotoxicity*. PYP1 (1-20), the synthetic 20-residue peptide of the N-terminus of PYP1, was used to investigate the effect of Chang liver cell proliferation, as determined by the CellTiter 96® aqueous non-radioactive cell proliferation assay. Cells were treated with various

concentrations (250 or 500 ng/ml) of PYP1 (1-20) for 24 h (Fig. 3A). PYP1 (1-20) did not induce cytotoxicity or proliferation in Chang liver cells. Subsequently, Chang liver cells were investigated for the protective effect of PYP1 (1-20) against hepatotoxicity following treatment with 15 mM APAP. As a result, cell viability decreased 80% when treated with 15 mM APAP. However, cell viability recovered ~38% when treated with PYP1 (1-20; Fig. 3B). Therefore, PYP1 (1-20) has a protective effect on hepatotoxicity caused by APAP in Chang liver cells.

## Discussion

As the PYP1 precursor has a putative chloroplast-targeting signal, PYP1 is most likely a chloroplast-localized protein. Although PYP1 was identified from the 10-kDa band following SDS-PAGE, the sequence mass of mature PYP1 was calculated as 5,763.3 Da. This observation indicates that PYP1 forms an SDS-resistant dimer, as reported in certain other proteins (22,23). As reported previously, PYP proteins are heat-soluble (7). The GRAVY of PYP1 is –1.086, and the amino acid composition is a preponderance of Ala (16%), Gly (13%), Lys (14%), Glx (13%), Asx (11%), Thr/Ser (9%) and Pro (9%), and lacks Trp and Cys. The hydrophilic N-terminal 33 residues and C-terminal 12 residues of PYP1 were predicted to be intrinsically disordered regions and the short internal hydrophobic region to be structured, respectively. Additionally, the physicochemical characteristic of PYP1 is similar to that of late embryogenesis abundant (LEA) proteins, which protect protein denaturation from desiccation, freezing, heat, salt and osmotic stress (24). A LEA-like protein from *Arabidopsis thaliana* also plays a role in the protection against oxidative stress (25). LEA proteins are heat stable, intrinsically disordered proteins, and have high hydrophilicity with GRAVY (often, <-1.0), a preponderance of Ala, Gly, Glu, Lys/Arg and Ser/Thr, and a lack or low proportion of Cys and Trp. Although structurally known motifs of the LEA family were not identifiable from the PYP1 sequence, PYP1 may be a novel LEA-like protein. PYP2 showed significant sequence similarity with PsbU (Fig. 2B). PsbU, an extrinsic protein localized in the luminal side of photosystem II, has been identified in the majority of cyanobacteria and red algae, but not in green algae and higher plants (26). Notably, PsbU contributes to the thermal stability of photosystem II (27) and enhances its structural stability, protecting it from reactive oxygen species (ROS), which are produced as an inevitable by-product of photosynthesis (28,29); therefore, PYP2 (i.e., *P. yezoensis* PsbU) could also have such functions. Since APAP-induced cell injury is caused by oxidative stress through ROS production (30), PYP1 (putative novel LEA-like) and PYP2 (PsbU) may also contribute to the chemoprotective activity though an uncharacterized protection mechanism from ROS attack.

For the first time, Hwang *et al* (7) reported that PYP demonstrated chemoprotective effects against APAP-induced liver injury. Also, antioxidant and anti-inflammatory effects of a glycoprotein from *P. yezoensis* were demonstrated in lipopolysaccharide-stimulated RAW 264.7 mouse macrophages (9). Despite various bioactive substances of *P. yezoensis*, no information was available regarding a specific peptide with bioactivity in PYP. In the present study, PYP1 (1-20), the synthetic 20-residue peptide of the N-terminus of PYP1, was used to investigate the effect of Chang liver cell proliferation. As a result, PYP1 (1-20) was shown to not induce cytotoxicity, as well as having the effect of the proliferation in Chang liver cells. These data indicate that PYP1 is a novel LEA-like protein with chemoprotective activity at the N-terminal portion. Taking into account the chemoprotective activity of PYP *in vivo* and *in vitro* (7), PYP1 may have an effect on protection against liver cell injury.

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