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# FEATURES OF PROTEIN ISOLATION FOR PEA *Pisum sativum* L. ROOT PROTEOME ANALYSIS DURING SYMBIOSIS WITH RHIZOBIA

**A.N. KIRIENKO, I.V. LEPPYANEN, E.S. GRIBCHENKO, E.A. DOLGIKH**

*All-Russian Research Institute for Agricultural Microbiology,* Federal Agency of Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail dol2helen@yahoo.com (corresponding author)
ORCID:
Kirienko A.N. orcid.org/0000-0002-2519-306X
Leppyanen I.V. orcid.org/0000-0002-2158-0855
Gribchenko E.S. orcid.org/0000-0002-1538-5527
Dolgikh E.A. orcid.org/0000-0003-3433-2102

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## Abstract

Pea *Pisum sativum* L. is a convenient model to study the molecular-genetic mechanisms of nitrogen-fixing symbiosis establishment with rhizobia, because a representative collection of mutants, blocked at different stages of symbiosis development was obtained. A comparative analysis of the proteomes of the wild type cultivars and lines of peas and mutants can be a useful approach for carrying out studies aimed on at identification and further analysis of regulators controlling the formation of nitrogen-fixing nodules. However as the review of modern literary data shows, studies of differential proteome changes in pea roots during symbiosis are almost not performed. Sample preparation is a key stage in proteomic studies. The quality of gels obtained after 2-D electrophoresis and the opportunity of following analysis depend on protein isolation efficiency from the tissues and purification from accompanying substances. Our work is aimed on finding the most effective method of protein isolation from *Pisum sativum* roots inoculated with rhizobia, which might be applied for carrying the 2-D electrophoresis. Special requirements aimed at separation stages minimization important for protein stability, as well as the efficient removal of contaminants which can negatively affect the quality of separation and the subsequent evaluation of qualitative and quantitative changes in the protein synthesis are necessary for proteomics. Analysis of data revealed a number of possible methods for the protein isolation from plant tissues. A comparison of three methods of the proteins isolation using the commercial protocol from Bio-Rad; the method based on treatment with phenol and ammonium acetate as well as the trichloroacetic acid application. Pea plants of cv. Frisson were used in our work, the strain *Rhizobium leguminosarum* bv. *viciae* CIAM1026 was used for inoculation. After protein isolation from the wild-type cv. Frisson roots of pea seedlings inoculated with rhizobia (1 day after inoculation) using three methods and consequent 2-D electrophoresis, it was shown that the best results are achieved using the method with phenol following by ammonium acetate precipitation. The gels were analyzed for trace presence that made it difficult to search for different proteins, the efficiency of total protein isolation and possible degradation products. Using this selected method, the differential 2-D electrophoresis of extracted proteins was carried out with fluorescent Cy2 and Cy5 labels based on isoelectric focusing of proteins using strips with a pH range of 3-10 and subsequent separation in a polyacrylamide (PAGE) gel. The analysis showed that when proteins were isolated using phenol and ammonium acetate, it was possible to obtain rather representative proteomes of the roots of pea seedlings. The differential 2-D electrophoresis allowed to see the differences between the control samples (non-inoculated roots) and the samples inoculated with rhizobia (inoculated roots). This method may be recommended for further proteomic studies in pea roots.

Keywords: *Pisum sativum* L., pea, legume rhizobium symbiosis, proteomics analysis, receptors, Nod factors, legumes, rhizobia

In the formation of a symbiosis between leguminous plants and nodule bacteria (rhizobia), the plant undergoes significant changes in metabolic processes, hormonal status, and structural rearrangements associated with the reorganization of the cytoskeleton [1]. These changes are aimed at the formation of new organs on the roots — symbiotic nodules, in which nitrogen fixation is carried out. In recent years, with the spread of complex analysis methods that allow

studying changes in the transcription activity of plant genes under the influence of various factors (transcriptomics), it has become possible to identify regulators involved in controlling the development of symbiosis. However, such studies are conducted mainly on model species of *Medicago truncatula Gaertn.* and *Lotus japonicus L.*, the genomes of which are deciphered due to relatively small sizes (~ 470-500 MB) [2, 3]. It is shown that during the development of symbiosis in the roots of leguminous plants, the transcription activity of several thousand genes changes [4-6]. However, the functional significance of such changes in gene expression remains quite far from understanding.

