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Lectins of *Erythrina poeppigiana* and *Erythrina steyermarkii* (Leguminosae): characterization and mitogenic effect

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Abstract: Erythrina species are widely distributed in Costa Rica and known popularly as "poró". In this study, two species were selected, *Erythrina poeppigiana* and *Erythrina steyermarkii*. Seed extracts were prepared in phosphate-buffered saline. The presence of lectins in the extracts was verified by hemagglutinating effect over suspensions of human erythrocytes. A selective hemagglutinating effect on erythrocytes of several mammal species, goat, horse and rabbit red cells was tested; only the latter were agglutinated by *E. steyermarkii*. The hemagglutinating effect of both lectins was inhibited with the following carbohydrates: D-galactose, N-acetyl galactosamine, D-lactose and D-raffinose. The lectin from *E. steyermarkii* was also inhibited with L-rhamnose. Both lectins were isolated with gel filtration and affinity chromatography using lactose as ligand. Fractions that proved positive were tested with the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gel filtration and SDS-PAGE showed that these lectins have an apparent molecular mass of 50kDa, and are formed by two subunits of approximately 25 kDa. *E. poeppigiana* had no mitogenic effect, but the extract of *E. steyermarkii* had a mitogenic effect on human mononuclear cells isolated from peripheral blood. The stability of the lectins was tested at different temperature and pH ranges (4 to 100 °C) and at pH 2 to 12. Both were stable at a pH range from 2 to 10, and at temperatures from 40 to 70 °C.

Key words: *Erythrina*, leguminosae, poró, lectins, mitogen.

Lectins are simple proteins or glycoproteins present in numerous plants, animal tissues and microorganisms that specifically bind to carbohydrates and have the capacity to agglutinate certain types of cells (Barondes 1981, Quiocho 1986). Despite their poorly understood biological functions, it has been suggested that they are involved in the symbiosis of nitrogen fixing microorganisms and legumes, and in the defense of plants against fungi and insects (Adar and Sharon 1996). The ability of lectins to recognize specific carbohydrates makes them valuable tools for taxonomic studies and for the isolation and purification of glycoconjugates (Van Leuven *et al.* 1993).

Lectins extracted from plants may function as lymphocyte polyclonal mitogens, by binding to glycoconjugates on the cell surface and activating a series of events that result in cellular activation and proliferation (Sansford

and Harris-Hooker 1990, Maennel *et al.* 1991). Some lectins, as Phytohaemagglutinin (PHA) extracted from *Phaseolus vulgaris* and Concanavalin A (Con A) from *Canavalia ensiformis*, are commercially available mitogens. However, the binding of lectins to the surface of lymphocytes does not necessarily induce cellular activation. Lectins have been used to demonstrate structural and functional differences in membrane composition between normal and tumoral cells (Slesak *et al.* 1989, Figols *et al.* 1991, Slupsky *et al.* 1993).

The biological importance of lectins has increased due to their potential for the purification, selection and cloning of subpopulations of cells involved in normal or pathological immune response (Cunnick *et al.* 1990, Tietz *et al.* 1991).

In this investigation, the lectins from the seeds of *Erythrina steyermarkii* and *Erythrina poeppigiana* were isolated, biochemically characterized, and screened for mitogenic activity on mononuclear leukocytes.

MATERIALS AND METHODS

Preparation of the crude extracts: The seeds extracts of *Erythrina steyermarkii* and *E. poeppigiana* were obtained as described in a previous work (Nanne-Echandi and Aragón-Ortiz 1991) with slight modifications. Briefly, 80 g of the seeds were homogenized with 500 mL of 0.15 M NaCl containing 3mM KCl, 0.05M sodium phosphate buffer, pH 7.2 (PBS), and centrifuged at 10000 g for 30 min at 4°C. The proteins in the supernatant were precipated with an equal volume of acetone and centrifuged at 10000 g for 10 min at 4 °C. The pellet was dissolved in PBS and lyophilized. The protein concentration of the extracts was determined according to Bradford (1976).

