

# Two-dimensional gel electrophoresis to identify arcelins from *Phaseolus vulgaris* with immuno-proteomic analysis

## Electroforesis bidimensional para la identificación de arcelinas en *Phaseolus vulgaris* mediante análisis inmuno proteómico

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### ABSTRACT

Arcelins are lectin-like proteins present in the common bean *Phaseolus vulgaris* that are believed to play a role in bruchid resistance. The present study used proteomics to analyze the expression of lectin-like proteins, specifically arcelins, in *P. vulgaris* cultivar varieties from Venezuela. A PAGE-SDS analysis of 30 commercial accessions of *P. vulgaris* showed significant differences in the molecular weight range of lectin-like proteins (arcelins). Eight different accessions were selected based on their electroforetic mobility for the proteomic analysis. Arcelin immuno-detection of two dimensional electrophoresed proteins was used to easily display the different arcelin proteomic profiles of the studied accessions. Mass spectrometry analysis confirmed the arcelin nature of these proteins. This is the first report on arcelin evaluation of the Venezuelan germoplasm of *P. vulgaris* with the aim of enhancing breeding programs by identifying accession materials with resistance to bean storage pests.

**Key words:** common bean, immunoassay, proteomics, lectin-like proteins, bidimensional gels, mass spectrometry.

### RESUMEN

Las arcelinas son proteínas tipo lectinas, presentes en el frijol común *Phaseolus vulgaris* que se piensa que están implicadas en la resistencia a bruquideos. El presente trabajo utiliza la proteómica para analizar la expresión de proteínas tipo lectinas, específicamente arcelinas, en variedades de cultivares de *P. vulgaris* en Venezuela. El análisis mediante electroforesis SDS-PAGE de 30 accesiones de *P. vulgaris*, mostró diferencias importantes en el intervalo de peso molecular de las proteínas tipo lectina (arcelinas). Se seleccionaron ocho accesiones diferentes en base a su movilidad electroforética para el análisis proteómico. Se utilizó la inmuno-detección de proteínas separados mediante electroforesis bidimensional, para mostrar fácilmente los diferentes perfiles proteómicos de las arcelinas entre las accesiones estudiadas. El análisis de espectrometría de masas confirmó la naturaleza de las arcelinas presentes. Este es el primer informe sobre la evaluación de las arcelinas en el germoplasma de *P. vulgaris* venezolano con el objetivo de colaborar con los programas de mejoramiento mediante la identificación de materiales con resistencia a las plagas de frijol almacenado.

**Palabras clave:** frijol común, inmunoensayo, proteómica, proteínas tipo lectina, geles bidimensionales, espectrometría de masas.

## Introduction

The common bean (*Phaseolus vulgaris* L) is a widely cultivated legume that originated in the New World, which has been domesticated in Meso and South America (Chacon *et al.*, 2005). Dry beans remain the primary source of dietary protein carbohydrates, minerals and fiber in most developing countries, making them the third most important food legume crop worldwide (Singh and Muñoz, 1999).

The small-scale agriculture of the common bean is constrained by the multiplicity of biotic and abiotic factors that

affect crop productivity and quality during the growing season. In addition, one of the most important biotic factors is predation of dry bean seeds by post-harvest pests (bruchids), which largely affect the overall food grain production and consumption. It is estimated that 13% out of a total loss of 37% in global agricultural production is caused by pests and diseases (Obeng-Oferi, 2007).

A cost effective strategy is required in order to provide an efficient and sustainable control of bean infestation by bruchids. A combination of genetic resistance and biological and cultural control methods presents a sustainable way

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to deal with this problem. In efforts to develop bruchid-resistant beans, researchers have identified and introgressed different genetic resistance factors from wild relatives into common bean. The principal resistance factors in mature bean seeds are arcelins (Osborn *et al.*, 1988a; Osborn *et al.*, 1988b; Zaugg *et al.*, 2013).

Arcelins (Arcs) belong to the lectin-like family described in eight allelic variants in various accessions of the wild common bean, *P. vulgaris*, which exhibit different levels of resistance to bruchid insect pests. They are seed storage proteins associated with lectin or the arcelin-phytohemagglutinin- $\alpha$  amylase inhibitor (APA) complex locus (Zaugg *et al.*, 2013).

