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Purification of an Antiproliferative Lectin from *Erophaca Baetica* (Leguminosae) Seeds

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Abstract A lectin has been purified from the seeds of *Erophaca baetica*, an endemic legume of the Mediterranean Region. The protein has been purified from an albumin extract by gel filtration chromatography, after realization that affinity chromatography using Sephadex G-50 did not retain any proteins. Characterization of this protein shows that it is a 60 kDa homodimeric glycoprotein with 235 mg sugars / g protein, and two 30 kDa subunits. Its amino acid composition is similar to those reported for the lectins of other related legumes such as *Astragalus mongholicus*. It agglutinates trypsinized erythrocytes, and inhibits proliferation of human leukemic THP-1 cells. Thus, this novel lectin may be of interest from a functional point of view due to its antiproliferative activity.

Keywords: *Erophaca baetica, lectin, antiproliferative, THP-1 cells, seed proteins, legumes*

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1. Introduction

The legume *Erophaca baetica* (L.) Boiss. is the only species included in the genus *Erophaca*. It is endemic to the Mediterranean Region where it has two disjunctive subspecies that are present at opposite ends of the Mediterranean Region, subsp. *baetica* and subsp. *orientalis*. This species has been historically included in the genus *Astragalus* as *A. lusitanicus* Lam. until it was transferred to the genus *Erophaca* by Podlech [1]. The genus *Erophaca* is included in the Tribe *Galegeae* together with other genera such as *Galega, Astragalus*, and *Oxytropis* [2]. *E. baetica* [3] and some species of the genera *Astragalus* and *Oxytropis* [4] are locally known as locoweeds because they produce toxic alkaloids that are responsible for locoism in livestock.

Lectins are non enzymatic proteins that can reversibly bind carbohydrates residues through specific domains, and are widespread in plants and animals. The function of lectins in plant seed is still unclear. Rüdiger and Gabius [5] proposed that lectins may have "internal" and "external" activities. The former include interactions with storage proteins and enzymes, while the latter include defensive roles against predators and diseases, as well as symbiotic interactions with bacteria and fungi. The antinutritional properties of lectins, characterized by digestion disorders that cause abdominal pain, nutrients malabsorption, and diarrhoeas [6] would be related to these external activities.

Lectins are abundant in legumes. Although the lectins that are present in the most common grain legumes have been the object of numerous studies, there are very few studies on the lectins from less common or wild legumes. Two lectins have been purified and characterized in two species belonging to the genus *Astragalus* that are used as traditional medicinal plants in China, *A. membranaceus* [7] and *A. mongholicus* [8]. These lectins induce apoptosis in human leukemia cells [9,10]. The goals of the present work was to determine whether a lectin similar to those reported in *Astragalus* species is also present in the taxonomically close *E. baetica* legume, and if this is the case, to determine whether it has antiproliferative activity.

2. Materials and Methods

2.1. Materials

Diethyl ethoxymethylenemanolate was from Fluka. Concanavalin, camptothecin and 2,5-diphenyltetrazolium bromide were from Sigma. Trypsinized, glutaraldehyde treated human erythrocytes (group B) from Sigma were used for agglutination experiments. Erythrocytes were supplied as a 4% (w/v) suspension in phosphate-buffered saline. Cell culture media, serum and other reagents for cell culture were from GIBCO. All other materials were of analytical grade. Fully mature *E. baetica* subsp. *baetica* seed samples were collected from several wild populations located in Andalusia (Southern Spain). The seeds were collected from ten different specimens in each population and stored at -20 ºC until use. Voucher specimens of these populations are deposited at the Instituto de la Grasa (C.S.I.C.).

2.2. Purification of *E. Baetica* Lectin

Albumin fraction - Albumins were extracted by stirring a suspension of flour (10% w/v in water adjusted to pH 4)

for 1 hour at 0ºC. The supernatant resulting from centrifugation at 15.000g for 15 min was taken to 80 % ammonium sulphate saturation, and stirred for 15 min at 0ºC. The pellet resulting from centrifugation at 15.000g for 15 min was redissolved in water prior to passing through a PD-10 column (GE) using phosphate 25mM, NaCl 0.2M, pH 7.5 buffer.

