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A GALACTOSE-SPECIFIC LECTIN FROM THE RED MARINE ALGA *PTILOTA FILICINA*

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Key Word Index—*Ptilota filicina*; Ceramiaceae; marine algal lectin; agglutinin; haemagglutinin; affinity chromatography; galactose specific lectin.

Abstract—A lectin from the red marine alga *Ptilota filicina* (PFL) was isolated by affinity chromatography on cross-linked guar gum. PFL agglutinated native and papain-treated human erythrocytes with preference for type O erythrocytes. The lectin was inhibited by galactose and its derivatives. The most potent inhibitors were *p*-Nitrophenyl-*N*-acetyl-α-and β-D-galactosaminide. Porcine stomach mucin, bovine submaxillary gland mucin and asialo bovine mucin were also inhibitory. The M_r of PFL, determined by gel filtration, was 56,900. SDS-PAGE gave one band with a subunit M_r of 19,320, indicating the native protein to be a trimer of apparently identical subunits. PFL was shown to be rich in acidic and hydroxyl amino acids but low in basic amino acids. The ten *N*-terminal amino acids were Asx-Thr-Lys-Thr-Tyr-Leu-Leu-Ala-.

INTRODUCTION

Lectins have been isolated from terrestrial plants and animals and their characteristics have been exploited extensively in many aspects of biochemistry and biomedicine. In contrast, few lectins from marine algae have been isolated and characterised in any detail [1]. Those which have been characterised, frequently show properties which are distinct from those of lectins obtained from other sources [2–4]. We report here the isolation and characterisation of a lectin from the red marine alga *Ptilota filicina*.

RESULTS AND DISCUSSION

The aqueous extract of *P. filicina* was exposed to 75% satd $(NH_4)_2SO_4$. The dialysed ppt. was subjected to affinity chromatography on cross-linked guar gum (Fig. 1). After elution with 0.1 M galactose the pooled lectin peak was dialysed extensively against distilled water and conc. by lyophilization. The purification protocol is summarised in Table 1. About 95-fold purification of the PFL was achieved by this method. Cross-linked guar gum, a galacto-mannan consisting of chains of β-D-mannose with α-D-galactose linked

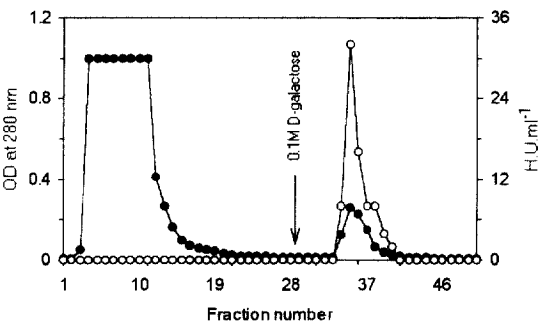

Fig. 1. Affinity chromatography on cross-linked guar gum of PFL. Ammonium sulphate fraction (0/75%) was applied to the column (1.8 × 7 cm), equilibrated and eluted with PBS, at a flow rate of 20 ml h^{-1}. The adsorbed lectin was eluted with 50 mol 0.1 M D-galactose in PBS. Fractions of 3 ml were collected and assayed for haemagglutinating activity using blood group O papain-treated erythrocytes. H.U. = haemagglutinating units. (●—●) *A* 280 nm, (○---○) haemagglutinating activity.

(1 → 6) as single unit side chains, has been used as an efficient, inexpensive and rapid general affinity medium for the purification of galactose-binding lectins from land plants [5–10].

The purified lectin appeared as a single band on SDS-PAGE (Fig. 2), corresponding to a M_r of 19,320 ± 450 (average of four determinations). The homogeneity of PFL was also observed by a single

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Table 1. Purification of *Ptilota filicina* lectin.

Fraction	Total protein (mg)	Titre*	Specific activity†	Purification (fold)
Crude extract	125.0	16	20	1.0
75% $(NH_4)_2SO_4$ ppt.	74.0	64	28	1.4
Affinity chromatography	2.3	1024	1900	95.0

*Titre is reported as the inverse of the last dilution with positive agglutination against blood group O papain-treated erythrocytes.

