Purification, Characterization, Molecular Cloning, and Expression of Novel Members of Jacalin-related Lectins from Rhizomes of the True Fern *Phlebodium aureum* (L) J. Smith (Polypodiaceae)*

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Hiroaki Tateno‡, Harry C. Winter, Jerzy Petryniak, and Irwin J. Goldstein§

From the Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109-0606

A lectin was purified from rhizomes of the fern *Phlebodium aureum* by affinity chromatography on mannosese-pharose. The lectin, designated *P. aureum* lectin (PAL), is composed of two identical subunits of ~15 kDa associated by noncovalent bonds. From a cDNA library and synthetic oligonucleotide probes based on a partial amino acid sequence, 5′- and 3′-rapid amplification of cDNA ends allowed the generation of two similar full-length cDNAs, termed PALa and PALb, each of which had an open reading frame of coding for a 486-atom amino acid residues. The two proteins share 88% sequence identity and showed structural similarity to jacalin-related lectins. PALa contained peptide sequences exactly matching those found in the isolated lectin. PALa and PALb were expressed in *Escherichia coli* using pET-22b(+) vector and purified by one-step affinity chromatography. Native and recombinant forms of PAL agglutinated rabbit erythrocytes and precipitated with yeast mannan, dextran, and the high mannos-containing glycoprotein invertase. The detailed carbohydrate-binding properties of the native and recombinant lectins were elucidated by agglutination inhibition assay, and native lectin was also studied by isothermal titration calorimetry. Based on the results of these assays, we conclude that this primitive vascular plant, like many higher plants, contains significant quantities of a mannos/glucose-binding protein in its storage tissue, whose binding specificity differs in detail from either legume mannos/glucose-binding lectins or monocot mannose-specific lectins. The identification of a jacalin-related lectin in a true fern reveals for the first time the widespread distribution and molecular evolution of this lectin family in the plant kingdom.

Lectins are proteins (or glycoproteins), other than antibodies and enzymes, that bind specifically and reversibly to carbohydrates, resulting in cell agglutination or precipitation of polysaccharides and glycoconjugates (1). They are ubiquitous in the biosphere, having been found in viruses, bacteria, fungi, plants, and animals (2). Among the divisions of the plant kingdom, the Pteridophyta, which includes the class Filicinae, or true ferns, have been largely overlooked in the study of lectins. Lectins of known specificity serve as valuable reagents in glycobiological research. They can be employed for the detection and preliminary characterization of glycocojugates on the surface of cells. Although many lectins belong to the same major specificity group of mannos- or mannos/glucose-binding lectins, their different reactivities toward more complex oligo- and polysaccharides render many of them specifically valuable for recognizing a particular type of saccharide structure and fuel the search for yet more novel lectins (3). Lectins are found in greatest quantity and are most readily purified from plant sources, especially storage tissues such as seeds, bark, bulbs, rhizomes, etc. Many lectins have been isolated and characterized from angiosperm subdivision of seed plants. On the basis of structural and evolutionary development, most of these plant lectins have been classified into seven families: legume lectins, chitin-binding proteins, type 2 ribosome-inactivating proteins, monocot mannos-binding lectins, amarathins, curcurbitaceae phloem lectins, and jacalin-related lectins (JRLs) (4). Each family has its own characteristic carbohydrate recognition domain. Jacalin, the prototype of JRLs, was isolated from seeds of jack fruit (*Artocarpus integrifolia*; Moraceae) (5). Subsequently, JRLs have also been isolated and characterized from various plant families of angiosperms such as Convolvulaceae (6), Asteraceae (7), Gramineae (8, 9), Musaceae (10–12), Fagaceae (13), and Mimosaceae (14). Although JRLs are widely distributed in higher plants, no information on JRLs outside of angiosperms is available except for a recently isolated lectin from the Japanese cycad (*Cycas revoluta*) of gymnosperm subdivision (15).

In an *ab initio* search for lectins in understudied groups of plants, we examined the large, fleshy, mesoterranean rhizomes of the tropical fern *Phlebodium aureum* for the presence of cell-agglutinating activity. We report herein the purification of *P. aureum* lectin (PAL), a mannos/glucose-specific lectin present in the rhizomes of this member of the Polypodiaceae family, as well as the cDNA cloning, expression, and characterization of this mannos/glucose-binding lectin and a closely related protein also having lectin activity. cDNA sequencing revealed that these fern lectins are novel members of the JRLs. This is the first report of the molecular cloning of JRLs from a lower plant, fern, which shows the structural and evolutionary relationship of JRLs in the plant kingdom. The two lectin genes were expressed in *E. coli*, and their physicochemical characterization is described and compared with the native lectin. This

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB099932 (PALa) and AB099933 (PALb).

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§ To whom correspondence should be addressed: Dept. of Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI 48109-0606. Tel.: 734-763-3511; Fax: 734-763-4581; E-mail: igoldste@umich.edu.

1 The abbreviations used are: JRL, Jacalin-related lectin; gJRL, galactose-specific JRL; mLJRL, mannose-specific JRL; PAL, *P. aureum* lectin; rPAL, recombinant PAL; nPAL, native PAL; PBS, phosphate-buffered saline; Man₅, Man₅[6]Man₃[3]Man; Heltuba, *H. tuberosus* lectin; HPLC, high pressure liquid chromatography; RACE, rapid amplification of cDNA ends; CRD, carbohydrate recognition domain.

This paper is available on line at http://www.jbc.org
expression system should also be useful for mutagenesis studies to elucidate the structure-function relationship of JRLs.