Affinity chromatography - Sephadex G-50 (Pharmacia) affinity chromatography was carried out using a Sephadex G-50 column (10cm x 2.5cm, void volume 50 mL), and phosphate 25mM, NaCl 0.2M, pH 7.5 as running buffer. The column was washed after loading the sample using running buffer until all non-bound proteins were eluted. The same buffer with the addition of 100mM glucose was used for elution of proteins bound to the Sephadex G-50 media.

Gel filtration chromatography - Gel filtration chromatography using a Superose 12 HR 10/30 column from GE was carried out as previously described [11]. The running buffer was phosphate 25mM, NaCl 0.2M, pH 7.5. Blue dextran 2000 (2000 kDa), α-amylase (200 kDa), bovine serum albumin (67 kDa), and ribonuclease A (13.7 kDa) were used as molecular weight standards (GE).

2.3. Amino Acid Analysis

Samples were hydrolyzed by incubation in 6 N HCl at 110ºC for 20 h. Amino acids were determined after derivatization with diethyl ethoxymethylenemanolate by high-performance liquid chromatography (HPLC) according to the method described by Alaiz et al. [12], using D L α-aminobutyric acid as internal standard. The HPLC system (Beckman-Coulter) consisted of a 126 solvent module, 166 detector and IBM personal computer. Data acquisition and processing were effected with 32 Karat 7.0 version software (Beckman-Coulter). Samples (20 µL) were injected in a reversed-phase column (Novapack C_{18}, 300 3.9mm i.d., 4 µm, Waters) that was kept at 18 ºC. Tryptophan was determined by HPLC-RP chromatography after basic hydrolysis according to Yust et al. [13].

2.4. Agglutination Test

A semi quantitative agglutination assay was carried out by incubating erythrocytes (40 µL, 1.6 mg, 4 % w / v in PBS buffer) in the presence of increasing concentrations of lectin in 96 U shape well microplates for 1 hour at room temperature. Concanavalin (1 µg / well) was added to erythrocytes as a positive control, and incubations of erythrocytes with no sample or concanavalin added were used as negative control.

2.5. Determination of Lectin Sugar Content

Sugar content was determined by reaction with phenol and concentrated sulphuric acid according to the colorimetric method of Dubois et al. [14] using standard curves of glucose.

2.6. SDS-PAGE Electrophoresis

Protein extracts were adjusted to 2 mg protein / mL, mixed (1:1 v/v) with solubilisation buffer (Tris 80mM, 0.57% EDTA, 0.26% DTT, 3.3% SDS, 0.008% bromophenol blue, 20% sucrose, pH 6.8), and heated at 100 ºC for 10 min. Tricine - sodium dodecylsulphate - polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Schägger and von Jagow [15] at a constant voltage of 60 V for the stacking gel, and 120 V for the separation gel. Gels were fixed in 20% methanol, 8% acetic acid for 15 min before staining using 0.25% Coomassie Brilliant Blue G in 45% methanol and 10% acetic acid. Molecular masses were determined using the low molecular weight standards from Pharmacia LKB Biotechnology.

2.7. Cell Proliferation Assay: THP-1 Cells Culture and Treatment

Human leukemia THP-1 cells were kept at 5 % (v/v) CO_2 in Dulbecco's Modified Eagle Medium (1.000 mg/L glucose, 110 mg/L pyruvate, and 580 mg/L glutamine) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) MEM non-essential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin. For routine maintenance, THP-1 cells were subcultured every 2–3 days by resuspension in fresh medium. Experiments were initiated by replacing the medium with fresh medium containing the *E. baetica* lectin. Cells (4 x 10^4 cells / well) were exposed to increasing lectin concentrations (0–12 µg/mL) for up to 5 days. The MTT viability assay was used to estimate cell viability at different times in order to determine cell proliferation [16]. The blue formazan crystals formed by reduction of MTT were dissolved by addition of 100 µL HCl (0.1 N) in isopropanol, and absorbance was measured at 570 nm using a background reference wavelength of 630 nm using a dual-wavelength plate reader.

