Erythroagglutinin from *Phaseolus coccineus* Var. Alubia: Chemical Characterization, Sugar Specificity, and Effect on Blood Coagulation Factors

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Purification of the erythroagglutinin from *Phaseolus coccineus* var. Alubia was achieved by affinity chromatography on human α_1-acid glycoprotein and by ion exchange chromatography. The lectin is a tetrameric glycoprotein of 31 kDa/subunit with 8% sugar by weight, which agglutinates erythrocytes without serological specificity and is devoid of mitogenic activity toward human peripheral lymphocytes. The specificity of the erythroagglutinin is directed toward the Gal (β1−4) or (β1−3) GlcNAc (β1−2) Man (α1-) saccharidic sequence present in bi- or triantennary *N*-acetyllactosamine-type *N*-glycopeptides or related glycans. Alubia erythroagglutinin inhibits the generation of human thrombin, very probably by protecting prothrombin from enzymatic cleavage.

Keywords: *Phaseolus coccineus lectin; glycoproteins; lectin sugar specificity; coagulation factors*

INTRODUCTION

Lectins from a number of cultivars of the common bush bean *Phaseolus vulgaris* have been isolated (Felsted *et al*., 1981; Vargas-Albores *et al*., 1993). Some varieties of these lectins correspond to a mixture of five tetramers derived from the combination of two subunits, namely, erythroagglutinin (E-PHA) and leukoagglutinin (L-PHA), which are noncovalently associated (Osborn *et al*., 1983). Other varieties are constituted by three, two, or one lectin subunit (Brown *et al*., 1982; Leavitt *et al*., 1977). The presence of homologous lectins has been suggested in other members of the *Phaseolus* genus, such as *Phaseolus coccineus*, but so far only a few examples have been found. Examples are the lectin from scarlett runner seeds *(Phaseolus coccineus* L.), which is specific for *N*-acetyl-D-galactosamine formed from four identical 34 kDa subunitss and possesses erythroagglutinating and mitogenic activities (Nowakova and Kocourek, 1974), and the new anti-A lectin from *P. coccineus* var. Oaxaca, which is also specific for *N*-acetyl-D-galactosamine (Feria *et al*., 1996). *P. coccineus* var. Rubronanus (Shi *et al*., 1993) and *P. coccineus* var. Alubia lectins (Ochoa and Kristiansen, 1982) are not blood group specific; Alubia lectin was shown to have mitogenic and immunosuppressive activities (Calderon and Cordoba, 1976).

The blood coagulation system, together with the complement reaction, is of interest because a series of zymogen activation reactions forms a cascade, and all of these zymogens are glycoproteins (Varki, 1993). The specific role of the carbohydrate moieties of glycoproteins as signals determining the rate of clearance from the circulation or the level of protection against hydrolysis has been documented; however, the role of glycans in prothrombin formation has not yet been clearly identified (Varki, 1993).

Until now, Alubia lectin has been considered a noninhibitable lectin (Ochoa and Kristiansen, 1982). The aim of this study was to identify the nature of the oligosaccharide specificity of th *P. coccineus* var. Alubia erythroagglutinin and its application in the evaluation of the functional role of glycans in clotting factors.

MATERIALS AND METHODS

Materials. *P. coccineus* var. Alubia seeds were obtained in a local market and classified at the Centro de Investigaciones Biológicas, Universidad de Morelos, Morelos, Mexico. Pronase (from *Streptomyces griseus* fraction XXV), neuraminidase (from *Vibrio cholerae* fraction V, EC 3.2.1.18), pig stomach mucin grade II, fetuin grade II, bovine serum albumin, β-galactosidase from *Canavalia ensiformis* (jack bean), and all sugars were purchased from Sigma Fine Chemicals (St. Louis, MO). Bio-Gel P-2 was from Bio-Rad (Richmond, CA). Glycopeptides and asialoglycopeptides from human transferrin and lactoferrin and bovine lactoferrin were gifts from Prof. Genevieve Spik; (α2−3) and (α2−6)sialyllactose from human milk, Gal(β1−3)Gal, and hen ovomucoid were provided by Prof. Gerard Strecker from the Université des Sciences et Technologies de Lille, France. The glycopeptides used in this study are currently used as standards for ^{1}H-NMR and mass spectrometry (Montreuil *et al*., 1986).