Hemagglutinating activity: Goat, horse, rabbit and human [A, B, O, (Rh_0 (D)+, (Rh_0 (D)-] erythrocytes were washed three times with NaCl 0.9 % at 3000 g for 5 min and resuspended at a 5% (v/v) concentration in the same solution.

The hemagglutinating activity was assayed in 96-well microtiter plates (U-shaped), using 100 µL of extract solution (5 mg protein/mL), and adding 50 µL of erythrocyte suspension to each well. The agglutination was screened visually after one hour of incubation at room temperature.

In order to determine the carbohydrate recognized by the lectin, 50 µL of a carbohydrate solution (0.1M) was added to 50 µL of the crude extract (5 mg of protein/mL) and incubated one hour at room temperature. Immediately, 50 µL of a suspension of human erythrocytes (A, Rh_0 (D)+) was added to each well, and incubated again for one hour at room temperature. The inhibitory effect of the carbohydrates on the agglutination was determined by titration using double serial dilutions.

Temperature stability: One mL of each of the seed extracts (5mg/mL of protein) was incubated for 10 minutes at different temperatures (4-100°C), and immediately placed in an ice bath. These extracts were then centrifuged at 3000 g for 5 minutes. The hemagglutinating activity of the supernatants was determined as described previously, using human group A, Rh_0 (D)+ erythrocytes.

pH stability: The extracts were dissolved in saline phosphate buffers of different pH values (2-12), adjusted to obtain a protein concentration of 5 mg/mL, and incubated for one hour at room temperature. The pH was readjusted to 7.0 with 0.1M NaOH or with 0.1M HCl. The precipitate was eliminated by centrifugation (3000 g, 5 min) and the agglutinating activity of the supernatant was then assayed with human group A, Rh_0 (D)+ erythrocytes.

Isolation of the lectins: The crude extracts of the lectins were adjusted to a protein concentration of 30 mg/mL in PBS and were run through a Sephadex G-100 column (2.5 cm x 100 cm), at a flow rate of 20 mL/hour. Fractions of three mL were collected, screened at 280 nm, and their agglutinating

activity determined with human group A, Rh_0 (D)+ erythrocytes. Fractions showing positive results were passed several times at 4 °C through a Lactose-Agarose column (Sigma, USA). After washing the column extensively with PBS, the bound material was eluted with 100 mL of 2M D-lactose in PBS. The eluate was dialyzed 48 hours at 4 °C against PBS, and concentrated through a stirred cell filter with a 30 kDa pore membrane.

Mitogenic activity: Human peripheral blood mononuclear cells were separated from venous blood of healthy adult volunteers by density gradient centrifugation on Ficoll (Bøyum 1971). Cell cultures were performed as previously described (González *et al.* 1992). Briefly, the mononuclear cells were cultured at a density of 1 x 10^5 cells/well in RPMI medium (Sigma, USA) with 10% fetal calf serum (FCS) in the presence of serial dilutions of the lectin fraction obtained from the gel filtration. PHA (6.5 µg/mL) and medium alone were used as controls. The cultures were incubated at 37°C for 48 hours in an atmosphere containing 5% CO_2, followed by a 24 hour pulse with 1 µg ^{3}H-thymidine/well. The cells were harvested on filter paper discs and the thymidine incorporation was assessed using a beta counter. All samples were run in triplicate.

RESULTS

The seed extracts obtained from *E. steyermarkii* and *E. poeppigiana* aglutinate all types of human erythrocytes of the ABO and Rh_0(D) positive and negative systems. Differences were found when erythrocytes from other mammalian species were tested with the extracts. The *E. steyermarkii* seed extract did agglutinate rabbit erythrocytes, but not goat or horse erythrocytes, and the *E. poeppigiana* seed extract did not agglutinate any of these types of erythrocytes (Table 1).