2.8. Statistical Analysis

All experiments consisted of a minimum of three independent replicates. Student *t*-test was carried out to determine statistical significance. Cluster analysis of legume lectins was performed according to the furthest neighbor method based on a euclidean distance matrix using the Stagraphics 5.1 software.

3. Results and Discussion

A first attempt for purification of the lectins that could be present in *E. baetica* seeds was carried out by affinity chromatography of an albumin extract. Legume seed proteins can be easily resolved according to their solubility in water [17]. Affinity chromatography using different Sephadex supports such as G-50, G-100, and G-200 has been used in the past for purification of many lectins, especially for lectins in plants belonging to the tribe *Fabeae* [18,19]. Because sugar residues in the Sephadex matrix interact with lectins, elution of bound lectins is accomplished by addition of a buffer containing glucose, which competes with the sugar residues of Sephadex matrix for lectins for binding sites.

Globulins in the *E. baetica* seed protein extracts were precipitated at the isolelectric pH and albumins were precipitated from the resulting soluble fraction using ammonium sulphate. This albumin extract was used for affinity chromatography using a Sephadex G-50 column that has been successfully used before in our laboratory

for affinity purification of lectins from legumes of the tribe *Fabeae* (unpublished results). Nevertheless, affinity chromatography in Sephadex G-50 did not result on retention of any protein. This does not necessarily mean that there are no lectin present in *E. baetica* seeds, but might indicate that the lectins possibly present in these seeds would have no affinity for the Sephadex support. Thus, while the lectins that have been previously described in the tribe *Fabeae* are mannose and glucose specific and interact with Sephadex matrices, including lectins in *Lens culinaris* [18], and *Lathyrus tingitanus* [19], a lectin that was purified from a taxa closely related to *E. baetica*, *A. mongholicus*, was a galactose binding lectin that showed low affinity for glucose and no affinity for mannose [8].

Gel filtration chromatography of the albumin fraction using a Superose 12 column revealed two major, not-completely resolved peaks (Figure 1A). The elution volume of the first peak corresponds to a molecular weight of 60 kDa, which is very close to the molecular weight reported for the homodimeric lectin purified from *A. mongholicus*, 62 kDa [8]. The second peak probably corresponds to low molecular weight 2S albumins. Fractions corresponding to the first peak were pooled together and re-chromatographed in Superose 12 (Figure 1B), showing the presence of some albumins from the second peak. One more chromatography of the fractions corresponding to the major peak in Figure 1B provided a chromatographically pure preparation (Figure 1C). Determination of sugar revealed that this preparation was a glycoprotein containing 235 ± 2.9 µg sugars / mg protein, which is similar to the sugar content reported for the lectin in *A. mongholicus*, 196 µg sugars / mg protein [8].

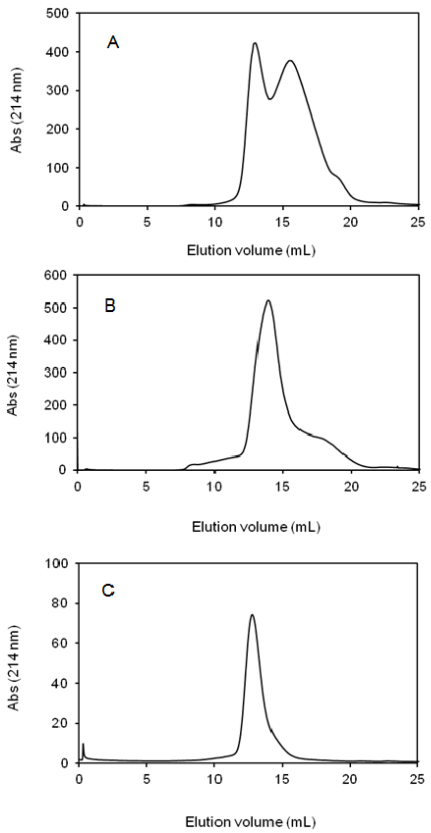

Figure 1. Gel filtration chromatography of *E. baetica* albumin extract using Superose 12 (A). Fractions corresponding to the first peak were pooled together and re-chromatographed twice more (B and C)