**MATERIALS AND METHODS**

*Phlebodium aureum* (L.) J. Smith (also classified as *Polypodium aureum* L.) were collected from a specimen plant growing in the greenhouse at the Matthaei Botanical Gardens of the University of Michigan. Positive identification was provided by Dr. David Michener, collections curator of the botanical gardens.

Unless stated otherwise, saccharides and their derivatives and glycoproteins were purchased from Sigma. Ovine submaxillary mucin was a gift of Dr. R. N. Knibbs (University of Michigan). Mannose-Sepharose was prepared by divinyl sulfone coupling of mannose to Sepharose CL-4B (16), and yeast invertase-Sepharose, prepared using cyanogen bromide-activated Sepharose, were available from previous studies.

**Purification of the Lectin**—All procedures were conducted at 4 °C. Pieces of rhizome from *P. aureum* were scraped to remove the soft, fuzzy layer and chopped into approximately 5-mm cubes. The light green chopped tissue (146 g fresh weight) was homogenized and extracted for 2–3 h with 600 ml of extraction buffer (PBS (10 mM sodium phosphate, 0.15 mM NaCl, 0.135 mM CaCl2, 0.04% sodium azide, pH 7.2) containing 10 mM thiourea, 0.25 mM phenylmethylsulfonyl fluoride, and 1 g/liter ascorbic acid), with the addition of 10 g of poly(vinylpolypyrrolidone). The homogenate was squeezed through four layers of cheesecloth and centrifuged at 20,000 g for 30 min. The supernatant was stored at −80°C until use.

**RNA Isolation and cDNA Cloning**—For RNA isolation, chopped rhizome tissue was immediately ground to a powder with a pestle under liquid nitrogen. Total cellular RNA was isolated with Concert Plant RNA reagent (Invitrogen), and subsequently poly(A)⁺ RNA was isolated with the Micro-FastTrack 2.0 kit (Invitrogen). Using this protocol, 1 μg of poly(A)⁺ RNA per 10 g of rhizome tissue was isolated.

A cDNA library for *P. aureum* lectin was constructed with the Marathon cDNA amplification kit (Clontech, Palo Alto, CA). Two degenerate forward primers (PALF1, CARGTGNTTYAGGNNAYGNACNAAR; PALF2, GCAAYGNCGARACNARGARAGTHGAYNTN) were designed from the amino acid sequence VNQLQVYGNGTKLHGXANGQKTEIDYV of a cyanogen bromide cleavage fragment of fern lectin for rapid amplification of cDNA ends (RACE). 3'-RACE was conducted with a combination of primers, adapter primer 1 (Invitrogen) and PALF1, and Platinum PfI DNA polymerase (Invitrogen) as follows. DNA was denatured at 94°C for 5 min, followed by three-step cycles (40 cycles) (92°C for 0.5 min, 50°C for 0.5 min, and 68°C for 1 min) and further extended at 65°C for 15 min. The amplified DNA fragment was subsequently amplified with a primer pair 2 (5'-TTGCCCTATGAGGAGCCTCGAG-3' and 5'-GTCGACCTTTTTCATAGAAACTATCAT-3'). The amplified 0.5-kbp fragment was cloned using Zero Blunt TOPO PCR cloning kit (Invitrogen). Inserted DNA was sequenced with T7 and SP6 primers by the DNA Sequencing Core Facility of the University of Michigan, and two similar but different genes (termed PALa and PALb) including poly(A)⁺ were obtained. Two specific reverse primers for each gene (PALaR1, CGCTATGAAACGGCTCGAGGCTCATACGCTACTGCACGCCTAC; PALbR1, GACATAAGAGCCCGGAGGATCCACAAGGTCCATGTCATCTC) were designed, and 5'-RACE was conducted with adapter primer 1 and PALaR1/PALbR1, respectively.

**Construction, Expression, and Purification of Recombinant Fern Lectin**—The full-length coding sequence PCR products of PALa and PALb including NdeI and XhoI sites into its forward and reverse primers, respectively, were cloned into pCR-Blunt II-TOPO vector (Invitrogen) and subsequently cloned into expression vector pET-22b (+) (Novagen) to generate carboxy-terminally His₆-tagged proteins, yielding pET-PALa and pET-PALb, respectively. The Nova Blue (DE3) strain of *E. coli* harboring expression plasmid pET-PALA and pET-PALB was precultured in 5 ml of LB medium containing 50 μg/ml ampicillin at 37°C for 3 h and was added to 1 liter of medium. After the optical density at 600 nm reached 0.4–0.6, 1 ml of 1 mM isopropyl-β-D-thiogalactoside was added to the medium, and the cells were further cultured at 25°C for 6 h. The induced cells were collected by centrifugation, suspended in a lysis buffer (PBS, containing 10 mM 2-mercaptoethanol, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride), and stored at −80°C.