Peas (*Pisum sativum* L.) has a very large genome (about 4300 MB), which has not yet been deciphered. Studies to identify genes that are differentially expressed in response to inoculation are a fairly complex task. In addition to the above, unique collections of mutants for *sym* genes, which control the development of symbiosis on the part of the plant, determine the interest in using this object. Proteomics serves as a complex approach for studying changes in the composition of proteins under the influence of biotic and abiotic factors. It allows to carry out comparative studies on peas and its mutants and to identify previously unknown regulators necessary for the formation of symbiosis. However, proteomic studies of pea proteins, which change in response to the treatment with rhizobia, were almost never carried out. The peribacteroid space of symbiosome in the development of symbiosis of peas with nitrogen-fixing bacteria was studied [7]. Pea proteomes at antagonistic interactions with crenate broomrape (*Orobanche crenata*) and mycosphaerella (*Mycosphaerella pinode*) [8, 9], at the development of vegetative organs and seeds [10-12], and the formation of resistance to temperature stress were analyzed [13]. The influence of salicylic and jasmonic acids on the protein composition of roots and leaves of peas was studied [14-16].

Sample preparation is a key step in proteomic studies using 2-D electrophoresis [17]. It is especially difficult when working with plants, the tissues of which are rich in proteases and substances that interfere with proteome analysis, i.e. polysaccharides, lipids and phenolic compounds [18]. Such compounds interfere with the separation of proteins and their analysis due to the occurrence of horizontal and vertical bands on the gels, blurs, reduced number of bright spots. In addition, plant tissues contain less protein than animal tissues and microorganisms, so effective protein extraction is important for obtaining successful results of 2-D electrophoresis [17, 18]. Proteomics allows qualitative and quantitative comparison of proteins in different samples, so the protein losses during analysis are crucial. When extracting proteins from the sample, it is necessary to preserve their quality and quantity, for which the chosen method should contain as few steps as possible in order to minimize losses [19, 20].

In this paper, we for the first time compared the effectiveness of three methods for isolating the total protein from the roots of the field pea. The method for isolating proteins using phenol and ammonium acetate proved to be most preferable for differential 2-D electrophoresis.

The purpose of the study was to optimize method for isolating the total protein pool from the roots of the field pea inoculated with rhizobia for obtaining better results during 2-D electrophoresis.

*Techniques.* Pea seeds (*Pisum sativum* L., variety Frisson) were sterilized for 5 min in sulfuric acid, washed 3 times with distilled water and germinated on 1 % aqueous agar in the dark for 4 days. Seedlings were transferred to the pots with sterile vermiculite impregnated with Jensen liquid medium [21]. The inoculation was carried out usinf strain *Rhizobium leguminosarum* bv. *viciae* CIAM1026 ($OD_{600}$ ≈ 0.5), 1 ml per seedling. Noninoculated seedlings were used for the control. The plants were grown in the phytotron Sanyo MLR-351H (Japan) at 21 °C, 16-hour

light day, 60 % humidity. For the analysis, fragments of the major roots corresponding to the zone of susceptibility to the rhizobia infection were selected 24 hours after inoculation with rhizobia. Samples were frozen in liquid nitrogen and stored at –80 °C. For isolating proteins, the roots (100 mg for each procedure) were crushed in liquid nitrogen in cooled mortars.