Agglutination assays were carried out in order to determine whether this glycoprotein behaves as a lectin. Human erythrocytes were agglutinated by this preparation. Two serial dilutions showed that the lowest lectin dilution agglutinating was 3 µg / 100 µL. Denaturing SDS-PAGE of the protein purified by gel filtration chromatography revealed one band corresponding to a molecular weight of 30 kDa according to the molecular weight calibration curve, although the band actually appeared lower than the 30 kDa standard (Figure 2). This indicates that the lectin purified from *E. baetica* seeds is most likely constituted by two subunits with the same molecular weight, which is also the case for the lectin isolated from *A. mongholicus* [8], and the grain legumes *Phaseolus sp.*, *Arachis hypogaea*, *Glycine max* and *Canavalia ensiformis*. Other lectins in Tribe *Fabeae* are characterized by the presence of two different subunits [5]. According to the nomenclature suggested by Van Damme et al. [20], the lectin purified from *E. baetica* seeds should be named EROBAEA. This name is derived from the first three letters of the generic name (*Erophaca*) followed by the first three letters of the specific name (*baetica*), followed by "a".

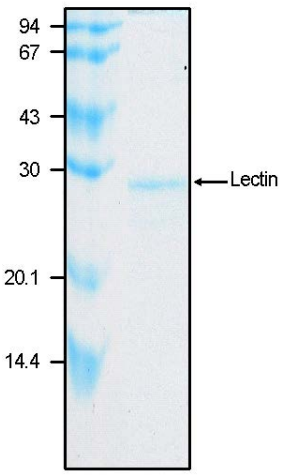

Figure 2. SDS-PAGE of the putative *E. baetica* lectin purified by gel filtration chromatography as shown in Figure 1. Molecular weight standards are shown on the left

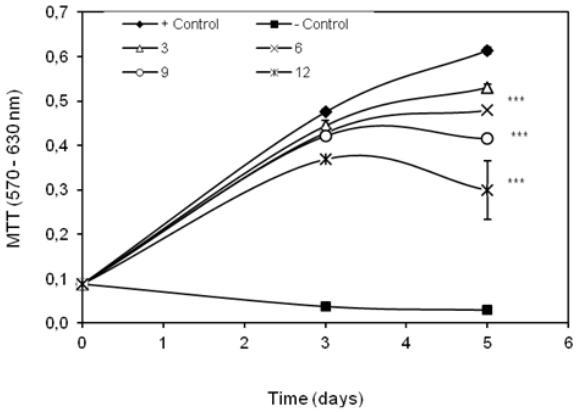

Figure 3. Effect of *E. baetica* lectin on the proliferation of human leukemic THP-1 cells. Cells were incubated in the presence of 3, 6, 9, 12 µg / mL lectin for up to 5 days. Positive control corresponds to cells treated with vehicle (PBS), negative control corresponds to cells treated with the apoptosis inducing agent camptothecin (20 µM). Results are the mean ± SEM for n = 6. *** ($p < 0.005$), significantly different as compared to positive control

Amino acid analysis revealed a composition similar to that of the lectin purified from *A. mongholicus* [8] (Table 1), which is characterized by relatively high content in Asp+Asn, Ser and Gly, and absence of Met, Cys, and Trp residues. Comparison with the amino acid composition of lectins from other related legumes by dendrogram analysis as sown in Figure 4 revealed a similarity with the lectin from the closely related *A. mongholicus*. Legumes included in Figure 4 belong to the denominated IRLC clade [21], which is characterized by the loss of one copy of a 25-kilobase inverted repeat in the plastid genome [22]. The monophyletic origin of this clade is well established [21]. Different subclades that are present in the IRLC clade are also revealed by the dendrogram. Thus, the two species in group A belong to the Astragalean clade. Species in group B belong to the Vicioid clade, which is the most distinctive subclade in the IRLC clade [21]. Finally, group C includes species that are taxonomically located between the Astragalean and Vicioid clades. It is concluded that the amino acid composition of these lectins correlates with the existing taxonomical classification of the corresponding legumes.