Lectin Purification. *P. coccineus* var. Alubia lectin (Alubia lectin) was purified from saline extracts of the ground seeds by affinity chromatography on a human α_1-acid glycoprotein−Sepharose 4B column (Pharmacia, Uppsala, Sweden) as previously described for the purification of phytohemagglutinin from red kidney bean (*Phaseolus vulgaris*) (Zenteno *et al*., 1994). The lectin was eluted from the column by adding 3% acetic

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acid in water. Four Alubia isolectins were purified from the affinity chromatography purified lectin by cationic exchange chromatography on a SP-Sephadex column (Pharmacia) as described by Felsted *et al.* (1977); the column was equilibrated with 5 mM sodium citrate, pH 5.5, and isolectins were eluted with a 0−0.5 M NaCl gradient. Homogeneity of the purified isolectins was analyzed by SDS−polyacrylamide gel electrophoresis according to the method of Laemmli (1970). The molecular mass standards (Pharmacia) used were bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

Analytical Methods. Protein concentration was determined according to the method of Lowry *et al.* (1951), using bovine serum albumin as standard. Total carbohydrate of glycans and modified glycoproteins was determined by using the phenol−sulfuric acid method (Dubois *et al.*, 1956), using lactose as standard. Carbohydrate composition analysis was carried out by methanolysis in the presence of *meso*-inositol (Sigma Chemical) as internal standard; the per-*O*-trimethylsilylated methyl glycosides (after *N*-reacetylation) were analyzed by gas chromatography using a capillary column (25 × 0.32 mm) of 5% Silicone OV 210 (Applied Science Laboratories, Buffalo, NY), in a Varian 2100 gas chromatograph (Orsay, France) equipped with a flame detector and a glass solid injector; the carrier gas was helium at a pressure of 0.6 bar, and the oven temperature was programmed from 150 to 250 °C at 3 °C/min as described by Montreuil *et al.* (1986).

Amino Acid Analysis. A 100 μg sample was hydrolyzed under vacuum with 2 mL of 6 M HCl at 110 °C in sealed tubes for 24, 48, and 72 h. The samples were analyzed on an automatic amino acid analyzer, Durrum 500, according to the procedure of Bidlingmeyer *et al.* (1984), using norleucine as internal standard.

Hemagglutinating Activity. Human erythrocytes types A, B, O, M, N, Le^a, Le^b, P, S, and Kell from healthy human donors were from the Central Blood Bank, IMSS, Mexico. The lectin hemagglutinating activity was assayed in microtiter U plates (Nunc, Roskilde, Denmark) by the twofold serial dilution procedure. The hemagglutinating activity was tested either with 2% (v/v) erythrocyte suspension in phosphate-buffered saline (PBS; with 0.01 M sodium phosphate, 0.14 M sodium chloride, pH 7.2) or with neuraminidase-treated (0.1 unit of *V. cholera* neuraminidase/0.5 mL of packed erythrocytes at 37 °C for 30 min) or Pronase-treated (100 μg/0.5 mL of packed erythrocytes at 37 °C for 30 min) erythrocytes.

Preparation of Glycans and Glycopeptides. Pronase digestion of glycoproteins (fetuin and α_1-acid glycoprotein) was performed by incubating 10 mg of glycoprotein with 1 mg of enzyme for 48 h at 37 °C. Glycopeptides were desalted on a Bio-Gel P-2 column (2 × 60 cm) equilibrated with water. Glycopeptides were desialylated by incubation at 100 °C for 1 h in the presence of 0.02 N sulfuric acid, as described by Spiro and Bhoyroo (1974), and desalted on a Bio-Gel P-2 column (2 × 60 cm) equilibrated with water. β-Galactosidase digestion of asialoglycopeptides from fetuin and α_1-acid glycoprotein was carried out at 37 °C for 2, 4, and 8 h with 10 mU of jack bean β-galactosidase for each 100 μM of glycan as described by Spik *et al.* (1975). Then the asialoagalactoglycopeptides obtained after 12 h of incubation were desalted on a Bio-Gel P2 column equilibrated with water and lyophilized until use.

Sugar Specificity. The sugar specificity of Alubia erythroagglutinin was determined by comparing the inhibitory activity of various sugars, glycans, and glycopeptides on the agglutination induced by the lectin against human type O erythrocytes. Results are expressed as minimal concentration required to completely inhibit four hemagglutinating doses (titer = 4). The molar concentrations of glycans and glycopeptides, as well as their asialo- or asialoagalacto- forms, were determined on the basis of their monosaccharide content as determined by gas chromatography.

Mitogenic Assays. Peripheral blood mononuclear cells from human health donors were prepared from heparinized peripheral venous blood by centrifugation over a Ficoll-Isopaque gradient (density = 1.067). The cells were washed with Hanks' balanced salt solution (GIBCO, Grand Island, NY), and the residual erythrocytes were lysed by the addition of Tris−0.83% NH_4Cl solution; the cells were then washed and suspended in Dulbecco's modified Eagle culture medium (GIBCO) containing 10% heat-inactivated fetal calf serum (Sigma Chemicals). Cultures were carried out in 96 well flat-bottom culture plates (GIBCO WARE, Grand Island, NY). Each well contained 4×10^5 cells in 0.2 mL of culture medium, and the optimal concentration of Alubia lectin, its isoforms, or Con A (Sigma Chemicals) as control; incubation was for 72 h in a humidified incubator at 37 °C in 7% CO_2 in air. One microcurie of methyl[^{3}H]thymidine (^{3}HTdr, specific activity = 6.7 Ci/mmol; New England Nuclear, Boston, MA) was added to each well 18 h before the incubation was ended, and its incorporation into DNA was measured in a Beckman LS6000 SE counter (Beckman Instruments, Fullerton, CA). All of the experiments were performed in triplicate.

