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Extraction and Solubilization Methods Suitable for Proteomic Analysis of Common Bean Seed of Ivory Coast

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Abstract

The extraction and solubilization of proteins from seeds of *Phaseolus vulgarisms* for two-dimension polyacrylamide gel electrophoresis (2D-PAGE) and analysis by mass spectrometry are very sensitive procedures. In this study, we used two methods of extraction and solubilization of proteins, the urea/thiourea method and the trichloroacetic acid (TCA)/acetone precipitation method, in order to determine their effectiveness in separating proteins from bean seeds by 2D-PAGE. In both methods, proteins were well separated by 2D PAGE with minor variations in the protein pattern. These two extraction methods showed that it was possible to separate hundreds of very resolvent proteins by 2D electrophoresis. A protein spot was selected on the 2D-PAGE gel, digested with trypsin and analyzed by mass spectrometry (LC-MS/MS). The results suggest that thiourea/urea and TCA methods were effective and reliable for the extraction and solubilization for 2D analysis of proteins from seeds of *Phaseolus vulgaris*.

Keywords

Proteomics, Bean Seeds, *Phaseolus vulgaris*, 2D-PAGE, LC-MS/MS

1. Introduction

The extraction and solubilization of proteins for proteomic analysis is a milestone for subsequent analysis by polyacrylamide two-dimension electrophoresis (2D-PAGE) [1]. By optimizing the methods of sample preparation, or the extraction of proteins resolubi-

lized in a buffer compatible with the electrophoresis, we improved the conditions so that separation by isoelectric focusing (IEF) is possible [1] [2].

Indeed, much progress has been made [3] [4] in sample application with the use of immobilized pH gradient strips (IPG) for isoelectric focusing (IEF). However, the extraction and solubilization are difficult to implement in legumes. The study of legume (soy) proteomics has already been reported [5]; however these authors have underlined the difficulty of extracting and solubilizing its proteins.

The search for new techniques of protein extraction remains a challenge for proteomic analysis because of the presence of contaminants affecting the performance in two-dimensional (2D) analyses. To separate a wide range of proteins, it is important to find effective methods of extraction and solubilization. This study aimed to test two different methods of extraction and solubilization of proteins from bean seeds (*Phaseolus vulgaris*) from Ivory Coast, in order to assess their compatibility with IEF and the quality of the electrophoretic separation.

2. Materials and Methods

2.1. Plant Material

Bean seeds (*Phaseolus vulgaris*) were collected at the National Center for Agricultural Research CNRA (Ivory Coast).

2.2. Methods

2.2.1. Protein Extraction

Bean seeds were crushed with a pestle in a mortar to a fine powder and subsequently extracted by one of the following methods: Urea/Thiourea and or Trichloroacetic acid (TCA)/acetone precipitation.

2.2.2. Extraction and Solubilization in Thiourae/Urea Buffer

One gram of crushed seeds was washed two times with hexane to remove lipids. After vacuum drying, Protein was extracted by vortexing 100 mg of seed powder with 1.5 ml of extraction buffer containing CHAPS (4%), urea (5 M), thiourea (2 M), (0.8%) ampholytes [pH 3 - 10] and DTT (65 mM) for 5 min at room temperature. The extract was centrifuged at 13,000 rpm for 30 minutes at room temperature. The supernatant was collected for the two-dimensional (2D) analysis.

2.2.3. Extraction and Solubilization in Trichloroacetic Acid/Acetone Buffer

For this method, bean seeds were powdered in liquid nitrogen using mortar and pestle. Bean seeds powder (100 mg) was homogenized with 5 ml of a solution containing 10% (w/v) trichloroacetic acid (TCA) in acetone with 0.07% (v/v) 2-mercaptoethanol for 2 hours 30 min at −20˚C and then centrifuged at 4000 rpm for 30 minutes at room temperature. Proteins of the crushed seeds were precipitated for 2 hours 30 min at −20˚C in an acetone/TCA buffer containing 10% of TCA, 0.07% of 2-mercaptoethanol and acetone, and the mixture was centrifuged at 4000 rpm for 1 hour at −20˚C. Then, the pellet was rinsed twice at −20˚C for 1 hour in 2-mercaptoethanol and acetone buffer

and centrifuged as previously done. After 2 hours and 15 min of vacuum drying, the pellet obtained was resuspended in a solution containing urea (9 M), CHAPS (1%), DTT (1%) and ampholytes [pH 3 - 10] (4%) and solubilized by stirring for 30 min. In order to eliminate insoluble compounds, a final centrifugation for 10 min at 13,000 rpm was performed and the protein solution recovered. This solution was ultracentrifuged (200,000 g, 30 min) for 1 hour at room temperature. The resulting supernatant was stored at −20˚C until use in 2D-PAGE analysis.