†Specific activity is expressed as titre ml^{-1} mg^{-1} protein.

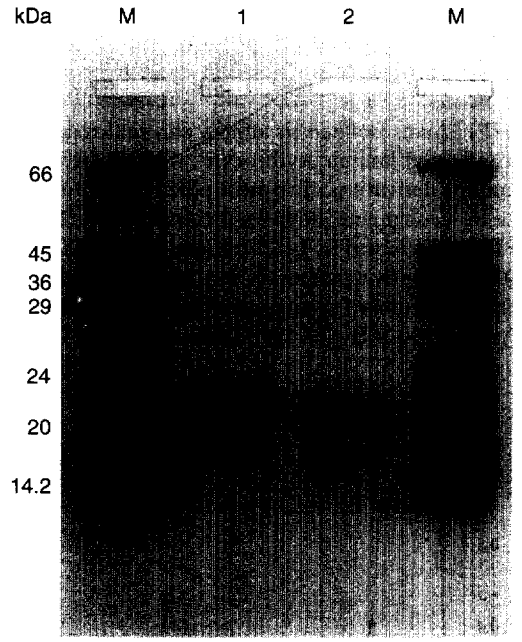

Fig. 2. SDS-PAGE of PFL. Lanes 1 and 2 represent PFL (10 and 20 μg ml^{-1}). Lanes M = Molecular weight markers. M_r markers: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate-dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa).

symmetrical peak obtained by molecular exclusion chromatography on Bio Geel P-100 in PBS (not shown), with apparent M_r of the native lectin of $56{,}900 \pm 670$ (average of four determinations). These results suggest therefore, that PFL is a trimer of apparently identical subunits. Although homotrimeric lectins are unusual, some have been reported, such as the lectin purified from the land plant *Sarothamnus welwitschii* [11]. We have also found lectins from the closely related species *P. serrata* (PSL) and *P. plumosa* (PPL), to be trimeric lectins, with subunits of 18,390 and 17,440 M_r, respectively (unpublished results).

This property of lectins from species of the same genus showing very close similarity with regard to their physical characteristics, is well established. The lectins from the red marine algae *Bryothamnion seafor-thii* and *B. triquetrum* [12] share similar characteristics (M_r of 4,500 and 3,500 respectively), as do the lectins isolated from various species of the green marine algal genus *Codium* [13].

The amino acid composition of PFL is shown in Table 2. The lectin is rich in threonine, aspartic acid and glycine and has a low content of histidine and methionine. Tryptophan was not detected. The amino acid composition of PFL is similar to the composition of other marine algal lectins, with large amounts of acidic and hydroxyl amino acids and small amounts of basic amino acids [14–16]. The *N*-terminal amino acid sequence of PFL was found to be Asx-Thr-Lys-Thr-Tyr--Leu-Leu-Ala--. The amino acids in positions 6 and 10 could not be detected. No homology was observed when the *N*-terminal was checked in the GENINFO®BLAST NEtwork–SwissProt sequences.

Most marine algal lectins are glycoproteins [1–4] but we had insufficient material to test PFL for carbohydrate.

PFL was able to agglutinate native and papainised human erythrocytes. Papain-treated erythrocytes from all blood groups gave higher titration values than those obtained with equivalent native cells. Human blood group O erythrocytes, native or papainised, were agglutinated at one or two dilutions higher than equivalent A or B cells. In contrast to many other marine algal lectins [1, 3, 17, 18] PFL requires metal ions for haemagglutinating activity. Treatment of

Table 2. Amino acid composition of PFL

Amino acid	Mol%	Amino acid	Mol%
Asx	12.0	Tyr	2.2
Glx	5.7	Val	7.2
Ser	7.7	Met	0.9
Gly	9.4	Cys	7.0
His	0.6	Ile	2.5
Arg	2.4	Leu	5.4
Thr	19.2	Phe	3.7
Ala	8.7	Trp	ND
Pro	3.2	Lys	2.0