Effect on Blood Coagulation Factors. The activated partial thromboplastin time (APTT) from normal or protein C resistant plasma was tested by a chromogenic assay in flat-bottom microtiter plates (Evergreen Sci., Richmond, CA), using the Neothromtin-CTS kit (Behringwerke AG, Marburg, Germany). The activity was measured photometrically at 405 nm using an ELISA microreader, with measurements every 5 s (Ponjee *et al.*, 1991).

The prothrombin time determination of normal plasma was tested by a chromogenic assay using the thromboplastin reagent Chromquick (Behringwerke AG). The activity was measured at 405 nm using an ELISA microreader (Francis *et al.*, 1985).

The thrombin time determination of normal plasma was performed at 37 °C with a photometric technique according to the method described by Penner (1974), with bovine α-thrombin (as control) and CTS thrombin reagent (Behringwerke AG). The activity was measured at 405 nm every 5 s.

The protein C activity was determined in an amidolytic assay, performed with normal plasma and *Agkistrodon contortrix* (Protrac, American Diagnostica Inc., Greenwich, CT). The reaction was developed with a chromogenic substrate, and the activity was measured at 405 nm (Francis and Seyfert, 1987).

The activity of protein S was determined in normal plasma and tested according to the method of Kamiya *et al.* (1986). The protein S deficient plasma free of C4bp, as control, activated human protein C, and the S starting reagent (venom of *Vipera russelli* and soybean-derived phospholipids) used in this test were obtained from Protrac, American Diagnostica Inc.; the coagulation time of these samples was determined in an ST4 semiautomatized coagulometer (Diagnostics Stago, Asnieres, France) (Kamiya *et al.*, 1986).

Normal plasma samples were obtained from Diagnostics Stago. Human activated protein C and protein C resistant plasma (with 42% sensitivity) were obtained from Behringwerke AG Diagnostic.

The effect of Alubia erythroagglutinin on the activity of coagulation factors was tested by adding, to each plasma sample, different concentrations of the lectin in the buffer used as diluent in each test. Specificity of the interaction was tested by adding the optimal lectin concentration previously incubated with 0.02 μM of fetuin *N*-glycopeptides. Neither *O*-glycopeptides from fetuin at 10 μM nor simple sugars at 200 mM induced modifications of the effect of Alubia erythroagglutinin on the coagulation factors tested.

RESULTS

Purification of the Alubia Erythroagglutinin. The lectin from *P. coccineus* var. Alubia was purified by affinity chromatography on an α_1-acid glycoprotein Sepharose 4B column. The lectin was eluted from the affinity matrix by decreasing the pH of the eluent to pH 2.8. The yield of protein is 8% from the total saline extract, and the hemagglutinating activity recovered in this fraction represents 78% of the initial activity applied onto the affinity chromatography column (Table 1). Alubia lectin is a 120 kDa glycoprotein composed of a mixture of isoforms, which were resolved into four

Table 1. Purification of Alubia Lectin and Isolectins[a]

fraction	protein (mg)	hemagglutinating specific activity[b]	mitogenic index[c]
crude extract	1728	1.5	3.4
affinity chromatogr			
nonretained	1640	0.0	0.0
Alubia lectin	31.1	6164	7.9
ion exchange chromatogr			
F I	8.2	140263	1
F II	5.4	9940	1
F III	3.1	4329	3.9
F IV	3.0	0.0	12.8

[a] From 10 g of Alubia flour. [b] Hemagglutinating activity/mg of protein determined in the presence of human erythrocytes type O. [c] Increase in the incorporation of ^{3}HTdr in stimulated human peripheral lymphocytes at optimal protein concentration of 10 μg/mL, compared with the nonstimulated lymphocytes.

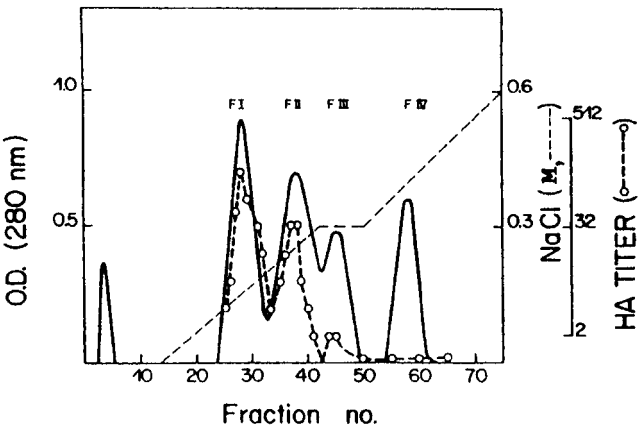

Figure 1. Purification of Alubia isolectins by ion exchange chromatography on Sp-Sephadex. The column was equilibrated with 5 mM sodium citrate, pH 5.5, and the isolectins were eluted with a discontinuous NaCl gradient (0−0.5 M).