Protein extraction according to the standard procedure of Bio-Rad Laboratories (USA) included homogenization of the material in the buffer for isoelectric focusing (IEF). The samples was transferred to eppendorfs and a buffer for the IEF was added, containing 25 mM Tris-HCl (pH 8.0), 9 M urea, 4 % 3-(3-cholamido-1-propyl-dimethylammonio)-1-propanesulfonate (CHAPS), 50 mM dithiothreitol (DTT), 0.2 % ampholytes (Bio-Rad Laboratories, USA). The mixture was incubated for 20 min on ice, and then centrifuged for 15 min at 12,000 g, +4 °C. The supernatant containing proteins (150 μl) was applied to the rehydration strips.

To isolate proteins with phenol and ammonium acetate, the extraction buffer cooled up to +4 °C was added, containing 0.1 M Tris-HCl (pH 8.0), 30 % sucrose, 10 mM of DTT, 2 % sodium dodecyl sulfate (SDS), and a cocktail of protease inhibitors (Sigma, USA). The mixture was centrifuged (Hettich 320R, Germany) for 15 min at 12,000 g and +4 °C. The supernatant was mixed in a ratio of 1:1 with phenol (pH 8.0) (Invitrogen, USA), shaken for 30 seconds (Vortex Genius, Germany) and centrifuged at 10,000 g for 5 min at +4 °C. The upper phase was transferred to a new eppendorf; the proteins were precipitated with 5 volumes of cooled 100 mM ammonium acetate in methanol for 30 min at –20 °C, and then centrifuged for 5 min at 10,000 g. The precipitate was washed twice with 100 mM ammonium acetate in methanol and twice with 80 % acetone. The protein precipitates were air dried and dissolved in isofocusing buffer containing 25 mM Tris-HCl (pH 8.0), 9 M urea, 4 % CHAPS, 50 mM DTT, and 0.2 % ampholytes (Bio-Rad Laboratories, USA).

For isolation of total proteins with trichloroacetic acid (TCA), its 10 % solution and 0.07 % β-mercaptoethanol, prepared with acetone, were added to the crushed roots. The resultant mixture was ultrasonically treated 3 times for 20 seconds at 10 μm amplitude (Soniprep 150 Plus, MSE, UK). The suspension was incubated for 1 hour at –20 °C, with stirring every 15 min in a shaker, and then centrifuged for 20 min at 9,000 g and +4 °C. The resulting precipitate was washed twice with 0.07 % β-mercaptoethanol in acetone, then dried in a vacuum evaporator (Concentrator Plus, Eppendorf, USA), dissolved in a buffer containing 25 mM Tris-HCl (pH 8.0), 9 M urea, 4 % CHAPS, 50 mM DTT, and 0.2 % ampholytes (Bio-Rad Laboratories, USA), incubated for 20 min on ice and centrifuged for 10 min at 12,000 g and +4 °C. The resulting supernatant containing proteins (150 μl) was applied to the rehydration strips.

Isoelectric focusing (IEF) was carried out using 7-cm-long strips with a pH gradient of 3-10 and a Protean IEF Cell (Bio-Rad Laboratories, USA). Preliminarily, rehydration of strips and the loading of the obtained protein samples (150 μl per strip) were performed for 12-14 hours. The IEF was carried out at a temperature of +20 °C, the samples were desalted at 250 V for 15 min, then the voltage was linearly increased up to 4,000 V for 2 hours, then the IEF was carried out (up to 10,000 V/h with a current limit of 35 mA per gel). After the IEF procedure, the strips were frozen and stored at –80 °C or immediately separated in the second direction.