Working with human A, Rh_0 (D)+ erythrocytes that were randomly selected since all types of human red cells were agglutinated by the lectin extracts, the inhibition of the agglutinating effect of the seed extracts was experimentally tested against D-galactose, D-lactose, D-N-acetyl galactosamine, L-rhamnose and other carbohydrates. The titers of the inhibition of haemagglutination tests using D-galactose, D-lactose, D-N-acetyl galactosamine and raffinose, against the lectin extracts of both *Erythrina* species, are shown in Table 2. D-galactose, D-lactose, D-raffinose and D-N-acetyl galactosamine inhibit the agglutination of the human erythrocytes on both seed extracts (Table 2). In the particular case of *Erythrina steyermarkii* extract, agglutination of human erythrocytes was inhibited by D-raffinose. This carbohydrate had no inhibitory effect when tested with rabbit red cells (Table 3).

TABLE 1

Agglutinating activity of the lectins from E. steyermarkii *and* E. poeppigiana *with different erythrocytes types*

	Human erythrocytes			Animal erythrocytes		
Extract	A	B	O	Rabbit	Goat	Horse
Erythrina steyermarkii	+	+	+	+	-	-
Erythrina poeppigiana	+	+	+	-	-	-

+ Haemagglutination
- No haemagglutination

The results of the mitogenic effect of both extracts against mononuclear cells isolated from peripheral blood of two healthy human donors are presented in Figure 1.

E. steyermarkii extract showed mitogenic effect on these cells, whereas *E. poeppigiana* extract did not have any effect on mononuclear cells.

The effect of temperature on the capacity of haemagglutination of both extracts was investigated. The extracts were heated at different temperatures, ranging from 40 to 90°C for ten minute intervals. It was found that the hemagglutinating capacity was stable between 40 and 70°C, and was lost completely at 80°C.

TABLE 2

Titer of the inhibitory activity of the carbohydrates on the haemagglutination of human erythrocytes (A Rh+)with the lectins of E. poeppigiana *and* E. steyermarkii

E. POEPPIGIANA	1:1	1:2	1:4	1:8	1:16	1:32	1:64
Galactose	+	+	+	±	-	-	-
Lactose	+	+	+	+	+	-	-
N-Acetyl Galactosamine	+	+	+	+	+	-	-
Raffinose	+	+	+	+	-	-	-
E. teyermarkii							
Galactose	+	+	+	+	-	-	-
Lactose	+	+	+	+	+	+	-
N-Acetyl Galactosamine	+	+	+	+	+	-	-
Raffinose	+	+	+	+	-	-	-
Rhamnose*	+	+	-	-	-	-	-

* hemagglutinating inhibition with rabbit erythrocytes.

TABLE 3

Inhibitory activity of carbohydrates (0.1 M) on the haemagglutination of human erythrocytes (A Rh+) and rabbit erythrocytes by the lectin of E. steyermarkii

Carbohydrate	A +	Rabbit
D-Lactose	+	+
D-N-Ac Galactosamine	+	+
D-Galactose	+	+
L-Rhamnose	-	+
D-Raffinose	+	-
D-N-Ac Glucosamine	-	-
Sorbose	-	-
D-Glucose	-	-
Inositol	-	-
D-N-Ac Mannosamine	-	-
Trehalose	-	-
D-Mannose	-	-
D-Saccharose	-	-
Sorbitol	-	-
D-Xylose	-	-

+ Inhibitory activity
- No inhibitory activity

Regarding pH, the activity of both extracts remained constant at pH 2 to 10, and was lost at pH 11.

Fractionation of crude extracts was performed by gel filtration on Sephadex G-100 column. In *E. poeppigiana* extract four fractions were observed whereas five fractions were separated in the extract of *E. steyermarkii*. In both extracts, the hemagglutinating activity was located in the second fraction (Fig 2). Further purification of the lectins was achieved by collecting the hemagglutinating fractions, concentrating and passing them through an affinity chromatography column with agarose-lactose.