fractions by ion exchange chromatography on an SP-Sephadex column (Figure 1). Characteristically, fraction F I (further termed Alubia erythroagglutinin) contained 60% of the hemagglutinating activity from Alubia lectin (Figure 1; and Table 1); no mitogenic activity was identified in this fraction. The Alubia fraction F IV is devoid of hemagglutinating activity toward human erythrocytes but shows strong mitogenic activity toward human peripheral lymphocytes, as determined by the increased rate of ^{3}HTdr incorporation (further termed as mitogenic Alubia). The Alubia isolectins are tetrameric proteins derived from the association of 31 kDa subunits, as determined in the SDS−polyacrylamide gel electrophoresis (Figure 2).

Amino acid analyses of the Alubia erythroagglutinin (F I) and the mitogenic (F IV) isolectin are very similar. They contained mainly aspartic acid, serine, glycine, threonine, and leucine residues but lacked methionine and cysteine residues. As indicated in Table 2, both isolectins show slight differences in the concentration of nonpolar amino acids such as phenylalanine, leucine, and tyrosine residues. Neutral sugars represent 8% of the FI and 5% of the F IV isoforms total weight. Gas chromatography analysis revealed the presence of mannose, *N*-acetyl-D-glucosamine, galactose, and xylose in molar ratios of 3:4:0.2:1 for F I and 3:4.7:1:0.5 for F IV, respectively.

Biological Activity of Alubia Isolectins. The Alubia erythroagglutinin (F I isoform) does not possess blood group specificity since it agglutinated human type A, B, and O erythrocytes independently of the phenotype (N, M, Le^a, Le^b, Kell, P, or S) at lectin concentrations

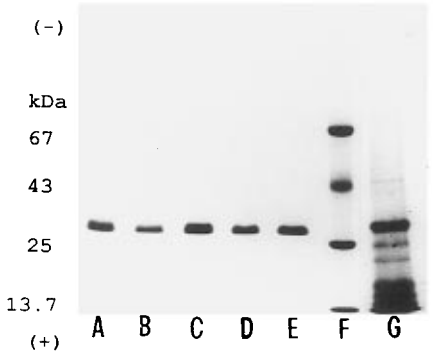

Figure 2. SDS electrophoresis of 5 μg of the purified *P. coccineus* var. Alubia lectin and its isolectins: (A) the affinity-purified Alubia lectin; (B−E) Alubia lectin isoforms I−IV, respectively; (F) molecular weight standards (Pharmacia) used were bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa); (G) 15 μg of Alubia crude extract.

Table 2. Amino Acid Composition of *P. coccineus* Var. Alubia Lectin and Isolectins F I and F IV[a]

	residues/100 amino acids		
		isolectin	
amino acid	lectin	F I	F IV
Asx	12	12	12
Glx	11	8	7
Ser	10	12	11
Gly	8	9	9
His	1	1	2
Arg	3	3	3
Thr	7	9	9
Ala	7	7	7
Pro	4	5	4
Tyr	1	1	2
Val	7	7	8
Met	0	0	0
Cys	0	0	0
Ile	5	5	5
Leu	9	9	10
Phe	6	6	5
Lys	5	4	5

[a] Calculated on the basis of 120 kDa.

as low as 1.5 μg/mL. Neuraminidase or Pronase treatment of the erythrocytes increased the lectin agglutinating activity 6-fold. Alubia erythroagglutinin is devoid of mitogenic activity toward human peripheral lymphocytes at a concentration of 50 μg/mL. However, parental Alubia lectin and Alubia fraction F IV obtained by ionic exchange chromatography (termed mitogenic Alubia isolectin), induce mitogenic indices of 3.4 and 12.8 at a concentration of 10 μg/mL, respectively.

Sugar Specificity of Alubia Erythroagglutinin (F I Isoform). Preliminary studies on Alubia lectin showed that simple monosaccharides are not recognized by the Alubia lectin; therefore, the carbohydrate specificity of the erythroagglutinin was determined by comparing the inhibitory ability of known oligosaccharide structures on the isolectin agglutinating activity. As shown in Table 3, the *N*-glycopeptides from calf fetuin, human serum α_1-acid glycoprotein, and human IgA were effective inhibitors, whereas hen ovomucoid, human and bovine lactoferrin, and pig stomach mucin, as well as the *O*-glycosidically linked oligosaccharides from fetuin, at concentrations as high as 100 μM failed to inhibit the agglutinating activity.