ND-not detected

PFL with 5 mM EDTA diminished haemagglutinating activity to 12.5% of the original level. The addition of the divalent cations Ca^{2+}, Mn^{2+} or Mg^{2+} totally restored haemagglutinating activity. The lectin was stable in the pH range 4 to 9, retaining 50% of its haemagglutinating activity at pH 3 and 10 and 25% at pH 2 and 11–12. In addition, the haemagglutinating activity of PFL rapidly declined when the lectin was heated above 50° for 30 min, and was totally abolished at 70° for 30 min, indicating that PFL activity depends on the native protein conformation of the lectin.

The results of inhibition tests by a large number of simple sugars and glycoproteins are shown in Table 3. In contrast to the great majority of algal lectins [1, 3, 4] PFL was strongly inhibited by simple sugars, such as galactose and its derivatives. However, glucose and its derivatives, L-fucose, rhamnose, trehalose, mannose, *N*-acetyl-mannosamine, galactose-6-phosphate, arabinose, NeuNAc, muramic acid or galacturonic acid had no effect on haemagglutinating activity. The most potent inhibitors were *p*-nitrophenyl-*N*-acetyl-α- and β-D-galactosaminide (0.04 mM). These results show that the lectin does not differentiate between α and β linkage, since lactose (β-anomeric linkage) inhibits the activity to the same degree as melibiose (α-anomeric linkage). Like some plant lectins [19–21], PFL seems to possess a hydrophobic region in the vicinity of the carbohydrate binding site, since the inhibition shown by nitrophenyl-galactosides was stronger than methyl-D-galactosides. The importance of a C-5 hydroxymethyl group in the pyranose ring was indicated by the failure of galacturonic acid and galactose-6-phosphate to act as inhibitors, as has been observed with peanut lectin [22]. Also, the configuration at C-4 is important, since neither glucose nor glucose derived compounds showed any inhibitory activity. Furthermore, replacement of the free hydroxyl group at C-2 by an acetamido group does not abolish the inhibitory capacity and, therefore, C2 configuration is probably not important in the sugars to which PFL binds. D-fucose has a very similar configuration to D-galactose and the results show that these sugars have a similar grade of inhibition. The addition of a nitrophenyl group at C-1 to both, increased the binding of these sugars to the lectin. This evidence supports the suggestion that there is a hydrophobic pocket in, or near, the sugar-combining site of PFL, as observed for the lectin from velvet bean (*Mucuna derrigiana*) [23].

Table 3. Substances inhibitory to the lectin from *Ptilota filicina*

Substances	Minimum inhibitory concentration*
p-Nitrophenyl-*N*-acetyl-α-D-galactoside	0.04 mM
p-Nitrophenyl-*N*-acetyl-β-D-galactoside	0.04 mM
o-Nitrophenyl-*N*-acetyl-α-D-galactoside	0.09 mM
o-Nitrophenyl-β-D-fucoside	0.09 mM
p-Nitrophenyl-α-D-galactoside	0.19 mM
p-Nitrophenyl-β-D-fucoside	0.19 mM
p-Nitrophenyl-β-D-galactoside	0.19 mM
o-Nitrophenyl-*N*-acetyl-β-D-galactoside	0.39 mM
o-Nitrophenyl-α-D-galactoside	0.39 mM
o-Nitrophenyl-β-D-galactoside	0.39 mM
Lactose	0.39 mM
N-Acetyl-galactosamine	0.39 mM
Melibiose	0.39 mM
D-Galactose	0.78 mM
Methyl-α-D-galactoside	0.78 mM
Galactosamine-HCl	1.56 mM
Methyl-β-D-galactoside	1.56 mM
Raffinose	1.56 mM
2-Deoxy-D-galactose	1.56 mM
D-Fucose	1.56 mM
Lactulose	1.56 mM
Fucoidan	625 μg ml^{-1}
Porcine stomach mucin	<4.8 μg ml^{-1}
Asialo bovine mucin	78 μg ml^{-1}
Bovine submaxillary gland mucin	310 μg ml^{-1}