Although homologous to lectins, arcelins have a different intrinsic specificity for complex sugars, creating a mechanism of toxicity to bruchids, as opposed to the monosaccharide-binding affinity of true lectins (Fabre *et al.*, 1998; Blair *et al.*, 2010). The toxic properties of arcelins may be related to their ability to recognize and interact with glycoproteins and other constituents of the digestive tract membrane, as well as to their capacity to directly bind to the intestinal cells of insects (Minney *et al.*, 1990; Fabre *et al.*, 1998; Paes *et al.*, 2000).

One or two dimensional sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) techniques have been used for seed protein characterization to distinguish Andean bean genotypes from those of Mesoamerica according to the electrophoretic mobility of phaseolins (Gepts *et al.*, 1986; Gepts and Bliss, 1986; Kami *et al.*, 1995).

Additionally, in spite of the genetic studies that have been carried out and the approaches that have been used to study the *P. vulgaris* proteome (Natarajan *et al.*, 2013; Parreira *et al.*, 2016), the unique identification of arcelins in the different varieties is complicated.

The present study provides the first immuno-proteomic approach used for the characterization of arcelins from the common bean, *P. vulgaris*, as a preliminary germplasm characterization.

## Materials and methods

### Seed material

Mature seeds from eight common bean (*P. vulgaris*) accessions from the germplasm bank of INIA-CENIAP (Maracay-Venezuela) (10°17'14" N, 67°36'02" W, and 480 m a.s.l.) were used. Information about the germplasm used

in the present study (passport data) was published by Gamero *et al.* (2010). The seeds were selected from different states of Venezuela, showing dissimilar commercial values, consumer preferences and seed color. Commercial seeds corresponded to the states of Victoria, Tacarigua, Montalban, and Tenerife, and study cultivars NAG-7S, DOR-500, MGM-08-02-026 and MGM-10-02-108 were also selected.

### Protein extraction and samples preparation

Dry beans were milled, and 100 mg of flour were suspended in 20 mM Borate buffer pH 9 with 30  $\mu\text{g mL}^{-1}$  of a protease inhibitor mix (Amersham Biosciences, UK) and stirred for 1 h at 37°C. The samples were then centrifuged for 15 min (14,000 g) at 4°C, and the supernatant was used for the assays.

### Protein determination

The protein concentration was determined with the Bradford method (1976) using the Bio-Rad assay reagent (Bio-Rad, USA) and bovine serum albumin as the standard.

### Polyacrylamide gel electrophoresis

The proteins were separated with SDS-PAGE according to the method of Laemmli (1970). Briefly, 20  $\mu\text{g}$  of proteins were dissociated with heating at 90°C for 5 min in the presence of a denaturing buffer (20 mM Tris-HCl pH 8.6 containing 1% SDS, 8.3% glycerol and 0.5% 2-mercaptoethanol) and separated with electrophoresis in 12% polyacrylamide gels. The proteins were stained with Coomassie Brilliant Blue and destained in 7% acetic acid.

### Two-dimensional electrophoresis (2-DE)

2-DE was performed as described by O'Farrell (1975); 1-D isoelectric focusing (IEF) was conducted in a ZOOM® IPGRunner™ (Invitrogen, USA), whilst the SDS-PAGE was carried out using a Mini-protean II (BioRad, USA). Each sample was run in triplicate. The protein samples were diluted in a rehydration solution (De Streak™, Amersham Biosciences, UK) with 1% IPG buffer pH 3-10 to a final volume of 155  $\mu\text{L}$  (ZOOM® IPGRunner™ Cassettes, Invitrogen, USA) in a 7 cm IPG strip (Amersham Bio-Sciences, UK), pH 3-10 overnight; the proteins were then focused at a maximum voltage of 2,000 V. After IEF, the proteins were reduced and alkylated by soaking the IPG strips in the equilibration buffer (6M urea, 2% SDS, 30% glycerol, 50 mM Tris-HCl, pH 8.8) containing 1% DTT for 15 min at room temperature and then equilibrated in 4% iodoacetamine (IAA) for 15 min. The equilibrated IPG strips were sealed on top of the SDS-PAGE gel (12% polyacrylamide) using 0.5% agarose. The 2-D SDS-PAGE was carried out using a Tris-glycine-SDS buffer system at 200 V until the

dye front reached the bottom edge of the gel. The protein spots were visualized with staining using PlusOne™ silver staining kit (Amersham Bio-Sciences, UK).