Desialylated glycopeptides from fetuin and α_1-acid glycoprotein inhibited the isolectin hemagglutinating activity, but the removal of all galactose residues from

Table 3. Minimum Concentration of Glycoproteins and Glycopeptides Necessary To Inhibit the Alubia Erythroagglutinin (F I)[a]

compound	concentration (μM)
human α_1-acid glycoprotein[b]	17
asialo α_1-acid glycoprotein[c]	4
asialoagalacto α_1-acid glycoprotein[d]	NI
fetuin *N*-glycopeptide[e]	19
fetuin *N*-asialoglycopeptide[f]	2
fetuin *N*-asialoagalactoglycopeptide[g]	NI
fetuin *O*-glycan[h]	NI
IgA-*N*-glycopeptide	28
IgA-*N*-asialoglycopeptide	5
IgA-asialoagalacto-*N*-glycopeptide	NI
IgA-*O*-glycan	NI

[a] The lectin concentration was adjusted to 3 μg/mL. Other substances with noninhibitory activity (NI) included the following: 100 mM concentration of lactose, (α2−3)- or (α2−6)-sialyllactose, Gal(β1−3)Gal or 100 μM of bovine lactoferrin, human serotransferrin and lactoferrin, hen ovomucoid and pig stomach mucin. Sugar composition of glycoproteins and derivatives were determined by gas−liquid chromatography and their composition are as follows: (*b*) $NeuAc_3Gal_5GlcNAc_5Man_3$; (*c*) $Gal_{4.6}GlcNAc_{4.4}Man_3$; (*d*) $GlcNAc_{4.4}Man_3$; (*e*) $NeuAc_2Gal_4GlcNAc_5Man_3$; (*f*) $Gal_{3.7}GlcNAc_{4.2}Man_3$; (*g*) $GlcNAc_4Man_3$; (*h*) $NeuAc_{1.6}Gal_{2.4}GalNac_1$.

the asialo forms from fetuin and α_1-acid glycoprotein glycopeptides by β-galactosidase treatment abolished the inhibitory capacity of these glycopeptides (Table 3). β-Galactosidase treatment after 2 and 4 h of incubation on asialo forms of fetuin and α_1-acid glycoprotein induced only partial diminution in the inhibitory capacity of these glycopeptides. *N*-Glycopeptides from α_1-acid glycoprotein with only one galactose residue are unable to inhibit the agglutinating activity of Alubia and its erythroagglutinating isoform (not shown), thus confirming that two to four galactose residues are necessary to inhibit the hemagglutinating effect of Alubia lectin and its erythroagglutinating isoform.

Effect of Alubia Erythroagglutinin on Human Coagulation Factors. Preliminary analyses of the effect of the Alubia erythroagglutinin (F I isoform) on the coagulation time of serum from healthy donors indicate that, at concentrations of 8.0 μg/mL, the lectin increases the time of coagulation by 30%. As indicated in Figure 3A, the generation of thrombin is diminished when normal plasma is incubated with 5 μg of Alubia erythroagglutinin (as at optimal concentration), and no effect was observed in the presence of plasma with 42% protein C resistance even at 25 μg of lectin (not shown). Alubia erythroagglutinin even at 25 μg/mL does not modify the activated thromboplastine (Figure 3B) and prothrombin time or the protein C and protein S activities on normal plasma or in the presence of 42% protein C resistant plasma. The modification of the generation of thrombin by the lectin was abolished when the lectin was previously incubated with 0.02 μM fetuin *N*-asialoglycopeptides (not shown).

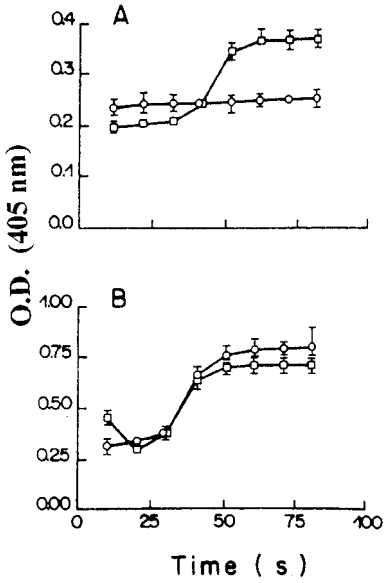

Figure 3. (A) Effect of Alubia erythroagglutinin (F I) on thrombin generation. This assay was performed with a chromogenic assay in normal plasma (□)and plasma incubated previously with 10 μg of Alubia erythroagglutinin (F I) (○). (B) APTT was tested by a chromogenic assay, using the Neothromtin-CTS kit, in normal plasma (□) or in plasma incubated with 10 μg of Alubia erythroagglutinin (○). In both determinations the activity was measured photometrically at 405 nm using an ELISA microreader, with measurements every 5 s. Each point is the mean of three individual samples, and ± SDs of the mean are indicated.