2.2.4. Isoelectric Focusing

The first dimension was performed on the Protean IEF Cell apparatus (Bio-Rad). Strips of immobilized pH gradient (IPG) 24 cm long covering a linear pH range from 3 to 10 linear were used. The samples were deposited with the strips rehydration solution during the first dimension (9 M urea, 4% CHAPS, 0.6% ampholytes and 20 mM DTT) for 13 h at 20˚C. The strips were protected from dehydration and oxidation by coating with mineral oil. The first dimension was held performed at 20˚C and was divided into five steps to obtain at least 100,000 Volts per hour. The strips were then directly used for the second dimension. Before the implementation of the second electrophoresis, the gels of the first dimension (IPG) were equilibrated in two different buffers. The strips were equilibrated for 15 min in 2 ml of buffer solution containing urea (6 M), SDS (2%), Tris-HCl (0.375%), glycerol (20%) and DTT 130 Mm. Then, these strips were placed for 20 min in the same buffer but by replacing DTT with 135 mM iodoacetamide.

2.2.5. Stage of the Second Dimension Gel 12% Acrylamide

The second dimension was performed at 20˚C. Balanced strips were placed by placing the equilibrated strips on a 12% acrylamide gel (Duracryl TM) without stacking gel. The strips were fixed at the top of the acrylamide gel with a solution containing 1% agarose with low melting point, diluted in a migration buffer. Migration was stopped after 7 h of migration at constant voltage of 200 V, that is to say 1400 V/Hour. The migration front was determined by the migration of bromophenol blue. At the end of the second dimension, the gels were rinsed three times for 1 min with MilliQ water and fixed overnight in a solution of methanol (30%) and acetic acid (10%). The gels were then rinsed twice in a solution of methanol (10%) for 10 min, placed for 1 minute in a solution of solubilization composed by sodium thiosulfate (0.02%), washed for 1 min with water and incubated for 30 min in a solution of silver nitrate (0.01%) at 4˚C with shaking. After washing twice for 1 minute with water, the gels were placed in the revealing solution (0.037%) (1.2% formaldehyde in sodium carbonate), under stirring, until the appearance of spots. The reaction was stopped by immersing gels in acetic acid, 1%, and kept them in a solution of 1% acetic acid.

2.3. Digestion of Proteins and Extraction of Peptides to Analyze

The proteins of interest were cut from the gel and transferred into eppendorf tubes. The pieces of gel underwent a first wash of 30 min at room temperature in 500 mL of 100 mM carbonate buffer and then were washed again in the same buffer but at 20 mM.

The pieces of gel were then subjected to a dehydration which facilitates the penetration of trypsin and favour digestion. Fifty µl of acetonitrile were added and after 15 minutes without agitation, drying was carried out in a lyophilizer (Savant UVS400A). The pieces of gel were rehydrated with 10 mL of digestive solution (trypsin 0.1 mg/mL) and after 15 minutes at room temperature, 50 mL of carbonate buffer (20 mM) were added and digestion was performed at 37˚C for 7 h. The digestive solution was then recovered and washing was performed in a mixture (50/50) acetonitrile/carbonate buffer, 20 Mm. A second washing with the mixture (100 uL) acetonitrile/TFA/water (01/10/1989) allowed extraction of the remaining peptides in the gel. The peptides were concentrated by drying in Speed Vac and resuspended in formic acid 0.1% and in acetonitrile 3% for analysis with mass spectrometry LC-MS-MS.

Sample Preparation and LC-MS-MS Analysis

Five µL of the peptide solution were injected for LC-MS-MS trap analysis. The ions formed in the source enter in a first analyzer that allows the selection of the ion which will be fragmented. This ion is the only one which comes out of the first analyzer and then will collide with neutral gas molecules placed in a collision chamber. The search in protein databases is carried out by the program Mascot 2.0 (www.matrixscience.com).