**Effect of Temperature and pH**—To examine their thermostability, lectin solutions (0.1 mg/ml) of PAS and PALB were incubated in a water bath at 40, 50, 55, 60, or 70°C. After 10 μl of lectin solution was cooled on ice, its hemagglutinating activity was assayed as described above. pH stability of the lectins was determined in the following buffers: 0.1 M glycine-HCl buffer (pH 2.0–3.0), 0.1 mM sodium acetate buffer (pH 4.0–5.0), 0.1 mM sodium phosphate buffer (pH 6.0–7.0), 0.1 M Tris-HCl buffer (pH 8.5–9.0), and 0.1 M glycine buffer (pH 10.0). Solutions of lectin (10 μl) were incubated with 10 μl of buffer at 4°C overnight. After adjusting to pH 8.0 by the addition of 30 μl of 1 M Tris-HCl buffer, pH 8.0, the hemagglutinating activity of the lectin was assayed as described above.
Purification of Native Fern Lectin

Because the hemagglutination activity of crude extracts of _P. aureum_ was inhibited by d-mannose, mannose-Sepharose was used as an affinity adsorbent for isolation of the lectin. After elution of nonabsorbed protein from the mannose-Sepharose column, 0.1 M Me α-Man eluted a sharp band of protein, which in a second affinity chromatography was totally bound and eluted in a similar manner (Fig. 1). The lectin was also bound to and eluted from a column of immobilized yeast invertase under the same conditions. As shown in Fig. 2A, upon SDS-PAGE at pH 8.3 with unheated samples, the lectin preparation obtained from affinity chromatography on mannose-Sepharose or invertase-Sepharose showed a major band at approximately 38 kDa. The low, broad band appearing before and after elution of the sharp lectin peak (Fig. 1A) appeared to contain several bands of nearly equal intensity. Upon rechromatography of the eluted lectin fraction (Fig. 1B), this contaminating broad band was allowed to wash off before commencing elution of the lectin with a haptenic sugar, yielding a slightly more purified material. When lectin samples were boiled in SDS with 2-mercaptoethanol, a single band of approximately 15 kDa was observed (Fig. 2B), suggesting that the native structure is a dimer of this monomer. The earlier and later contaminating material gave a single band at slightly lower mass. We have not investigated the nature of this apparent contaminant further.

Molecular Mass and Subunit Structure

The molecular mass was also estimated by size exclusion chromatography on a silica-based matrix. The purified lectin migrated as a single, nearly symmetrical band of approximately 31 kDa, based on standardization with known proteins (data not shown). Together with the SDS-PAGE analysis, these results indicate that the lectin exists as a dimer of approximately 15-kDa subunits that requires boiling in SDS to dissociate completely.

Amino Acid Composition and _N_-terminal Amino Acid _Sequence_

The amino acid composition of the purified fern lectin (data not shown) indicated that it contains a single residue each of methionine and histidine and the typically large amounts of aspartic acid/asparagine, serine, and glycine observed in many other lectins. Attempts to sequence the _N_-terminal region were not successful; however, after cyanogen bromide cleavage, a large peptide having the _N_-terminal sequence VNGLQVYVGNGTKLHGXANGTKEIIDV was detected. Cleavage with _Achromobacter_ protease I yielded a peptide with the partial sequence LGPGGSGGDSFDDS-N.

**RESULTS**

**Hemagglutinating Activity**—Crude extracts from _P. aureum_ rhizomes weakly agglutinated formaldehyde-stabilized rabbit erythrocytes (titer = 32–64) but not sheep nor any type of human erythrocytes. After purification as described below, the lectin agglutinated rabbit erythrocytes at a minimum concentration of 1 μg/ml (titer at 4 mg/ml = 4000), whereas sheep or any type of human erythrocytes required approximately 150 μg/ml for agglutination (titer at 4 mg/ml = 64). Lectin that was dialyzed extensively against metal-free, EDTA-containing buffer and assayed in the same buffer had identical hemagglutination titer against rabbit erythrocytes, indicating that it has no requirement for metal ions.

**Purification of Native Fern Lectin**—Because the hemagglutination activity of crude extracts of _P. aureum_ was inhibited by d-mannose, mannose-Sepharose was used as an affinity adsorbent for isolation of the lectin. After elution of nonabsorbed protein from the mannose-Sepharose column, 0.1 M Me α-Man eluted a sharp band of protein, which in a second affinity chromatography was totally bound and eluted in a similar manner (Fig. 1). The lectin was also bound to and eluted from a column of immobilized yeast invertase under the same conditions. As shown in Fig. 2A, upon SDS-PAGE at pH 8.3 with unheated, without reducing agent; B, samples in SDS buffer plus 2-mercaptoethanol, heated 5 min in boiling water. Lanes 1 and 10, benchmark protein standards; lane 2, crude extract; lane 3, 80% precipitate; lane 4, mannose-Sepharose unbound; lane 5, bound fraction from first mannose-Sepharose chromatography; lane 6, bound fraction, rechromatography; lane 7, invertase-Sepharose bound fraction; lane 8, PAL preparation II; lane 9, mannose-Sepharose retarded fraction, pooled, concentrated.
Phlebodium aureum Lectin