### Immunoassays

The proteins from the 2-D SDS-PAGE were transferred at 100 V for 1 h to a nitrocellulose membrane for Western blot analysis. The membrane was Ponceau stained for 1.5 min to visualize the transferred protein and air dried. The membranes were re-wetted in a blocking solution composed of 2% non-fat dry milk in tris-buffered saline (TBS) with 0.5% Tween-20 (TTBS) and finally incubated for 1 h. The membrane was then probed for lectin-like proteins during 1 h of incubation with rabbit anti-lectin polyclonal antibodies (Sparvolli and Bollini, 1998) diluted at 1:6000 in a blocking mixture at 1:5 in TTBS. The immune serum was pre-absorbed overnight with 100 ng of bean flour from arcelin-null *P. vulgaris* in 1 mL of TBS to eliminate any unspecific cross-reactivity (Lioi *et al.*, 2003). The bound antibodies were detected with horseradish peroxidase conjugated anti-rabbit IgG antibodies (1:2000 in TTBS) using the ECL™ Western Blotting Detection Reagents (GE Healthcare Life Sciences, UK), following the manufacturer's instructions. The image of the chemiluminescent signals was recorded on an X-ray film and developed according to standard procedures in a darkroom.

### Image analyses

2-DE protein patterns were obtained as digitalized images using a high-resolution system (ProXCISION, PerkinElmer, Inc., USA). Spot detection, quantitation, and analysis were performed using Phoretix 2D (Nonlinear Dynamics, UK).

### Protein identification

Spots of interest were excised, picked and placed in a 96-well plate using the protein picking workstation ProXCISION (PerkinElmer, Inc., USA). The MultiPROBE II PLUS HT EX (PerkinElmer, Inc., USA) Robotic Liquid Handling System was used for enzymatic digestion. Briefly, the method involves the following steps: (i) reduction with 10 mM DTT; (ii) alkylation with 55 mM IAA; (iii) overnight tryptic digestion (6 ng  $\mu\text{L}^{-1}$  enzyme); (v) sample loading onto MALDI target using 1  $\mu\text{L}$  of the tryptic digests mixed at 1:1 with a matrix solution (a saturated solution of  $\alpha$ -cyano-4-hydroxyxinnamic acid in 0.1% TFA-acetonitrile 50:50).

A MALDI prOTOF™ 2000 (PerkinElmer, Inc., USA) was used for peptide analysis. Calibration was performed using a commercial mixture of peptides, PepMix (Perkin Elmer, Inc., USA), of known molecular masses. TOFworks™ 1.0 (PerkinElmer, Inc., USA) was used for the data processing

and for the peak list generation. The most intense peaks of the obtained spectra were identified with MASCOT PMF searching (<http://www.matrixscience.com>) using the Swiss-Prot and NCBI databases.