DISCUSSION

Lectins from the *Phaseolus* genus hold great interest due to their use as tools in the study of the physiology of the nutrition [reviewed by Liener (1986); Pusztai, 1991]. Lectins from the common bush bean *P. vulgaris* have erythroagglutinating and mitogenic activities; these effects are due to their structural organization. These lectins correspond to a mixture of five tetramers derived from the combination of two subunits: erythroagglutinin and leukoagglutinin, which are noncovalently associated (Osborn *et al.*, 1983). Other varieties are constituted by three, two, or one lectin subunit, and their biological activity is well correlated with the type of subunit present in such varieties (Brown *et al.*, 1982; Leavitt *et al.*, 1977). Interestingly, reports concerning lectins from *P. coccineus* indicated that all of them are composed by four identical subunits and show mitogenic and erythroagglutinating activities (Nowakova and Kocourek, 1974; Shi *et al.*, 1993; Feria *et al.*, 1996). As we show in this work *P. coccineus* var. Alubia is also composed by four identical subunits with different biological activities. Although the subunits purified from Alubia lectin seem to be identical, slight differences were found mainly in the concentration of aromatic amino acids, as well as in the glycan composition. Our results suggested that Alubia F I (the erythroagglutinin isoform) contains oligosaccharides of high-mannose type, substituted by xylose, but the F IV (the mitogenic isoform) shows lactosaminic type, oligosaccharides. Our results strongly suggest that *P. coccineus* var. Alubia as well as *P. vulgaris* lectins are composed by four different tetramers derived from the assembly of the two main subunits. Although at the present status of the work it is difficult to confirm that their differences in biological activities are due to the microheterogeneity showed by each isoform, this hypothesis should not be ruled out.

The carbohydrate specificity of *P. vulgaris*, red kidney bean, isolectins has been extensively studied using different techniques. Hammarstrom *et al.* (1982) examined L-PHA specificity by means of quantitative precipitation and precipitation−inhibition assays, whereas Yamashita *et al.* (1983) as well as Cummings and Kornfeld (1972) determined E-PHA specificity by affinity chromatography on immobilized lectin, identifying the retained oligosaccharides or glycopeptides. Their results demonstrated that E-PHA recognizes *N*-glycosidically linked oligosaccharides with a minimal oc-

tasaccharide structure with the Gal(β1−4)GlcNAc(β1−2) Man(α1−6)**[**GlcNAc(β1−2)Man(α1−3)**]**Man(β1−4) sequence and bisecting GlcNAc residues (Cummings and Kornfeld, 1972; Hammarstrom *et al*., 1982; Yamashita *et al*., 1983; Green and Baenziger,1987). Previous studies on the sugar-binding specificity of *P. coccineus* var. Alubia showed that this lectin is not inhibitable by monosaccharides (Ochoa and Kristiansen, 1982). Our results show that Alubia erythroagglutinin is specific for glycoproteins, which characteristically possess bi- and triantennary *N*-glycosidically linked glycans of the *N*-acetyllactosaminic type, such as fetuin and α_1-acid glycoprotein. Fetuin is a powerful inhibitor of the Alubia erythroagglutinin, as is the α_1-acid glycoprotein. The α_1-acid glycoprotein is a human plasma globulin with an important microheterogeneity of different bi-, tri-, and tetraantennary *N*-acetyllactosamine-type glycans, which are distributed at five glycosylation sites (Fournet *et al*., 1978). The importance of galactose (β1−3)- or (β1−4)- in the interaction with fetuin and α_1-acid glycoprotein is suggested by the lack of affinity of the Alubia erythroagglutinin to structures devoid of this oligosaccharide determinant, such as bovine lactotransferrin and hen ovomucoid (Montreuil, 1984) or asialoagalacto-*N*-glycopeptides from α_1-acid glycoprotein and fetuin (Montreuil, 1984), indicating that a peripheral Gal(β1−3 or 4)GlcNAc(β1−2)Man sugar sequence is essential for the binding. The absence, in the putative Alubia lectin receptor, of fucose and sialic acid as in fetuin *N*-glycopeptides (Cumming *et al*., 1984) is neither important nor essential for an adequate lectin receptor interaction.

Functional roles for carbohydrate groups of oligosaccharides have been frequently associated with protection of glycoproteins against proteolysis (Varki *et al*., 1993; Montreuil, 1984), and coagulant proteins have provided several examples of the functional importance of glycans (Varki *et al*., 1993; Mitzuochi *et al*., 1980; Owen *et al*., 1974; Hemker *et al*., 1989). We decided to use Alubia erythroagglutnin as a tool to evaluate the role of the glycans in coagulation factors since many evidences indicate that all coagulant zymogens are glycoproteins (Varki, 1993) and previous papers indicated the occurrence of Gal(β1−3)GlcNAc on human thrombin and prothrombin grouped in biantennary glycans linked to asparagine (Mitzuochi *et al*., 1980). Thrombin is a serine protease generated during clotting zymogen prothrombin. This factor is particularly interesting since it plays a central role in blood coagulation, and its generation from prothrombin (a 70 kDa glycoprotein) implicates its cleavage by factor X into four intermediate fractions (51 to 13 kDa molecular mass) (Owen *et al*., 1974); thus, the inhibition of thrombin generation by Alubia erythroagglutinin is mediated by the interaction with specific lactosaminic structures present on thrombin, which inhibited, very probably, the proteolytic effect of factor X, since factor Xa is on the main pathway of thrombin generation (Hemker *et al*., 1989). These results indicate that Alubia erythroagglutinin could represent a tool to study the role of glycans in the coagulation factors, as was demonstrated for other lectins such as Con A (Solis *et al*., 1987; Perez-Campos *et al*., 1994) or wheat germ agglutinin (Freyssinet, 1981).