Table I

| Saccharide | Native PAL | rPALa P<sub>r</sub> | rPALb P<sub>r</sub> |
|------------|------------|--------------------|--------------------|
| Mannose    | 1.6        | 1                  | 0.5                |
| Me α-Man   | 1.6        | 1                  | 0.25               |
| p-Nitrophenyl α-Man | >8.8 | <0.2 | <0.32 |
| p-Nitrophenyl β-Man | 6.4 | 0.25 | 0.13 |
| 1,5-Anhydrido-D-mannitol | 0.8 | 2.0 | 2.0 |
| Glucose    | 6.4        | 0.25               | 0.25               |
| Me α-Glc   | 25         | 0.06               | <0.06              |
| Me β-Glc   | >25        | <0.06              | <0.06              |
| 2-Deoxy-Glc | 6.4 | 0.25 | 0.13 |
| GlevNaC    | 12.8       | 0.13               | 0.13               |
| Fructose   | 12.8       | 0.13               | 0.06               |
| Me β-Fruct | 12.8       | 0.13               | 0.06               |
| Man₁,5Man  | 1.25       | 1.3                | 1.3                |
| Man₁,3Man  | 0.2        | 8.0                | 8.0                |
| Man₁,6Man  | 1.6        | 1.0                | 1.0                |
| Man₁,3ManOMe | 0.31 | 5.2 | 5.2 |
| Man₁,4ManOme | 6.2 | 0.26 | 0.26 |
| Man₁,6ManOme | 1.6 | 1.0 | 1.0 |
| Man₃       | 0.3        | 5.2                | 5.2                |
| Man₄       | 0.08       | 20                 | 20                 |
| Man₁,6(Glc₁,3)Man | 1.5 | 1.0 | 1.0 |
| Manβ₁,4Man | >25        | <0.06              | <0.06              |
| Gal₁,3ManOMe | >12.5 | <0.13 | <0.13 |
| Glev₁,3ManOme | 3.1 | 0.5 | n.d. |
| Glev₁,2Glc(kojibiose) | 6.4 | 0.25 | 0.25 |
| Glev₁,3Glc(nigerose) | 12.8 | 0.13 | 0.13 |
| Glev₁,4Glc(maltose) | 50 | 0.03 | 0.03 |
| Glev₁,4Glc₁,4Glc(maltotriose) | 25 | 0.06 | 0.06 |
| Glev₁,6Glc(isomaltose) | 12.5 | 0.13 | <0.13 |
| Glev₁,2Glc(sophorose) | 6.2 | 0.25 | 0.25 |
| Glev₁,2Glc(gentiobiose) | 25 | 0.06 | <0.13 |

- Minimum concentration causing visible inhibition of agglutination of 4 arbitrary units of lectin. D-Galactose, L-mannose, L-fucose, L-rhamnose, N-acetyl-D-galactosamine, L-xylose, D-lyxose, 2-deoxy-D-ribose, melibiose, lactose, laminaribiose(Glc₂,3Glc), Me α-Fruct, and Me α-Gal exhibited no inhibition at 50 mM.

- Potency relative to Me α-Man with nPAL.

Carbohydrate Analysis—No periodate-Schiff staining bands were observed on SDS-polyacrylamide gels of the purified native lectin, although a small amount of neutral sugar (approximately 0.8–1.5 hexose units/subunit) was detected in some preparations at high concentration by the phenol/sulfuric acid assay. Analysis of such PAL preparations by the method of Fu and O’Neill (19) indicated primarily mannose to be present. Although the partial sequence shown above contains a putative glycosylation sequence (NGT), the asparagine residue must be largely or totally nonglycosylated to be detected by automated sequencing. Most likely, the mannose arises from trace amounts of endogenous mannan or high mannose glycoprotein contaminants, which remain associated with the lectin during the purification procedures, but the lectin itself is not glycosylated.

Inhibition of Agglutination—The ability of a number of mono- and oligosaccharides to inhibit agglutination of formaldehyde-treated rabbit erythrocytes is shown in Table I. It is evident that branched mannose oligosaccharides are the best inhibitors, whereas glucose-containing disaccharides were much less effective. This result indicates that PAL is a mannose/glucose-binding lectin.

Quantity Precipitation and Precipitation Inhibition—In qualitative precipitin tests in capillary tubes, mannans from *Saccharomyces cerevisiae* and *Saccharomyces rouxii* and dextran B-1355-S from *Leuconostoc mesenteroides* (27) gave visible precipitation, whereas many other polysaccharides, glycoproteins, and neoglycoproteins did not. Quantitative precipitin assays (Fig. 3) confirmed that the two yeast mannans precipitated to a significant extent with an equal weight of lectin; dextran B-1355-S at 3–4-fold higher concentration by weight also strongly precipitated the lectin. Two other dextrans from *L. mesenteroides*, B-742-S and B-742-L (27), partially precipitated the lectin at much higher concentrations. Among glycoproteins, only yeast invertase, a high mannose glycoprotein, partially precipitated the lectin. Soybean agglutinin, which also contains a high mannose glycan, was inactive. Precipitation of yeast mannan was inhibited 50% by Man₃ at 3.1 mM, mannose at 19 mM, Me α-Man at 80 mM, or Me β-Man at 130 mM (data not shown). These results are in qualitative agreement with the inhibition of agglutination but show that the precipitation of mannan involves a considerably stronger interaction than does rabbit erythrocyte agglutination, since higher concentrations of inhibitory sugars are required.

Titration Calorimetric Determination of Carbohydrate Binding—Thermodynamic parameters for the binding of various mannose and glucose-containing saccharides are shown in Table II. These results also agree with the previous data, confirming that the branched mannose trisaccharide and pentasaccharides are the best ligands, with the additional mannose units strongly enhancing the binding.

Molecular Cloning of Fern Lectin—3’-RACE with the adapter primers and the degenerate primers that were designed from the cyanojen bromide fragment yielded a 0.5-kbp product. Of eight clones sequenced, seven were identical (PALa), whereas one clone contained a similar but apparently different sequence (PALb). Cloning and sequencing of 5’-RACE products generated two full-length nucleotide sequences including polyadenylation. PALa contains a 24-bp 5’-untranslated region, followed by a 438-bp open reading frame encoding 146 amino acid residues and 272 bp 3’-untranslated region, whereas PALb contains a 5-bp 5’-untranslated region, followed...
by a 438-bp open reading frame encoding 146 amino acid residues and 275-bp 3′-untranslated region (Fig. 4). Neither contain an adenylation signal sequence. Since the isolated lectin contains only one methionyl residue, the N-terminal methionine of mature lectin has been removed, resulting in N-terminal serine. The calculated molecular masses of PALa (14,854 Da) and PALb (14,895 Da) without N-terminal Met are in good agreement with the molecular mass of native fern lectin (nPAL) (14,903 Da) determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Since nPAL appears to be blocked at its N terminus, the slightly higher molecular mass of nPAL than the calculated molecular mass of PALa is probably due to the presence of a blocking group, such as an N-acetyl moiety (M = 42 kDa) present on the native lectin.