## Results and discussion

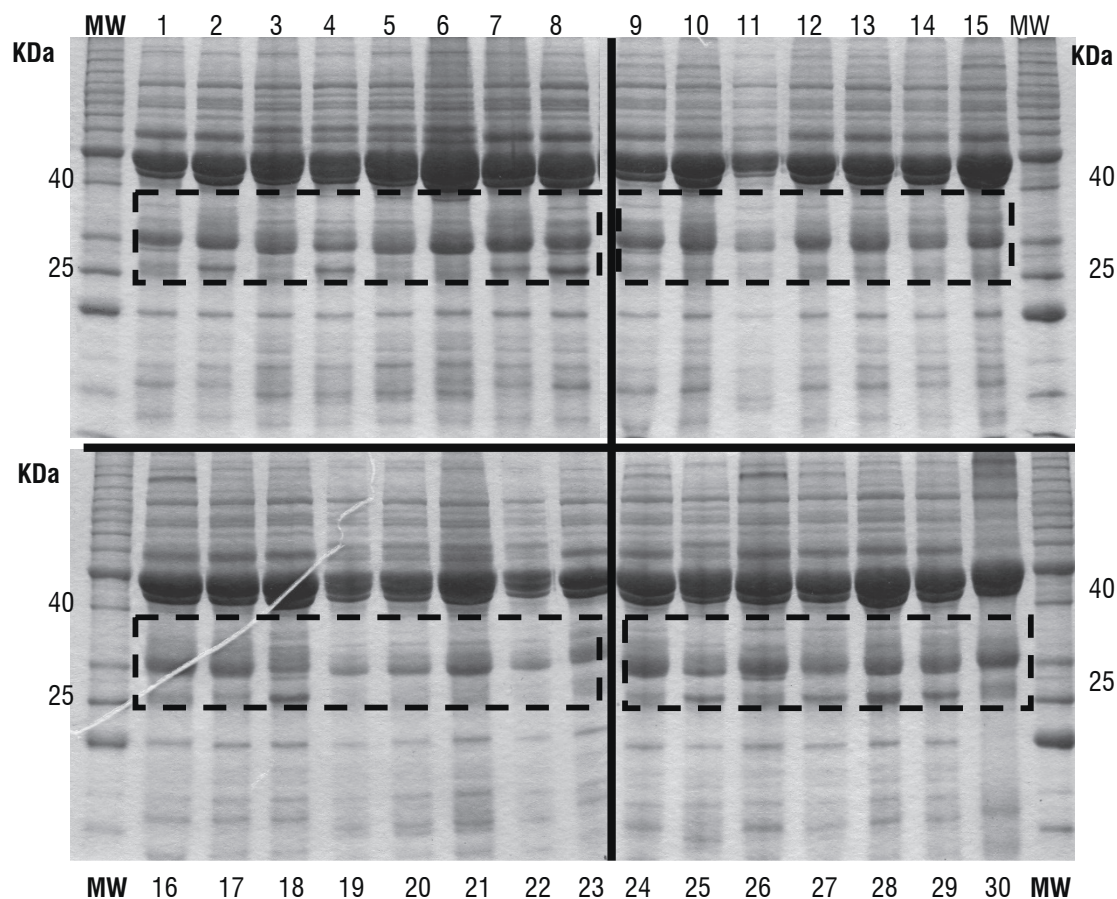
Different methods have been used for the identification and characterization of seed storage proteins in beans, including protein chemistry for purification of storage proteins for further structural and functional studies, proteomics protocols, and immunoassay techniques (Osborn *et al.*, 1988a; Osborn *et al.*, 1988b; Fabre *et al.*, 1998; Sparvolli and Bollini, 1998; Paes *et al.*, 2000; Lioi *et al.*, 2003; Zaugg *et al.*, 2013).

Moreover, in a previous study (Bernal *et al.*, 2006), an approximation of the comparative immunoassay technique with antibodies against the lectin family was used; however, the technique was nonspecific for the identification of proteins with mass spectrometry. The present study proposes a combination of proteomics and immunoassays techniques to successfully display the entire Arcelin catalogue of *P. vulgaris* for comparative purposes.

From the lectin family, arcelins were described as proteins associated with the inhibition of some bruchid species development. Although initially found in a limited number of wild common bean accessions from Mexico, approximately 10% of wild common bean accessions from Mesoamerica express Arcs. Until recently, Arcs were thought to be restricted to wild accessions of common bean, but arcelin-like sequences were obtained from other *Phaseolus* species (Osborn *et al.*, 1988a; Osborn *et al.*, 1988b).

Proteins of 30 *P. vulgaris* commercial accessions from a germplasm bank were first analyzed with SDS-PAGE to select the varieties that presented greater differences in the regions corresponding to the proteins of interest, in this case arcelins; these differences were visualized with image analysis programs (Fig. 1). Figure 1 shows the first selection of seeds with important differences in the 25-40 kDa range, where the majority of arcelins resolved (dotted boxes on the image, Fig.1).