LITERATURE CITED

Bidlingmeyer, B. A.; Cohen, S. A., Tarvin T. L. Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr.* **1984**, *33*, 93−104.

Brown, J. W. S.; Osborn, T. C.; Bliss, F. A.; Hall, T. C. Bean lectins. Part I: Relationships between agglutinating activity and electrophoretic variation in the lectin containing G2/albumin seed proteins of French bean (*Phaseolus vulgaris* L.). *Theor. Appl. Genet.* **1982**, *62*, 263−271.

Calderon, R. A.; Cordoba, F. Immunosuppressive activity of *Phaseolus coccineus* and *Phaseolus vulgaris* extracts in mice. *Eur. J. Immunol.* **1976**, *6*, 522−555.

Cumming, D. A.; Hellerqvist, C. G.; Harris-Brandts, M.; Michnick, S. W.; Carver, J. P.; Bendiak, B. Structures of asparagine-linked oligossaccharides of the glycoprotein fetuin having sialic acid linked to *N*-acetyl-D-glucosamine. *Biochemistry* **1984**, *28*, 6500−6512.

Cummings, R. D.; Kornfeld, R. Characterization of the structural determinants for the high affinity interaction of the asparagine-linked oligosaccharides with immobilized *Phaseolus vulgaris* leukoagglutinin and erythroagglutinating lectins. *J. Biol. Chem.* **1972**, *257*, 11230−11235.

Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350−356.

Felsted, R. L.; Egorin, M. J.; Leavitt, R. D.; Bachur, N. R. Recombinations of subunits of *Phaseolus vulgaris* isolectins. *J. Biol. Chem.* **1977**, *252*, 2967−2971.

Felsted, R. L.; Li, J.; Pokrywka, G.; Egorin, M. J.; Spiegel, J. Comparison of *Phaseolus vulgaris* cultivars on the basis of isolectin differences. *Int. J. Biochem.* **1981**, *13*, 549−557.

Feria, M.; Pérez Santiago, A.; Cuevas, D.; Martínez, M.; Córdoba, F. Purification and partial characterization of a new anti-A1 lectin of *Phaseolus coccineus* collected in Oaxaca, México. *Prep. Biochem. Biotechnol.* **1996**, *26*, 31−46.

Fournet, B.; Montreuil, J.; Strecker, G.; Dorland, L.; Haverkamp, J.; Vliegenthart, J. F. G.; Binette, J. P.; Schmid, K. Determination of the primary structures of 16 asialocarbohydrate units derived from human plasma α_1-acid glycoprotein by 360 ^{1}H NMR spectroscopy and permethylation analysis. *Biochemistry* **1978**, *17*, 5206−5214.

Francis, C. W.; Malone, J. E.; Marder, V. J. Comparison of a chromogenic prothrombin time with clotting prothrombin time in the assessment of clinical coagulation deficiencies. *Am. J. Clin. Pathol.* **1985**, *84*, 724−729.

Francis, R. B.; Seyfert, U. Rapid amidolytic assay of protein C in whole plasma using an activator from the venom of *Agkistrodon contortrix*. *Am. J. Clin. Pathol.* **1987**, *87*, 619−625.

Freyssinet, J. M. Wheat germ lectin, a tool to investigate metal ion induced structural changes of bovine blood coagulation factor XI. *FEBS Lett.* **1981**, *124*, 48−52.

Green, E. D.; Baenziger, J. U. Oligosaccharide specifities of *Phaseolus vulgaris* leukoagglutinin and erythroagglutinin phytohemagglutinins. Interactions with *N*-glycanase-released oligosaccharides. *J. Biol. Chem.* **1987**, *262*, 12018−12029.

Hammarstrom, S.; Hammarstrom, M. L.; Sunblad, G.; Arnarp, J.; Longren, J. Mitogenic leukoagglutinin from *Phaseolus vulgaris* binds to a pentasaccharide unit *N*-acetyllactosamine-type glycoprotein glycans. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 611−615.

Hemker, H. C.; Choay, J.; Beguin, S. Free factor Xa is on the main pathway of thrombin generation in clotting plasma. *Biochim. Biophys. Acta* **1989**, *992*, 409−411.

Kamiya, T.; Sugihara, T.; Ogata, K.; Saito, H.; Suzuki, K.; Nishioka, J.; Hashimoto, S.; Yamagata, K. Inherited deficiency of protein S in a Japanese family with recurrent venous thrombosis: a study of three generations. *Blood* **1986**, *67*, 406−410.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

Leavitt, R. D.; Felsted, R. L.; Bachur, N. R. Biological and Biochemical properties of *Phaseolus vulgaris* isolectins. *J. Biol. Chem.* **1977**, *252*, 2961−2966.