The mass spectra were analyzed using the Mascot software in the database NCBInr 20,090,324 (8,097,822 sequences; 2,786,930,639 residues) with the Taxonomy: Viridiplantae (Green Plants) (632,568 sequences) with the following parameters:

Type of search: MS/MS Ion Search Enzyme: Trypsin
Variable modifications: Carbamidomethyl (C), Carboxymethyl (C), Oxidation (M)
Mass values: Monoisotopic
Protein Mass: Unrestricted
Peptide Mass Tolerance: ±1.6 Da
Fragment Mass Tolerance: ±0.8 Da
Max Missed Cleavages: 1
Instrument type: ESI-TRAP

3. Results and Discussion

The thiourea/urea and the acetone/TCA methods of extraction and solubilization were compared in this study on *Phaseolus vulgaris* seed proteins. The Thiourea/Urea method (**Figure 1(a)**) showed a good proteins solubilization for proteomic analysis of *Phaseolus vulgaris* seed proteins. Indeed, this increase in solubility of the thiourea/Urea method could be explained by the combined effect of chaotropic and reducing agents as well as detergents. Furthermore, the urea and thiourea, two chaotropics solubilize proteins. Regarding the CHAPS, which is also a detergent, it also solubilizes proteins. It is not charged, that makes it compatible with IEF. Furthermore dithiothreitol (DTT), a reducing agent, has been shown to improve the proteins solubility during IEF and to increase the protein transfer during the second dimension [6]. Thus, in our study, the combination of two chaotropic agents with a detergent and a reducing agent has increased the solubility and resolution of proteins (**Figure 1**). The red circles highlight a

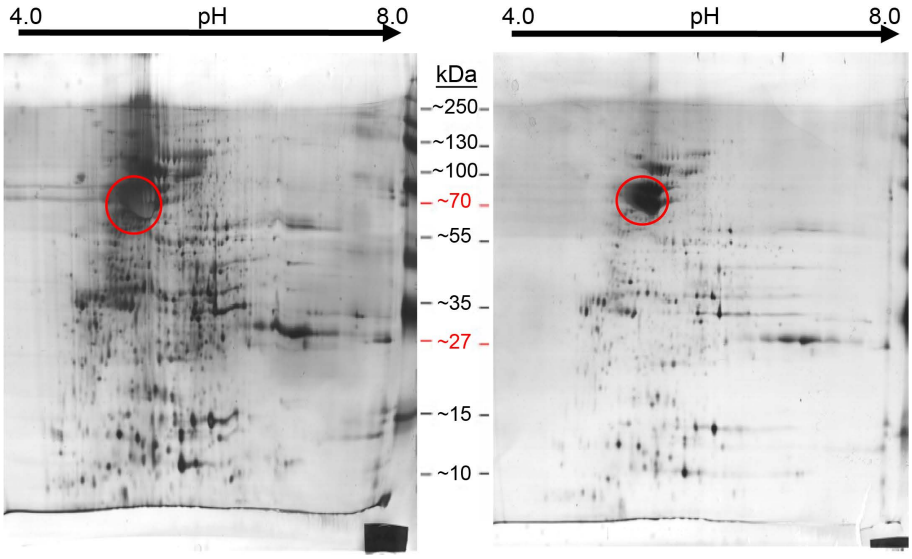

Figure 1. Two-dimensional electrophoresis gel of *Phaseolus vulgaris* proteins extracted using various methods: (a) Thiourea/urea methods and (b) TCA/acetone precipitation methods.

group of abundant proteins which were not well separated because they were present in excessive amounts.

This result is consistent with the findings of Mechin [7] Damerval [8]. The works [9] showed that the addition of thiourea to urea increased the chaotropic nature and led to a better solubilization of many proteins. Other studies showed that using this extraction method provided better solubilization of soybean proteins [10]. Sample preparation by the Thiourea/Urea method for isoelectrophoresis (IEF) is complex. Indeed, IEF imposes two constraints: to respect the protein charge and work at low ionic strength. The first constraint is obvious because of the separation principle applied, and disclaims any charged detergent which, by its binding to proteins, could modify their charge. The second constraint is, however, less obvious because in the case of IEF, the electric charge of proteins is initially low and gradually decreases as they approach their isoelectric points. Accordingly, strong fields must be used, and for long enough times. At these values, the presence of salt induces a huge joule effect, which therefore requires the preparation of the sample in environments of low ionic strength. The acetone/TCA precipitation method (**Figure 1(b)**), also improved the solubilization of *Phaseolus vulgaris* seed proteins.