Before electrophoresis of the proteins in the polyacrylamide gel (PAAG), the strips were incubated for 10 min in a buffer containing 0.375 M Tris-HCl (pH 8.8), 6 M urea, 2 % SDS, 2 % DTT, 20 % glycerol, then alkylated for 10 min in buffer with 0.375 M Tris-HCl (pH 8.8), 6 M urea, 2 % SDS, 20 %

glycerol, 2.5 % iodoacetamide. Then the strips were laid on a concentrating gel, and melted 0.5 % agarose (25 mM Tris-HCl, 192 mM glycine, 0.1 % SDS) with bromophenol blue (BFS) were layered down on top to control the separation. Electrophoresis was performed in 15 % PAAG using 4 % gel (35 mA per gel until the BFS was completely released from the gel) and Tris-glycine buffer (25 mM of Tris-HCl, pH 8.3, 192 mM glycine, 0.1 % SDS). After the electrophoresis completed, the gels were washed with deionized water and stained in SimpleBlue™ (Invitrogen, USA) according to the manufacturer's protocol. The gels washed from the dye were photographed on G:BOX-CHEMI-XX9 (Syngene, Great Britain).

To perform differential 2-D electrophoresis (D-D fluorescence difference gel electrophoresis, DIGE), the total protein obtained after isolation with phenol and ammonium acetate was dissolved in 200 μl of IEF buffer without DTT and ampholytes. The protein samples were then conjugated with the fluorophores Cy2 and (or) Cy5 (Lumiprobe, Russia) in different combinations by incubation of protein extract (50 mg) with 400 pM of dye dissolved in dimethylformamide for 30 min on ice in the dark. The labeling reaction was stopped with 10 mM of L-lysine (Sigma-Aldrich, USA) for 10 min on ice. Control and test samples labeled with different dyes were mixed and, after adding DTT and ampholytes, used for rehydration of strips. After separation of the proteins, the gels were visualized with a laser scanner Typhoon FLA 9500 (GE Healthcare, Germany). To analyze photos of gels, the program ImageJ (https://imagej.nih.gov/ij/) was used.

*Results.* In the last decade, proteomics has established itself as a method that makes it possible to rather effectively estimate the changes occurring in a plant under the influence of various biotic, abiotic and anthropogenic factors. The success of the method depends on the preparation of samples and obtaining the original extract of proteins [22]. This is especially important for differential proteomics, which is related to the study of the differences between the control samples and the samples obtained after the treatments. The choice of the optimal method for extraction of the total protein pool is a key factor for obtaining reliable experimental results [23]. The biochemical properties of proteins, such as solubility, overall charge, as well as localization features (e.g., membrane proteins) and a low amount of protein in the origin material, can negatively affect the study of full protein spectrum. In addition, plant cells contain significant amounts of non-protein substances — polysaccharides, lipids and organic acids [24], and the cell wall consists of a large amount of fiber and pectin. These substances have a significant effect on the quality of protein extracts and, consequently, on the results of 2D-electrophoresis [25, 26]. The optimal method of sample preparation is necessary in order to effectively remove non-protein substances from the sample.

We tested three methods for the total protein extraction from pea roots after 24 hours of incubation with rhizobia. In all cases, the destruction of tissue was carried out in liquid nitrogen, which made it possible to efficiently homogenize the material and destroy the cell wall. After additional washing (when phenol and ammonium acetate or TCA were used), all samples were dissolved in a buffer containing urea, detergent CHAPS, reducing agent DTT and a cocktail of protease inhibitors. These substances allow avoiding degradation, modification, loss and precipitation of proteins [27].

The preparation of the extract of pea proteins using the standard method was the least suitable for analysis. 2D-electrophoresis of samples detected a small amount of isolated protein in the control and processed samples (Fig. 1, A, B). The gel also showed traces of contamination, i.e. bands and background noise, which prevented the detection of differences in the spectrum of the separated proteins.