The deduced amino acid sequence of PALa contains the same sequences as those found in the native fern lectin, but PALb contains a slightly different sequence (Fig. 4), suggesting that the PALa gene encodes the native PAL. No signal sequence could be discerned in the deduced amino acid sequences of either PAL gene, indicating their syntheses on free polysomes. PALa and PALb have two and one potential N-linked glycosylation site (NX(S/T)), respectively in the sequences.

**Construction, Isolation, and Characterization of Recombinant Fern Lectin—**To express recombinant PALa (rPALa) and PALb (rPALb), the entire open reading frames of each were cloned into expression vector pET22b(+) and introduced into *E. coli* strain Nova Blue (DE3) cells. Active recombinant lectins expressed as carboxyl-terminal His6-tagged fusion proteins were purified from *E. coli* extract by a single chromatographic step on a mannose-Sepharose 4B column. The yields of rPALa and rPALb purified by affinity chromatography were each approximately 4 mg from 1 liter of culture.

The N-terminal amino acid sequences of rPALa ([H-N-SSAGSEVAKLGPGWGGSGGD]) and rPALb ([H-N-SSASSEVAKLGPWGGSGGD]), determined by a gas phase protein sequencer without pretreatment, indicated that the initial methionine of these proteins was also removed in the bacterial expression system but that no N-terminal blocking occurred.

SDS-PAGE analysis of the recombinant lectins was compared with that of native lectin (Fig. 5; cf. Fig. 2). The two recombinant lectins gave virtually the same pattern of bands as did the native lectins in both unheated and heated samples, except for the absence of minor contaminating or isolectin bands in the recombinant samples. After boiling for 5 min either in the presence or in the absence of 2-mercaptoethanol in SDS sample buffer, the three forms each gave a single band at about 14 kDa (nPAL) or 14.5 kDa (rPALa), respectively. The slightly greater apparent size of the recombinant subunits is accounted for by the presence of the His6 tags. Likewise, size exclusion chromatography of intact recombinant lectins in solution gave elution profiles indistinguishable from that of the native lectin, indicating each to have a molecular mass of ~31 kDa.

The minimum concentration for hemagglutination activity of rPALa and rPALb against formaldehyde-treated rabbit erythrocytes was estimated to be 0.6 μg/ml, which is not significantly different from the native fern lectin (1 μg/ml). Sugar-binding specificities (estimated by inhibition of hemagglutination) of recombinant fern lectins, especially rPALa, were very similar to the native lectin (Table I). The branched oligomannosides M2 and M3, as well as Man1–3Man, were the best inhibitors in

**Table II**

| Saccharide | Subunit concentration | n | Kₐ | Kₐ | –ΔH° | –ΔG° | –ΔS° |
|-----------|----------------------|---|----|----|------|------|------|
| Me α-Man | 0.135 [0.75]a | 0.978 | 1.02 | 7.90 | 4.07 | 3.8 |
| Man₁ | 0.416 | 0.135 | 7.43 | 10.33 | 5.23 | 5.1 |
| Man₂ | 0.244 | 0.025 | 39.47 | 12.29 | 6.21 | 6.09 |
| Man₁,Man₃ManOMe | 0.30 | 0.069 | 14.51 | 8.53 | 5.63 | 2.9 |
| Man₁,3Man | 0.244 | 0.076 | 13.11 | 8.16 | 5.57 | 2.6 |
| Man₁,6ManOMe | 0.21 | 0.308 | 3.35 | 8.38 | 4.75 | 3.6 |
| Man₁,6Man | 0.244 | 0.269 | 3.71 | 11.0 | 4.82 | 6.12 |
| Man₁,2Man | 0.32 | 0.183 | 5.46 | 5.63 | 5.09 | 0.54 |
| Me α-Glc | 0.248 [0.75]a | 7.19 | 1.39 | 5.22 | 2.90 | 2.3 |

* Based on 1 binding site for mono- or oligosaccharide per subunit.
* Brackets denote data fitting with value of n fixed.
each case. Most α- and β-diglucosides were weakly inhibitory, but it is noteworthy that maltose (Glcα1–4Glc), laminaribiose (Glcα1–3Glc), and gentiobiose (Glcα1–6Glc) were very weak or noninhibitory at the maximum concentration tested. This pattern of specificity distinguishes these lectins from the mannose/glucose-binding banana lectin, a member of the JRLs (11, 12).

**DISCUSSION**

The so-called “primitive” vascular plants (ferns and fern allies) have only occasionally been investigated for the presence of lectins, despite the widespread occurrence of high levels of lectins in storage tissues (seeds, bark, rhizomes, bulbs, etc.) of flowering plants and the carpophores of fungi and the almost universal occurrence of low levels of lectins and related pro-

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**FIG. 4. Nucleotide sequences and the deduced amino acid sequences of PALa and PALb.** Nucleotides and amino acid residues are numbered on the sides. Identical nucleic acids in the sequence of PALb gene are denoted by a dot; breaks for maximum alignment are shown by a hyphen. Identical amino acids are denoted by an asterisk. The solid and dotted underlines denote the sequences determined by amino acid sequence analysis of the isolated peptides generated by cleavage of the native fern lectin with cyanogen bromide and *Achromobacter* protease I, respectively. The circled asparagine residues denote the putative N-glycosylation sites. #, stop codons.