The molecular weight of the hemagglutinating fractions, determined by gel filtration in Sephadex G-100 was estimated as 50 kDa (Fig 3). By polyacrylamide gel electrophoresis (SDS-PAGE), under reducing conditions, the hemagglutinating fraction of both lectins obtained by affinity chromatography have a molecular weight close to 25 kDa. These finding suggest that each lectin is formed by two subunits of similar molecular weight.

DISCUSSION

The extracts of *E. poeppigiana* and *E. steyermarkii* agglutinated all types of human erythrocytes tested. These findings agree with

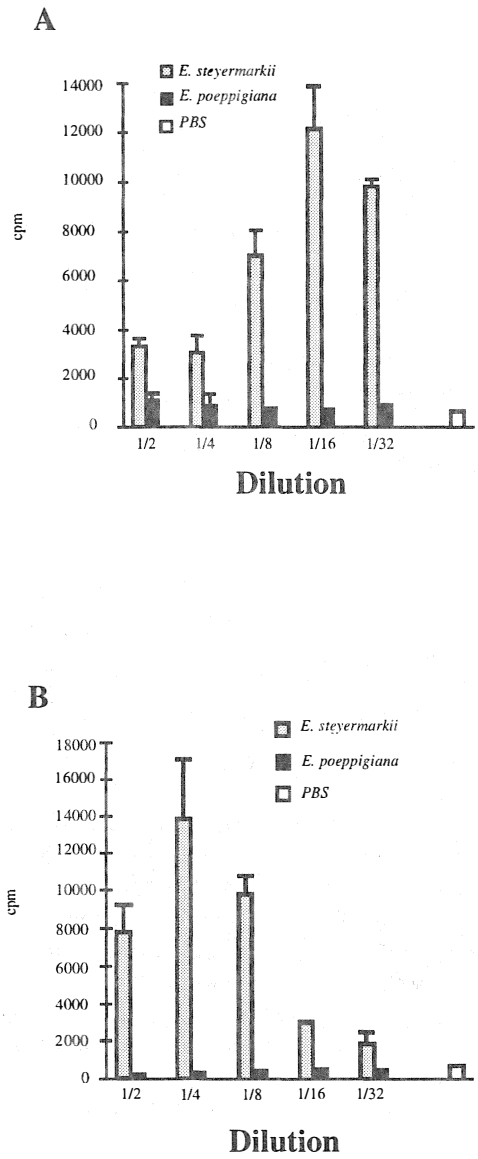

Fig. 1. Mitogenic response of human mononuclear cells from two healthy donors (A, B) to hemagglutinating fractions obtained by gel filtration of extracts of *E. steyermarkii* and *E. poedppigiana.*

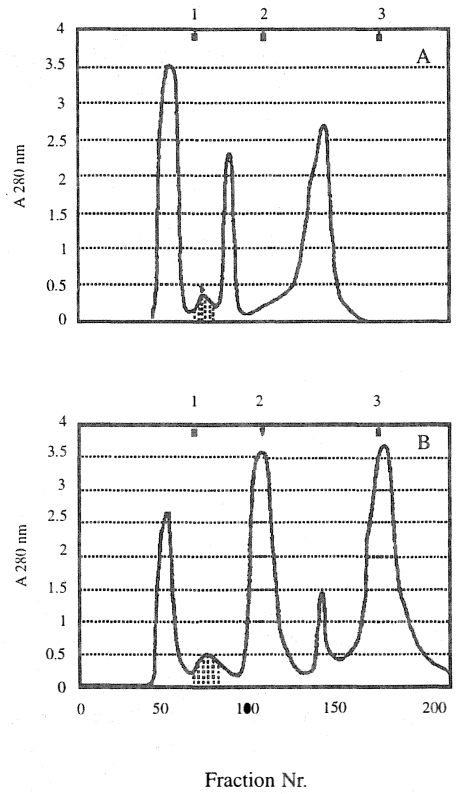

Fig. 2. Gel filtration of *E. poeppigiana* (A) and *E. steyermarkii* (B) extracts on Sephadex G-100 column. Agglutination of human erythrocytes was observed in the fractions of peak 2 from both extracts (*). Protein markers: 1. Bovine serum albumin (66 kDa). 2.Carbonic anhydrase (29 kDa). 3. Lysozyme (14.3 kDa).