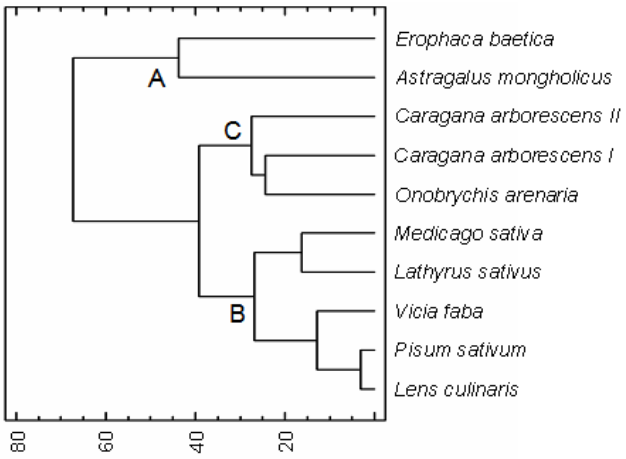

Figure 4. Dendrogram produced by cluster analysis of the amino acid composition of different legume lectins. *A. mongholicus* (Yan et al., 2005), *C. arborescens* (Bloch et al., 1976), *O. arenaria* (AN.: AAL79163.1), *M. sativa* (AN.: CAA76366.1), *L. sativus* (AN.: CAD27485.1), *V. faba* (AN.: CAD27484.1), *P. sativum* (AN.: AAA33675.1), *L. culinaris* (AN.: CAC42124.2)

Table 1. Amino acid composition of *E. baetica* seed lectin. Results are the average ± SD of two determinations

	% amino acids (g / 100 g protein)
Asp + Asn	16.7 ± 0.11
Glu + Gln	6.1 ± 0.00
Ser	16.0 ± 0.04
His	1.6 ± 0.07
Gly	13.1 ± 0.04
Thr	9.1 ± 0.04
Arg	1.9 ± 0.07
Ala	7.2 ± 0.07
Pro	2.3 ± 0.00
Tyr	0.9 ± 0.00
Val	5.0 ± 0.04
Met	0.0 ± 0.00
Cys	0.0 ± 0.00
Ile	3.6 ± 0.04
Trp	0.0 ± 0.00
Leu	7.4 ± 0.04
Phe	5.3 ± 0.04
Lys	3.8 ± 0.00

Legume seed proteins are usually divided into globulins and albumins according to the classification of Osborne [23]. Globulins are salt soluble proteins and represent the major protein fraction in legume seeds. Albumins are soluble in water and include most of the functional proteins such as enzymes, protease inhibitors, and lectins. However, this traditional classification that correlates physicochemical properties with functionality is being questioned in recent years. Thus, globulins may also have antinutritional and defensive properties in addition to represent a source of N and energy during seed germination [24,25]. Conversely, storage-like functions have been proposed for albumins such as lectins [20]. Hence lectins, which are abundant in legumes, would play both defensive- and storage-related roles depending on the requirements of plants [20]. The lectin that has been purified from *E. baetica*, representing 35% of albumins in the seeds, could be a good example of that.

In addition to their storage and antinutritional properties, plant lectins may also have health promoting properties such as antiproliferative and pro-apoptoptotic effects on tumor cells. Thus, concanavalin [26], and lectins from *Viscum album* [27], *Phaseolus* [28], and soybean [29] inhibit the in vitro growth of different human tumour cells. The possible effect on cell proliferation of the lectin from *E. baetica* has been determined by investigating the effect on the proliferation of human leukemic THP-1 cells. As shown in Figure 3, concentrations between 6 and 12 µg lectin/mL have a significant antiproliferative activity after incubation with the cells for 5 days. The highest lectin concentration, 12 µg/mL, inhibited proliferation by more than 50%. Interestingly, it has been described that the lectin from *A. mongholicus* inhibits the growth of tumour cells through cell cycle arrest at the S phase [9], while the lectin from *A. membranaceus* induces apoptosis through a caspase-dependent mechanism, probably following the mitochondrial pathway [10].

E. baetica is a long-lived perennial plant that produces abundant pods up to 7cm long, with seeds up to 1cm long containing 35.6 % protein (unpublished results). As shown here, the new lectin isolated from these seeds is easily prepared and could be of interest as a functional component because of its ant proliferative effect on cancer cells.

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