**FIG. 5. SDS-PAGE of native and recombinant fern lectin.** nPAL, rPALa, or rPALb (5 μg) was mixed with SDS sample buffer with boiling for 5 min and in the presence of 2-mercaptoethanol as indicated. Lanes 4 and 8 contain Benchmark premade molecular mass standards.

rPALa generated quantitative precipitation curves with the same yeast mannans and dextran 1355-S as did native PAL (Fig. 3). Interestingly, rPALa also gave a modest precipitin curve with rabbit liver glycogen, whereas native PAL gave no appreciable precipitation. rPALb precipitated neither dextran 1355-S nor glycogen (Fig. 3B). Native and recombinant fern lectins showed similar stability to pH and temperature. No appreciable change was observed in the hemagglutinating activity of lectins preincubated in a pH range from 4 to 9, and they retained more than half of their activity at pH 2.0, 3.0, and 10.0. In contrast, the lectins are less stable to heat; all forms retained their activity up to 50 °C but lost half of their activity within 15 min at 60 °C.
teins in all types of plant tissues, animals, and microorganisms. Mellor and co-workers (28) purified a hexameric 120-kDa lectin from the Azolla-Anabaena symbiosis and, at lower levels, from nonsymbiotic Azolla (Salviniaceae) plants. This lectin was inhibited by galactose and more weakly by GalNAc but not by lactose. A brief report by Vasheka et al. (29) showed the presence of agglutinating activity toward rat erythrocytes in the rhizomes of three species of Dryopteris (Polypodiaceae) ferns; in one of the species, an extract of the fronds also had high activity. These activities were not purified; nor was any carbohydrate specificity established. Both of these genera are in the class of true ferns, Filicinae. To our knowledge, no other reports of lectins in true ferns or fern allies have been made. This report thus represent the most detailed and extensive investigation of a lectin from the plant division Pteridophyta to date.

Agglutination inhibition, precipitation, and calorimetric titration data all indicate that PAL is a mannose/glucose-binding lectin, although its affinity for glucose is considerably weaker than typical mannose/glucose-binding legume lectins. Mannose-binding legume lectins typically have affinities for glucose of 20–50% that of mannose, whether comparing the free sugars or the Me α-glycosides (2). In the case of PAL, however, affinity for Me α-Glc is ~10% or less that of Me α-Man (Tables I and II). The very weak interaction of PAL with maltose is also in sharp contrast to legume Man/Glc-binding lectins (2). Monocot mannose-binding lectins, however, exhibit no detectable binding to glucose-containing mono- or oligosaccharides (3, 30). PAL may thus be considered to be a new class or subclass of mannose-binding lectins exhibiting weak but measurable activity toward glucose structures.

By definition, a lectin is a sugar-binding protein or glycoprotein of nonimmune origin that agglutinates cells and/or precipitates glycoconjugates (1). In order to form detectable precipitate, however, both the lectin and the glycoconjugate must be multivalent and must be mixed in an appropriate stoichiometric ratio. Furthermore, in order to form the cross-linked aggregated necessary for precipitation, at least one of the components (lectin or polymeric ligand) must possess three or more binding sites; otherwise, only linear, nonprecipitating aggregates can form. The strong precipitation of yeast mannian is readily understandable. Likewise, yeast invertase contains 9–10 N-linked glycan structures/polypeptide, each containing 26–54 mannose residues/residue of N-acetylglucosamine (31, 32), which provides a high density of branched mannose structures for cross-linking. On the other hand, soybean agglutinin contains one Man$_9$GlcNAc$_2$ structure per subunit of a tetrameric protein and is readily precipitated by concanavalin A (2). It fails to precipitate PAL, however, suggesting that this lectin does not bind as tightly, either because of differing fine specificity or poorer accessibility of the binding sites than in the case of concanavalin A. Bovine ribonuclease B possesses a single Man$_6$GlcNAc$_2$ structure per 15-kDa monomer (33) and thus would not be expected to precipitate with the lectin in any case. Although glucose and its oligosaccharides are very poor ligands, highly branched glucan structures might provide sufficient binding interactions to precipitate the lectin. The highly branched dextran B-1555-S, which contains almost equal amounts of α-1,6- and α-1,3-linked glucose units and a small amount of α-1,4 linkages (27), is an especially good precipitant, and those designated B-742-L and -S, which contain about 20% α-1,4 linkages and 0% (L) or 26% (S) α-1,3 linkages, are moderate precipitants. Dextrans B-1208 and B-512 (clinical dextran), almost exclusively (95%) α-1,6-linked, are essentially linear structures. Hence they are not precipitated by the lectin, again in sharp contrast to legume Man/Glc-binding lectins such as concanavalin A. Isolichenan and elisian, which also failed to precipitate the lectin, are linear α-glucans of maltose and maltotriose units linked by α-1,6 or α-1,3 bonds, respectively (11). Interestingly, the banana lectin, a mannose/glucose-binding member of the jacalin-related lectin family, is precipitated by elisian by virtue of reacting with its internal α-1,3-glucan structures but is unreactive with isolichenan (11). As discussed below, PAL is also related to the jacalin family of lectins.