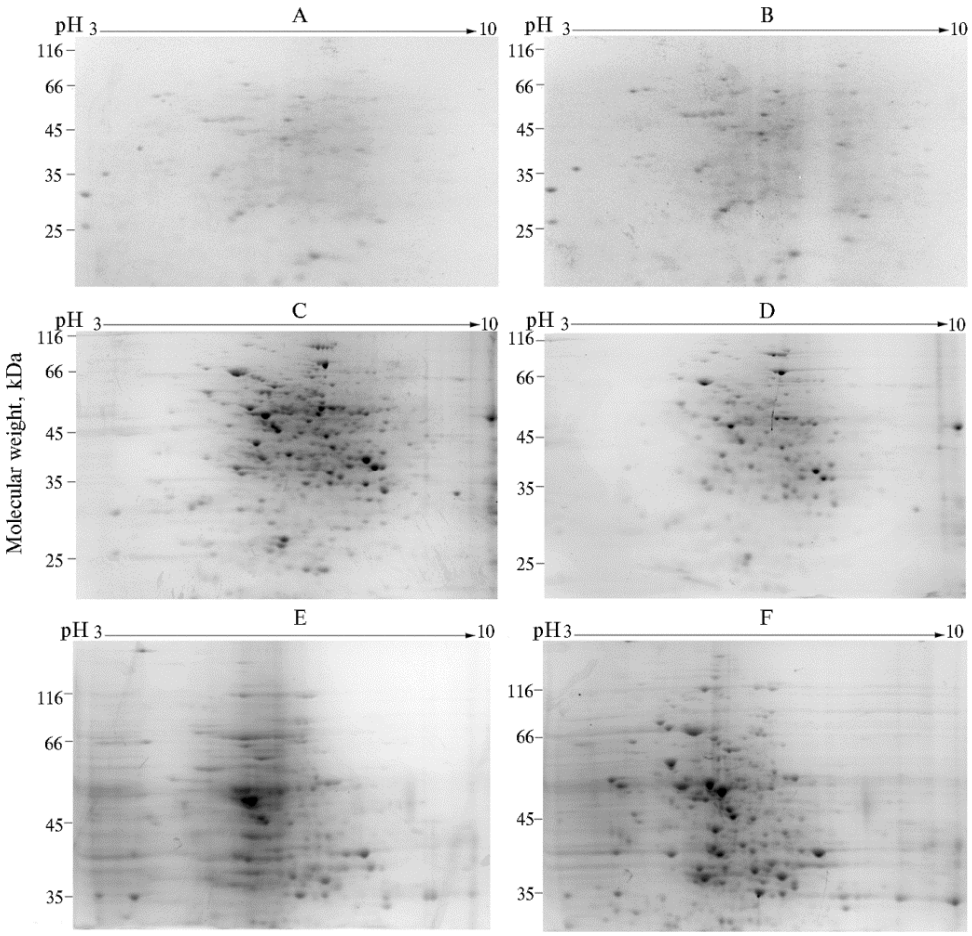


**Fig. 1. 2-D electrophoregrams of proteins isolated from roots of pea (*Pisum sativum* L.) Frisson variety according to the standard procedure of Bio-Rad Laboratories (USA)** (A, B), **using phenol and ammonium acetate** (C, D)**, using trichloroacetic acid** (E, F)**:** on the left — control samples, on the right — samples from roots inoculated with rhizobia (1 day after inoculation). Strips with pH 3-10 were used. Separation in the second direction was carried out in 12 % PAAG.

Isolation of proteins with TCA proved to be more effective. We were able to extract greater amount of protein compared to the standard method, due to a more saturated spectrum of spots in PAAG. However, 2-D electrophoresis of samples obtained using TCA (see Fig. 1 E, F) showed the impossibility of removing background noise (horizontal bands), the occurrence of which is associated with the residues of nucleic acids, polysaccharides and phenolic compounds [28, 29]. Polysaccharides were also the cause of protein aggregation in the sample. Such protein complexes can block the PAAG pores and make it impossible for the peptides to pass through the gel and to focus in the desired zone [30]. According to reported findings, the disadvantages of this method include small number of extractable proteins, which was noted earlier in experiments on obtaining a protein extract from cells of sugar beet *Beta vulgaris* L., cactus *Mammillaria gracilis* Pfeiff. and Jupiter's-beards *Sempervivum tectorum* L. Such a disadvantage can be eliminated using a larger amount of the origin material, but this, in turn, will increase the amount of polluting agents.