With this method, proteins were first precipitated. After the precipitation, we used a single chaotropic agent, a detergent and a reducing agent. So, the fact of precipitating the proteins makes the solubilization easier. Our results confirm the work [11]; who found that the resolution of acetone/TCA precipitation improves the solubility of protein spots. These authors explain this by the inhibition of proteolytic activity. [2] [12] had also shown a high resolution of proteins by 2D electrophoresis using a precipitation method with acetone and trichloroacetic (TCA) with soy proteins and Arabidopsis thaliana on wheat seedlings. A study of Natarajan [13] showed that the trichloroacetic

acid (TCA) precipitation and the thiourea/urea methods are quite similar, efficient and reliable for 2D separation of soy proteins. Other studies have also shown that the use of these extraction methods allows a better solubilization of proteins. In order to denature, reduce and well solubilize various proteins found in a complex biological sample such as *Phaseolus vulgaris*, these solubilization media contain a reducing agent of disulfide bonds, a neutral chaotropic agent (urea) at a high concentration, an electrically neutral surfactant and a buffer at low concentration, which must meet the chemical and electrical requirements of the IEF [3]. This buffer system often contains carrier ampholytes which have the advantage to increase the proteins solubility and smooth the conductivity of immobilized pH gradients.

The extraction and solubilization is the first and therefore crucial step in proteomic analysis. Indeed, an inadequate extraction and solubilization of protein causes poor migration in the first dimension and leads to poor resolution of 2-D gels. It is important to note that the terms of sample extraction and solubilization will determine the success of proteomic analysis.

Since the aim is to obtain individualized proteins after separation by two-dimension electrophoresis for identification by mass spectrometry, it is necessary to make a good extraction. The cells destruction must be achieved while preserving the integrity of proteins [14]. The extraction is fairly difficult with proteins of legumes [3]. Thus, in our study we were able to extract and solubilize proteins from a sample of *Phaseolus vulgaris* seeds that might be explained by the fact that the seed of *Phaseolus vulgaris* does not contain large quantities of fat. The two solubilization methods used allowed to remove and eliminate non-protein components that interfere with IEF as non-protein impurities can affect the quality of the 2D separation. The choice of the revealing method depends on the desired resolution and the type of analysis to be performed on proteins after separation. To identify specific proteins, the gels from both experiments were stained with silver nitrate following a method compatible with subsequent mass spectrometry. However, there are many other methods for detecting separated proteins [15]. With this silver nitrate revealing method, a total of 971 spots were identified for the Urea/Thiourea method (**Figure 2(a)**) and 923 spots detected for the acetone/TCA precipitation method (**Figure 2(b)**). Then, a spot of interest was excised from each method gel, digested with trypsin and analyzed by mass spectrometry LC-MS/MS. The results gave us high quality spectra (**Figure 3** and **Figure 4**).

Interest spots identified as number 1453 and 1527 with molecular weight 18 kDa and 18.5 kDa, and with pI 4.7 and 4.9 respectively. The peptide profiles of the spot 1453 and 1527 (**Figure 3** and **Figure 4**), do not correspond to peptidic protein profiles in the consulted databases. Each peptide profile analyzed by the Profound software provides recovery rate from 10% to 36% for 1453 spot and 63% for 1527 spot.

We were however unable to identify the specific proteins of interest because of a lack of data on *Phaseolus vulgaris* in protein databases. However protein families can be identified by homology to other plant species protein. Therefore, based on the molecular weight (MW) of fragmented ion son, isoelectric point (pI) and the recovery rate, the