Estimates of relative binding (or dissociation) constants for mono- and oligosaccharides by inhibition of erythrocyte agglutination, direct calorimetric titration, and inhibition of mannan precipitation are in good agreement, although absolute values vary with the assay system used. To the limited extent that precipitation inhibition assays have been carried out, inhibition constants are considerably higher (e.g. 3 versus 0.1 m for Man$_3$ and 80 versus 1–1.6 m for Me α-Man). Inhibition of rabbit cell agglutination also requires somewhat higher concentrations than indicated by dissociation constants determined calorimetrically. These quantitative differences reflect the fact that the lectin’s affinity for mannian is quite strong and involves multiple interactions that are difficult to reverse or inhibit, as compared with the rabbit erythrocyte cell surfaces or binding of monovalent ligands in solution.

It is clear from Tables I and II that the most favorable structural element for binding is the Man$_1$3Man structure. Man$_1$4Man is a much poorer inhibitor than the other dimannosides, as is the case also with the corresponding diglucosides, where maltose requires at least 50 m, whereas the other three gluosidic linkages show somewhat stronger inhibition. Man$_1$2Man, whose linkage involves the axial hydroxyl group of the reducing mannose unit and thus has a considerably different conformation than the other disaccharides, exhibits poorer inhibition or binding than the α-1,3-disaccharide or the monosaccharide, and also appears from the calorimetric data (Table II) to have considerably different entropy and enthalpy contributions to binding. Man$_1$6Man shows enhanced binding over the monomannoside, although this structure in Man$_1$3 has a small variable effect on α-1,3-linked disaccharide binding, depending on the form of the lectin and assay system used. A second α-1,3-linked moiety in the Man$_1$3 structure further enhances the binding by almost the same factor as the first α-1,3-linked residue. On the other hand, the O-methyl glycoside moiety has little effect in the case of the disaccharides as seen in Table II. Also, as noted in the agglutination inhibition studies, mannose may actually be a slightly better inhibitor than Me α-Man, and the bulkier p-nitrophenyl aglycone appears to interfere significantly with binding when in the α- but not the β-anomeric position.

The complete amino acid sequences of two closely related gene products were deduced from clones of two full-length cDNAs, termed PALa and PALb, obtained by 5′-3′-RACE procedure using primers designed from the amino acid sequence of a cyanogen bromide cleavage fragment. The subunits of these proteins were composed of 146 amino acid residues each, with a cyanogen bromide cleavage fragment. The subunits of these proteins were composed of 146 amino acid residues each, with a cyanogen bromide cleavage fragment.
platycephala (Pk) of Mimosaceae (14). Multiple sequence alignment of PALa and PALb with previously isolated JRLs indicated that only 10 amino acid residues are invariant within amino acid sequences of isolated JRLs (Fig. 6). These key residues, consisting mainly of glycine and aromatic residues, are important for making the compact β-prism fold, which is the common structural feature of this family (24). The prism consists of three four-stranded β-sheets (β1-β12), which possess an approximate internal 3-fold symmetry.

The mannos-binding site of Heltuba consists of five residues (Gly118, Gly135, Asp138, Val137, and Asp139) linked to O-3, O-4, O-5, and O-6 of mannose by creating a network of eight hydrogen bonds as shown by X-ray crystallographic studies (24). In addition, Met92 mediates hydrophobic interaction with the pyranose ring of mannose. Four of the six amino acid residues are conserved in the PAL polypeptide (Gly118, Gly132, Asp133, Asp136), although the replacement of Met92 and Val137 of Heltuba with Gly69 and Arg134 of PAL was observed (Fig. 6). The difference in sugar binding specificity between Heltuba and fern lectin might be caused by the replacement of these key residues. Mano1–2Man and Mano1–3Man are equally good inhibitors for the hemagglutination activity of Heltuba. On the other hand, among the α-linked mannosace disaccharides, Mano1–3Man is the best inhibitor of PAL, with the α1,2 and α1,6 being approximately one-fifth as active, whereas Mano1–4Man is essentially a noninhibitor.

JRLs have been classified as galactose-specific (gJRLs) and mannose-specific (mJRLs) according to their sugar specificities, which relate to a specific structural difference. gJRLs including Jacalin and M. pomifera lectin are built up of cleaved proterons consisting of a β-chain (20 amino acids) and an α-chain (133 amino acids). Proteolytic processing of the proproteins generates an N-terminal Gly, whose amino group mediates a hydrogen bond with O-4 of galactose, which is responsible for the galactose-binding specificity of jacalin and M. pomifera lectin. In contrast, mJRLs, including all other JRLs, consist of uncleaved proterons of about 150 amino acid residues. Another major difference between gJRLs and mJRLs is their biosynthesis, processing, and localization. Jacalin (gJRL) is synthesized on the endoplasmic reticulum as a preproteon and is targeted in the vacular compartment after a complex series of processing steps. In contrast, C. sepium lectin (mJRL) is synthesized and localized in the cytoplasm without processing due to the absence of a signal peptide. The mature protein corresponds to the entire open reading frame. A phylogenetic tree constructed based on the amino acid sequences of

**Fig. 6.** Multiple sequence alignment of the CRD of jacalin-related lectins. Hyphens show gaps inserted to achieve maximum alignment. Invariant and conserved amino acid residues in all sequences are boxed with solid lines and dotted lines, respectively. Closed circles, amino acid residues involved in the mannos-binding site of Heltuba. The arrows above the sequence are 12 β-sheets identified in the crystal structure of Heltuba. Jacalin is the galactose-binding lectin from A. integrifolia; Calsepa, C. sepium lectin; Orysata, O. sativa lectin; BanLec, M. acuminata lectin; Pk, P. platycephala lectin. Tandemly repeated JRL-CRDs of C. crenata lectin (CCA-N and CCA-C) and P. platycephala lectin (Pk1, Pk2, and Pk3) were separately aligned for comparison.