* Minimum concentrations required for inhibition of 4 haemagglutinating units of the lectin. D-arabinose, L-fucose, galactose-6-phosphate, D-galacturonic acid, D-glucose, *N*-acetyl-glucosamine, 2-deoxy-D-glucose, glucuronic acid, glucosamine-HCl, *p*-nitrophenyl-α-D-glucoside, *p*-nitrophenyl-β-D-glucoside, mannose, *N*-acetyl-β-mannosamine, muramic acid, *N*-acetyl-neuraminic acid, rhamnose were not inhibitory at concentrations up to 50 mM. Egg albumin, fetuin, α-acid glycoprotein, lactoferrin, ovomucoid, thyroglobulin, apo-transferrin were not inhibitory at concentrations up to 2.5 mg ml^{-1}.

Remarkably, PFL showed no inhibition by L-fucose, but was inhibited by fucoidan, a polysaccharide composed of sulphated L-fucose. Similar results have been observed for the lectin from *P. serrata* (unpublished results) and the lectins from the green marine algae *Ulva lactuca* and *U. laetevirens* [24, 25]. Possibly, the lectins react with a more extended structure than the monosaccharide. It has been reported that larger, more complex polysaccharides interact with secondary sites on the lectin surfaces as well as with the primary binding site [26].

From the glycoproteins tested, porcine stomach mucin (Table 3), a glycoprotein with terminal GalNAc residues, as well as having fucose and galactose as internal residues [27], exhibited the highest inhibitory effect. Bovine submaxillary gland mucin, which has NeuNAc as a terminal residue linked to GalNAc, was 64 times less inhibitory. In fact, elimination of sialic acid from bovine mucin rendered this molecule four times more inhibitory than the parental glycoprotein. Asialo bovine mucin has mainly terminal β-galactosyl residues [28]. The neuraminic acid residues occur at C-6 on the sub-terminal galactose residue in bovine mucin, showing that the hydroxyl group at C-6 has influence in the binding of PFL to galactose residues

of this glycoprotein. A similar observation has been made with the lectin from the sea worm *Chaetopterus variopedatus* [29]. Moreover, sialoglycoproteins with terminal NeuNAc groups (fetuin, α_1-acid glycoprotein, transferrin and lactoferrin) and the high-mannose type glycoproteins (ovomucoid, thyroglobin and ovalbumin) failed to inhibit the activity of PFL.

EXPERIMENTAL

Materials

Ptilota filicina J. Ag. was collected on the Oregon coast, U.S.A., stored at −40° and transported to our laboratory in dry-ice. Guar gum and ephychlorohydrin were purchased from Sigma. Bio Gel P-100 was obtained from Bio Rad Lab. All sugar and glycoproteins used for the sugar inhibition assays and the MW-SDS-70L kt, were from Sigma. All the other reagents were of analytical grade.

Preparation of aqueous extract

P. filicina was ground to powder in liquid N_2, homogenised in two vol of 0.17 M NaPi buffered saline (PBS), pH 7.3, for 18 h with stirring at 4°. The suspension was filtered through gauze. This step was repeated twice. The combined filtrates were centrifuged at 15,000 *g* for 30 min at 4° and the aq. phase pptd with 75% satd $(NH_4)_2SO_4$ for 18 h. After centrifugation at 15,000 *g* for 30 min at 4°, the pellet was resuspended in PBS, dialysed against dist. H_2O then PBS. The dialysate was clarified by centrifugation at 15,000 *g* before further purification.

Purification of PFL

The supernatant was subjected to affinity chromatography on a column (7 × 1.8 cm) of cross-linked guar gum, prepared as described in Ref. [6], equilibrated and washed with PBS. The lectin was eluted with 0.1 M galactose in PBS. The active fractions were pooled, dialysed extensively against H_2O, concd by ultrafiltration (Amicon, Ltd-membrane size exclusion 10,000), then lyophilised.