those of Allen and Brillantine (1969), who found that the vast majority of lectins agglutinate in a non specific way all types of human red cells.

Horse and goat red cells were not agglutinated by the lectin extracts of both species studied. In the particular case of the extract of *E. steyermarkii*, agglutination was observed with rabbit red cells. These observations suggest that this lectin is similar to the one isolated from *E. edulis* by Pérez (1984). This lectin agglutinated rabbit red cells, but had no effect on horse or goat erythrocytes.

The mitogenic effect of both lectins was evaluated with mononuclear cells isolated from the blood of two healthy donors. Results varied in each case. With donor A the mitogenic effect increased with higher dilution. This could be explained by assuming that interfering substances present in the extract partially inhibited cell proliferation. This

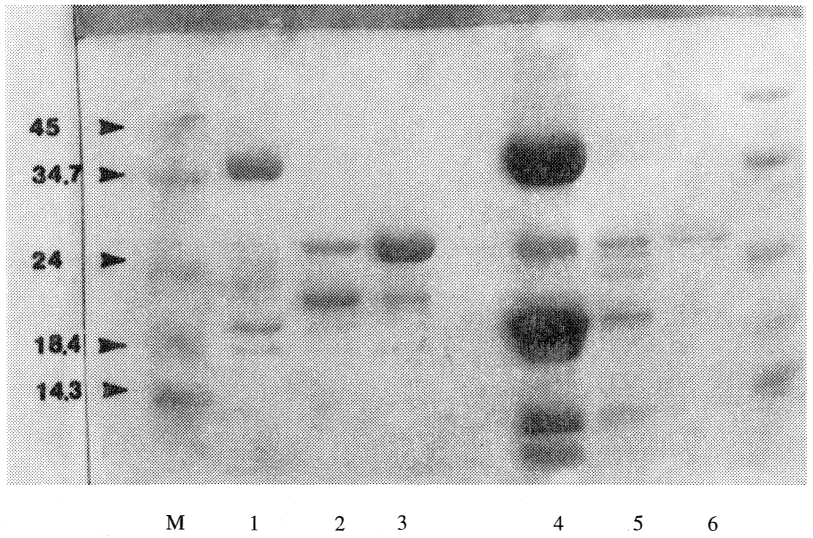

Fig. 3. SDS-PAGE run under reducing conditions of the extracts from *E. steyermarkii* and *E. poeppigiana.* M: protein markers. 1: *E. poeppigiana* crude extract. 2: Positive fraction from gel filtration of *E. poeppigiana.* 3: Lectin of *E. poeppigiana* isolated from affinity chromatography. 4: *E. steyermarkii* crude extract. 5: Positive fraction from gel filtration of *E. steyermarkii.* 6: Lectin of *E. steyermarkii* isolated by affinity chromatography.

effect was lost at higher dilutions. On donor B not such effect was observed, and the mitogenic effect was more intense at lower dilutions (1/4 and 1/8). Nevertheless, it was evident that only the extract *of E. steyermarkii* has mitogenic effect. In a previous investigation by Lis *et al.* (1985) it was found that among a group of lectins investigated, six of them agglutinated rabbit red cells and had mitogenic on lymphocytes isolated from peripheral human blood, whereas three weakly agglutinated rabbit erythrocytes and two of them displayed a poor mitogenic effect.