**Fig. 7.** Phylogenetic tree of the CRD of jacalin-related lectins. A phylogenetic tree was constructed by the neighbor-joining algorithm based on an evolutionary distance matrix constructed by Kimura’s method. Jacalin is galactose-binding lectin from A. integrifolia; KM+, mannose-binding lectin from A. integrifolia; Calsepa, C. sepium lectin; Orysata, O. sativa lectin; BanLec, M. acuminata lectin; Pk, P. platycephala lectin; MornigaG, galactose-binding lectin from M. nigra; MornigaM, mannose-binding lectin from M. nigra; Calsepa, C. sepium lectin; Orysata, O. sativa lectin; BanLec, M. acuminata lectin; CCA-N, C. crenata lectin (residues 1–154); CCA-C, C. crenata lectin (residues 155–309); Pk-1, P. platycephala lectin (residues 1–147); Pk-2, P. platycephala lectin (residues 150–293); Pk-3, P. platycephala lectin (residues 296–442). Barley is a jacalin-like protein from Hordeum vulgare (residues 148–306).
18 jacalin-related lectin carbohydrate recognition domains (JRL-CRDs) from 15 lectins showed their evolutionary relationships (Fig. 7). The cluster of JRLs is in good agreement with the taxonomic classification of angiosperms, because JRLs from dicots are separated from JRLs from monocots (O. sativa lectin, barley, and M. acuminate lectin). In this tree, JRLs from monocots are evolutionarily closer to PALs from ferns. Two tandemly repeated JRL-CRDs from C. crenata and three tandemly repeated JRL-CRDs from P. platycepha might have been duplicated after the divergence of these plant families. The only other mRNA from a nonangiosperm is that recently isolated and partially characterized from a gymnosperm, Japanese cycad (C. revoluta), but only a partial amino acid sequence is available (15). The identification of mJRLs in a nonperomysotoyte, fern, reveals that JRLs are not restricted to Spermatophyta but rather are widely distributed in the plant kingdom. In contrast, gJRLs, which were believed to be a major subgroup of JRLs, have been found exclusively in the dicot Moraceae. It appears that gJRLs were evolved from mJRLs by the insertion of signal peptide and vacuolar targeting signal after the divergence of Moraceae from other flowering plants. In other plant families, the molecular evolution of this group of lectins can also be observed. The subunits of JRLs from C. crenata and P. platycepha consist of two or three tandemly repeated jacalin-related lectin domains, indicating that these JRLs have evolved by gene duplication and/or exon shuffling. In addition, chimeric JRLs have been identified in Brassica napus. Therefore, it is likely that an ancestral protein of JRLs has evolved to play diverse roles in each plant family. A relatively small number of invariant amino acid residues among JRLs and different sugar binding specificity support this hypothesis.

Although the physiological function of the JRLs is not known, there are several possibilities. mJRLs have been thought to be stress-responsive proteins. A JRL from rice is identical to a salt and drought stress-inducible saNt gene product, and this lectin is expressed only after induction by either jasmone or NaCl. Myrosinase-binding proteins from B. napus, which contain one or two jacalin-related lectin domains in the sequences, are also stress-inducible. mJRLs are also assumed to be self-defense proteins because of their binding affinity to mannos, which is a relatively scarce sugar in plants but is widely distributed in microorganisms, insects, and animals.

The expression system of PALa and PALb containing carboxy-terminal His_{6} tags in E. coli was constructed, and their sugar-binding specificity and physicochemical characteristics were compared with those of the native lectin isolated from fern rhizomes. The studies indicated that rPALa and rPALb resemble the native lectin in most respects. It is not surprising that rPALb had similar sugar binding specificity to rPALa, because they share high (88%) sequence identity and conserve all key amino acids among JRLs (Figs. 4 and 6). rPALa gave precipitin curves with the highly branched α-mannan from yeast and α-D-glucan (dextrans B-1355-S) as expected (Fig. 3B). Interestingly, rPALa also precipitated moderately with rabbit liver glycoprotein, whereas native PAL did not. The carboxy-terminal His_{6} tag and/or the free N terminus of rPALa might account for the difference in binding affinity with glycoprotein. Although rPALa gave a precipitin curve with mannan, in contrast to rPALa, it precipitated neither with dextran nor with glycoprotein, indicating that even small differences in noncritical residues can affect the lectin’s reactivity with polysaccharides.

The identification of a JRL in a true fern further expands the widespread distribution of JRLs in the plant kingdom. Because fern lectin is probably the closest known relative of the common ancestor of JRLs, the finding of JRLs from ferns might help to explain the original function of JRLs in plants. Although the specificity of PAL as detailed here does not suggest any unique utility of this particular lectin at this time, its distinct character is nevertheless of interest in understanding lectin binding interactions. Recombinantly expressed fern lectins will be useful for such investigations by site-directed mutagenesis. Their applications in biological and medical research by virtue of their specificities toward mannose and oligomannosides and their ease of preparation as recombinant expression proteins from E. coli by one-step affinity chromatography might also be significant.

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