Preparation of human red blood cells

Human red blood cells were obtained from Wessex Regional Transfusion Centre, Southampton, U.K. Native erythrocytes were prepared by washing the red cells 3 times with PBS, then resuspending at a final concn of 5% in PBS. Enzyme-treated erythrocytes were prepared by first washing native cells 3 times with PBS. Washed packed red cells were suspended in an equal vol of papain (BDH Chemicals Ltd) (0.1%; w/v), left at 37° for 30 min and then washed again 3 times with PBS and adjusted to a 5% suspension in PBS.

Haemagglutination and haemagglutination-inhibition assays

The haemagglutinating activity was determined using serial 2-fold dilutions of lectin in small glass tubes [30]. The activity was expressed as the reciprocal of the highest dilution showing positive macroscopic agglutination, using human blood group O erythrocytes, unless otherwise stated. For the haemagglutination-inhibition studies, aliquots of sugar or glycoprotein (100 μl) were serially diluted with PBS, mixed with equal vol of lectin soln containing 4 haemagglutinating units (HU). After 1 h at room temp., 100 μl of 5% human red cell suspension was added and further incubated for 1 h. The minimum conc. of sugar of glycoprotein showing inhibition of haemagglutination was recorded [30].

Effect of EDTA and divalent cations

The effect of EDTA and divalent cations on haemagglutinating activity was carried out on serial 2-fold dilutions of lectin prepared in 0.15 M NaCl alone or 0.15 M NaCl containing 5 mM EDTA. Papain-treated human erythrocytes (5%) were prepared in 0.15 M NaCl with 5 mM EDTA and used an indicator cells. Equal vols. of 10 mM $CaCl_2$, $MgCl_2$ or $MnSO_4$ were added later to haemagglutination tests performed in the presence of EDTA to evaluate their capacity to restore haemagglutination.

Effect of temperature and pH

The haemagglutinating activity was determined after incubating aliquots of lectin at different temps (40–90°). The heated soln was rapidly cooled in ice and assayed for agglutinating activity. Results were expressed as a percentage of the haemagglutination shown by a control kept at 20° for 30 min. The buffers used to study the stability of PFL under different conditions of pH were: 0.2 M NaOAc buffer, pH 3–6; 0.2 M Tris-HCl buffer, pH 7–9 and 0.2 M glycine-NaOH buffer, pH 10–12. Identical vols of purified lectin were dialysed from 18 h against buffers, then 18 h against PBS and assayed for haemagglutinating activity. Results are expressed as the reciprocal of the highest dilution showing positive agglutination. The control values were the agglutination titre of purified lectin in PBS.

M_r *of the lectin*

This was determined by gel filtration on a Bio Gel P-100 column in PBS (60 × 1.6 cm), calibrated with BSA (66,000), ovalbumin (45,000), carbonic anhydrase (29,000), myoglobin (18,000) and cytochrome C (12,400).

Polyacrylamide gel electrophoresis

The M_r of PFL was determined by SDS-PAGE carried out at pH 7, using LKB-Multiphor II electrophoresis equipment with a gel containing 10% (w/v) acrylamide. Samples and standards were prepared in 20% SDS and 1% 2-mercaptoethanol, then heated at 100° for 2 min. A standard picrate Coomassie-blue method was used for staining the gel following electrophoresis. Protein markers used were BSA (66,000), ovalbumin (45,000), carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (20,100) and α-lactalbumin (14,200).

Amino acid analysis was carried out on an Applied Biosystems 420H Amino Acid Analyser with automatic hydrolysis and derivatisation, employing a C18 reverse phase narrow bore cartridge.

N-terminal amino acid analysis of PFL was analysed on an Applied Biosystems ABI 477A Protein Sequencer.

Protein determination was by the method of Ref. [31], using BSA as standard. Eluates of the columns were monitored by A 280 nm.

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