A method using phenol and ammonium acetate was first proposed by W.J. Hurkman and C.K. Tanaka for proteomic analysis [19]. Phenolic extraction was used to isolate proteins from potatoes, rape, apples, banana and olive leaves,

tomatoes, alfalfa, avocado and bananas [17, 18, 24, 31-33]. When studying plant-microbial interactions, it was used to analyze the *Lotus japonicus* proteome in the late stages of symbiosis with *Mesorhizobium loti* [34, 35]. The method is more time-consuming than others, but in our case it turned out to be most effective for obtaining high-quality 2-D electrophoregrams. There were no clearly expressed horizontal bands on the gel that are typical of polysaccharide contamination (see Fig. 1, C, D). The high quality of the gel was achieved due to the large amount of isolated protein, which makes possible a more reliable comparative analysis of the control sample and the sample after treatment. The advantages of the method include efficient extraction of membrane proteins from the sample [19].

Based on the results obtained, this method was selected for isolation of proteins when performing differential 2-D electrophoresis (DIGE). The obtained protein samples were incubated with fluorescent dyes; IEF and separation in PAGE were performed (Fig. 2).

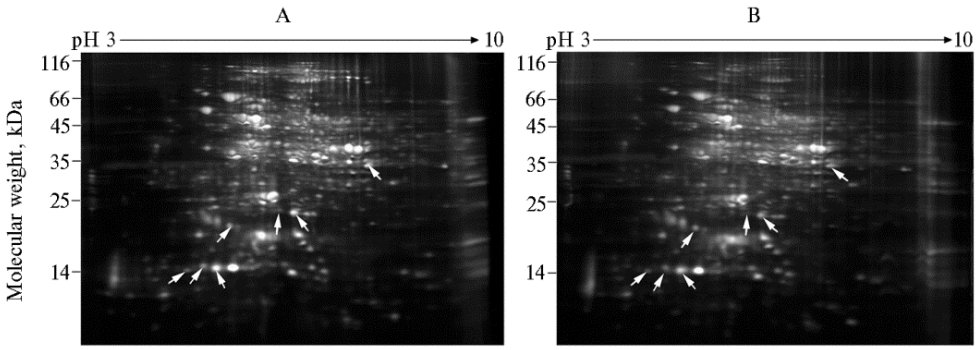


**Fig. 2. 2-D differential electrophoregrams of proteins isolated from roots of pea (*Pisum sat*ivum L.) Frisson variety using phenol and ammonium acetate:** A — control sample (Cy2, blue fluorescent label) and a sample after treatment with rhizobia (Cy5, yellow fluorescent label); B — control sample (Cy5, yellow fluorescent label) and sample after treatment with rhizobia (Cy2, blue fluorescent label). Strips with pH 3-10 were used. Separation in the second direction was carried out in 12 % PAAG. White spots indicate the absence, color indicate the presence of a difference in the level of protein synthesis. Arrows indicate proteins, the synthesis of which is enhanced after treatment.

Thus, extraction of the total protein pool of pea roots inoculated with rhizobia using phenol and ammonium acetate proved to be the most effective and qualitative in preparing samples for carrying out both standard electrophoresis in PAAG followed by SimpleBlue™ staining and for differential 2-D electrophoresis. This method made it possible to isolate a sufficient amount of protein and to eliminate impurities that interfere with qualitative electrophoresis, to perform effective labeling and to obtain a picture of high resolution, which is necessary for determining the difference in the spectrum of synthesized proteins. In the future, the method will be used to study the differences in the spectrum of synthesized proteins at successive stages of development of symbiosis between the field pea and *Rhizobium leguminosarum* bv. *viciae* CIAM1026.

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