Liener, I. E. Nutritional significance of lectins in diet. In *The Lectins, Properties, Functions and Applications in Biology*

and Medicine; Liener, I. E., Sharon, N., Goldstein, I. J., Eds.; Academic Press: New York, 1986.

Lowry, O. H.; Rosenbrough, N. J.; Lewis-Farr, A. L.; Randall, R. S. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

Mizuochi, T.; Yamashita, K.; Fujikawa, K.; Titani, K.; Kobata, A. The structures of the carbohydrate moieties of bovine blood coagulation factor X. *J. Biol. Chem.* **1980**, *255*, 3526−3531.

Montreuil, J. Spatial structures of glycan chains in relation to metabolism and function. *Pure Appl. Chem.* **1984**, *56*, 859−877.

Montreuil, J.; Bouquelet, S.; Debray, H.; Fournet, B.; Spik, G.; Strecker, G. In *Carbohydrate Analysis, A Practical Approach*; Chaplin, M. F., Kennedy, J. F., Eds.; IRL Press: Oxford, U.K., 1986; pp 143−204.

Nowakova, N.; Kocourek, J. Studies on phytohemagglutinins. XX. Isolation and characterization of hemagglutinins from scarlet runner seeds (*Phaseolus coccineus* L.). *Biochim. Biophys. Acta* **1974**, *359*, 320−333.

Ochoa, J. L.; Kristiansen, T. Purification and partial characterization of an agglutinin from *Phaseolus coccineus* var. alubia. *Biochim. Biophys. Acta* **1982**, *705*, 396−402.

Osborn, T. C.; Ausloos, K. A.; Brown, J. W. S.; Bliss, F. A. Bean lectins. III. Evidence for greater complexity in the structural model of *Phaseolus vulgaris* lectin. *Plant Sci. Lett.* **1983**, *31*, 193−203.

Owen, W.; Esmon, C. T.; Jackson, C. M. The conversion of prothrombin to thrombin. Characterization of the reaction products formed during the activation of bovine prothrombin. *J. Biol. Chem.* **1974**, *249*, 594−605.

Penner, J. A. Experience with a thrombin clotting time assay for measuring heparin activity. *Am. J. Clin. Pathol.* **1974**, *61*, 645−653.

Pérez-Campos, E.; Pérez-Ortega, E.; Córdoba, F.; Zenteno, E. Inhibition of protein C activity with plant lectins *in vitro*. *Rev. Iberoam. Tromb. Hemostasia* **1994**, *7*, 45−46.

Ponjee, G. A. E.; Vader, H. L.; De Wild, P. J.; Janssen, G. W. T.; Van der Graaf, F. One step chromogenic equivalent of activated partial thromboplastin time evaluated for clinical application. *Clin. Chem.* **1991**, *37*, 1235−1244.

Pusztai, A. *Plant Lectins*; Cambridge University Press: Cambridge, U.K., 1991.

Shi, W. X.; Shen, Z. M.; Sun, C.; Yang, J. T. Conformation and activity of *Phaseolus coccineus* var rubronanus lectin. *J. Protein Chem.* **1993**, *12*, 153−157.

Solis, D.; Albert, A.; Diaz-Maurino, T. Effect of lectin binding to fibrinogen D and E domains on coagulation and fibrinolysis. *Biochim. Biophys. Acta* **1987**, *926*, 61−69.

Spik, G.; Bayard, B.; Fournet, B.; Strecker, G.; Bouquelet, S.; Montreuil, J. Studies on glycoconjugates. LXIV. Complete structure of two carbohydrate units of human serotransferin. *FEBS Lett.* **1975**, *50*, 296−304.

Spiro, R. G.; Bhoyroo, V. D. Structure of the *O*-glycosidically linked carbohydrate units of fetuin. *J. Biol. Chem.* **1974**, *249*, 704−711.

Vargas-Albores, F.; Hernández, J.; Córdoba, F.; Zenteno, E. Isolation of an immunosuppressive lectin from *Phaseolus vulgaris* cv. cacahuate using stroma. *Prep. Biochem.* **1993**, *23*, 473−483.

Varki, A. Biological roles of oligosaccharides: all theories are correct. *Glycobiology* **1993**, *3*, 97−130.

Yachnin, S.; Allen, L. W.; Baron, J. M.; Svenson, R. H. The potentiation of phytohemagglutinin-induced lymphocyte transformation by cell-cell interaction; a matrix hypothesis. *Cell. Immunol.* **1972**, *3*, 569−589.

Yamashita, K.; Hitoi, A.; Kobata, A. Structural determinants of *Phaseolus vulgaris* erythroagglutinating lectin for oligosaccharides. *J. Biol. Chem.* **1983**, *258*, 14753−14755.

Zenteno, E.; Ortega, M.; Qin, Z.; Montreuil, J.; Debray, H. Fast purification of *Phaseolus vulgaris* isolectins. *Prep. Biochem.* **1994**, *24*, 175−183.

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