After this analysis, only 8 accessions (Fig.1: 1, 2, 4, 8, 15, 18, 22 and 25) were selected and further analyzed with two-DE. The comparative analysis of the proteome images using Phoretix 2D revealed an average of 104 spots on the different proteomes from *P. vulgaris* (Fig. 2 B-I). These experiments were performed in triplicate for each accession.



**FIGURE 1.** SDS-PAGE of extracted proteins from 30 *P. vulgaris* accessions.

The immuno-detection of the arcelins was obtained from the 2-DE gels of extracted proteins from a commercial variety by using a specific immune serum against this family of proteins. Figure 2A shows the results of the immunoblot, with only a few spots detected in the range of 33-35 kDa and 5.5-5.7 pI, which correspond to the characteristics of arcelins. The immunoblot image was used as a reference to identify the same group of proteins in each proteome with Phoretix 2D (Fig. 2 B-I). The protein spot patterns were different in each *P. vulgaris* proteome, suggesting the presence of different arcelins in each case, although it was not possible to distinguish between subgroups of arcelins with this approach. We observed the presence of two, three, four and five spots related to the range of molecular weight and immuno-detection of arcelins (Fig. 2).

Some authors have stated that the more promising arcelin variants that confer insect resistance are arcelin-1, arcelin-5 and arcelin-8 (Osborn *et al.*, 1988a; Zaugg *et al.*, 2013); however, there is not much information on the presence of other variants and the role of these proteins in bruchid toxicity is not clear. Moreover, genetic studies suggest that resistance to storage pests in fact depends on a combination

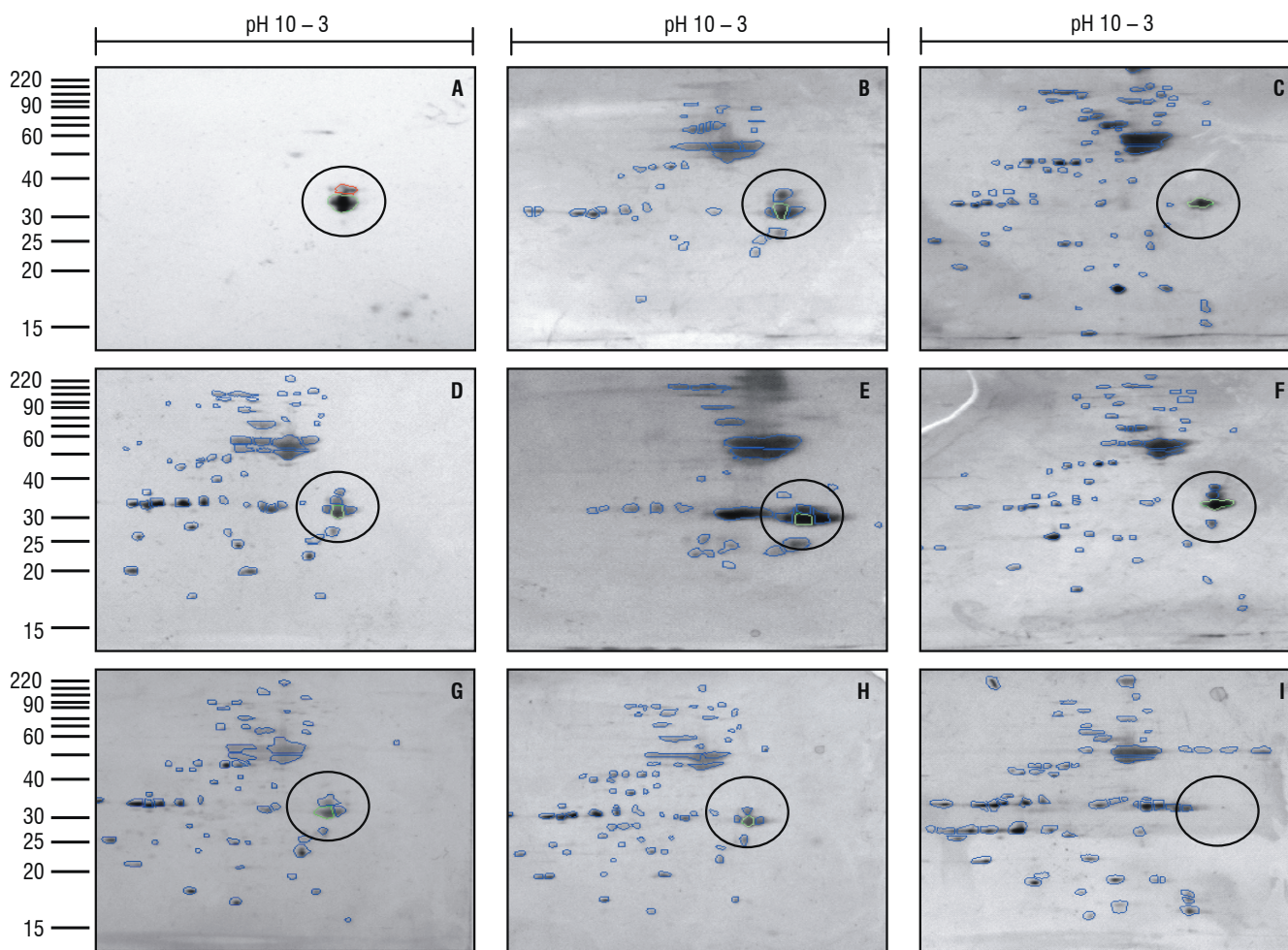
of proteins from the APA locus (arcelin, phytohemagglutinin and  $\alpha$ -amylase inhibitor), in which case the participation of the arcelin family can play a fundamental role (Blair *et al.*, 2010).