D-galactose, D-N-acetygalactosamine and D-lactose inhibited the agglutinating activity of both extracts in human red blood cells. Of these carbohydrates, D-lactose was the most potent, inhibiting at a minimum concentration of 3.1 mM. All of the *Erythrina* extracts that have been studied in the past by various authors, show the same pattern of inhibition with these carbohydrates (Bhattacharyya *et al.* 1981, Lis and Sharon 1985, Nanne and Aragón 1991).

The inhibitory activity of raffinose of haemagglutination by both extracts can be explained by the recognition of the galactose moiety of this trisaccharide. L-rhamnose only inhibits the agglutination of rabbit red cells with the *E. steyermarkii* extract. Lis *et al.* (1985) proposed that the differences in behavior of the different lectin extracts with rabbit red cells and mononuclear human cells is caused by variation in or close the combination site of the carbohydrate to the red cell membrane.

Incubating the lectin extracts at different temperatures (40-90°C) the hemagglutinating capacity remained intact in a 40 to 70°C range, but was lost at 80 °C. Similar results were reported by Bhattacharya *et al.* (1981) and Nanne and Aragón (1991). The stability of the extracts at various pH ranges demonstrated that these remained constant between pH 2 and pH 10, completely loosing hemagglutinating capacity at pH 11. Roberson and Strenght (1983) and Cammue *et al.* (1985) reported that

the lectins from the seed of *Vigna unguiculata* and the tubercle *Eranthis hyemalis* maintained their hemagglutinating activity in a pH range of 2 to 12 for the *Vigna* extract, and from 3 to 11 for the *Eranthis* extract. Nanne and Aragón (1991) found that the lectin of *Erythrina costaricensis* is stable at a pH range of 2 to 10.

The molecular weight of the lectins studied, determined by gel filtration, was approximately 50 kDa, similar to other *Erythrina* lectins (Pérez 1984, Lis and Sharon 1985). By polyacrylamide gel electrophoresis (SDS-PAGE), a molecular weight close to 25 kDa was determined, suggesting that each lectin is formed by two subunits of similar molecular weight.

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RESUMEN

Las diferentes especies de *Erythrina* se encuentran ampliamente distribuidas en Costa Rica y se las conoce popularmente con el nombre de "poró". En el presente estudio, se seleccionaron dos especies: *Erythrina poeppigiana* y *Erythrina steyermarkii*. Se prepararon extractos de las semillas en solución tampón salina de fosfatos y se verificó la presencia de lectinas en ellos mediante la técnica de hemaglutinación, utilizando eritrocitos humanos. Se trató de demostrar un efecto selectivo de la hemaglutinación empleando eritrocitos de varias especies de mamíferos, específicamente de carnero, caballo y conejo. Solo los eritrocitos de conejo fueron aglutinados con la lectina de *E. steyermarkii*. El efecto hemaglutinante de las dos lectinas fue inhibido con los siguientes carbohidratos: D-galactosa, N-D-acetil galactosamina, D-lactosa y D-rafinosa. La lectina de *E. steyermarkii* también fue inhibida con L-rhamnosa. Las dos lectinas fueron aisladas por filtración en gel y cromatografía de afinidad, usando lactosa como ligando. Las fracciones que resultaron positivas se analizaron mediante la técnica de electroforesis en gel de poliacrilamida y duodecil sulfato de sodio (SDS-PAGE). Con la filtración en gel y el SDS-PAGE, se comprobó que las dos lectinas tienen una masa molecular aparente de 50 kDa y que están formadas por dos subunidades de 25 kDa, aproximadamente. Se buscó un efecto mitogénico en las dos lectinas y se encontró que sólo *E. steyermarkii* lo manifestaba sobre células mononucleares humanas aisladas de sangre periférica. Se determinó la estabilidad de las lectinas en diferentes ámbitos de temperatura (4 a 100°C) y de pH (2 a 12). Las dos lectinas se mantuvieron estables en un rango de temperatura de 40 a 70°C y en un pH de 2 a 10.

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