More profound mass spectrometry analysis is needed to elucidate which arcelin variants are expressed in these common bean accessions. It is worth noting that assayed commercial varieties have shown spots related to arcelins (Fig. 2). It is interesting to observe this high frequency of arcelins in the Venezuelan commercial varieties of *P. vulgaris*.

As shown in Table 1, it was possible to identify a group of peptides related to arcelins (98% identity) with mass spectrometry.

Despite the tools described in this paper, it was not possible to determine the type of arcelins present in each variety. These results further confirmed the arcelin nature of the immuno-detected spots.

The large-scale study of the *P. vulgaris* proteome has been described in previous studies (Natarajan *et al.*, 2013;



**FIGURE 2.** (A) 2-DE immunoblot of extracted proteins from a commercial variety of *P. vulgaris*. (B-I) 2-DE of extracted proteins from eight different accessions. Immuno-detection of the arcelin region with a specific antibody (A). Circles highlight the Two-DE region corresponding to arcelins in the different proteomes according to the immunoblot and the Phoretix 2D profile analysis.

**TABLE 1.** Peptide sequences and mass-specs for arcelin identification. Theoretical pI: 6, 16 / Mw (average mass) 29375.87 / Mw (monoisotopic mass): 29357.62.

Mass	Position	Peptide sequence
4564.2340	125 - 165	DYFLGLFNKPDDPEAHIVAVVFDTSNQEIDMNSISPMAR
3718.7547	215 - 248	VLNDWVSVGFSATSGLYDPTSETHDVLVSWFSK
3401.7627	6 - 36	LLSLALFLVLLTHANSASETSFNFTSFDNTK
1369.5875	203 - 214	CNFSTSSVHMEK
1217.6736	37 - 48	LILQGDASVSSK
1205.5215	58 - 69	GNGDPTVDSMGR
1092.5068	249 - 257	FSQHTTSER
1022.5517	193 - 202	ASLVYPSGTK
813.3989	128 - 188	ITYDSSK
772.4927	49 - 55	GQLLLTK
610.2831	173 - 177	YNGEK
608.2886	80 - 85	DSTTGK
516.2889	189 - 192	NNLR
502.2983	178 - 181	VEVR

Parreira *et al.*, 2016); however, it does not present a useful tool for the study of protein families of interest, such as lectins. The immuno-proteomic approach described in this study is a reliable alternative technique to easily localize/identify a subset of proteins from complex bidimensional gels. This is the first immuno-proteomic approach used for the characterization of arcelins from *P. vulgaris*.

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