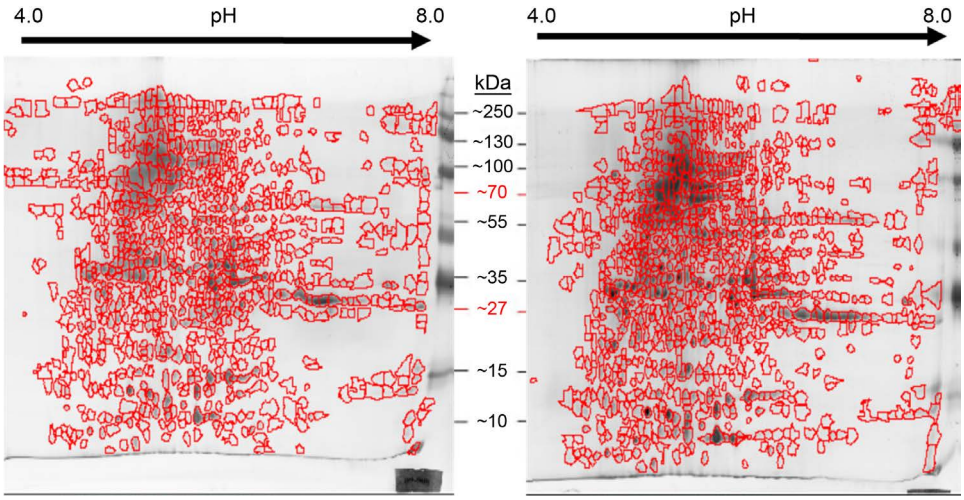

Figure 2. Two-dimension electrophoresis gel of *Phaseolus vulgaris* proteins extracted using Thiourea/urea methods and TCA/acetone precipitation methods after coloration.

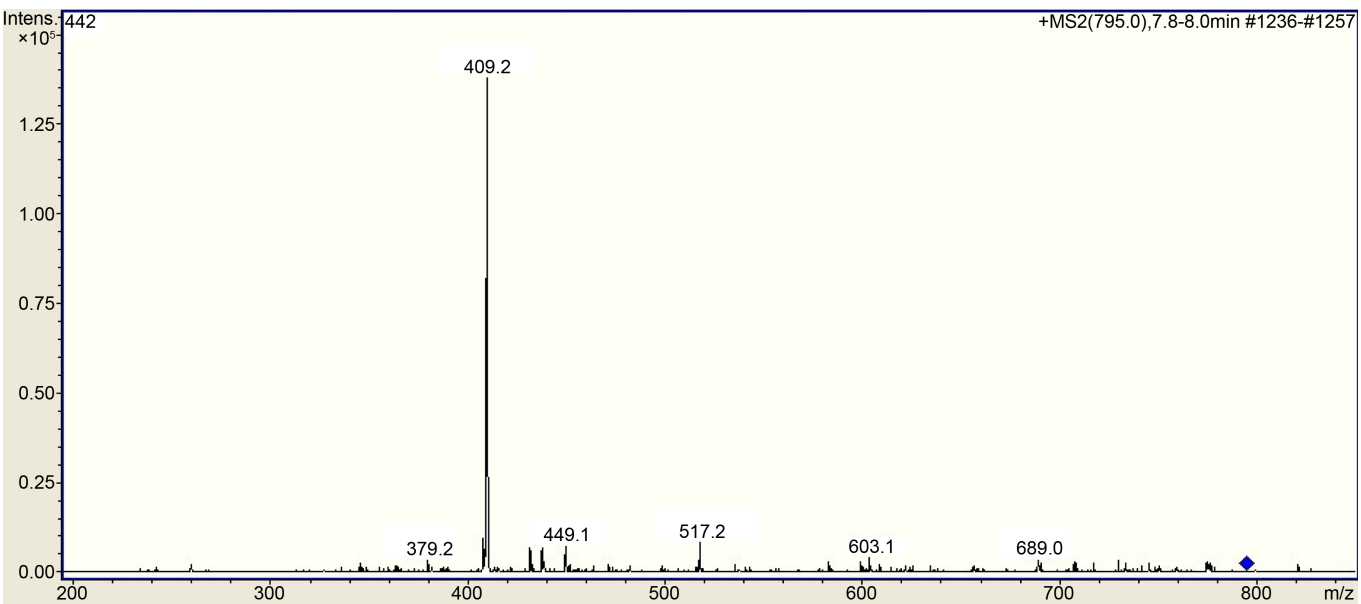

Figure 3. Peptide profile obtained by liquid chromatography-mass spectrometry in tandem or MS/MS of trypsin digestion of spot BS_1453_MS1 peptide 1.

1453 spot could correspond to the 11S globulin and the 1527 globulin.

4. Conclusion

These two extraction methods allowed satisfactory separation of proteins by 2D electrophoresis, even though these separation techniques do not allow seeing all the proteins. It is possible to make comparative electrophoresis between two varieties (or two conditions of seeds storage) of *Phaseolus vulgaris* for example to highlight proteins that differ (quantitatively or qualitatively). The mass spectrometry analysis is limited by the

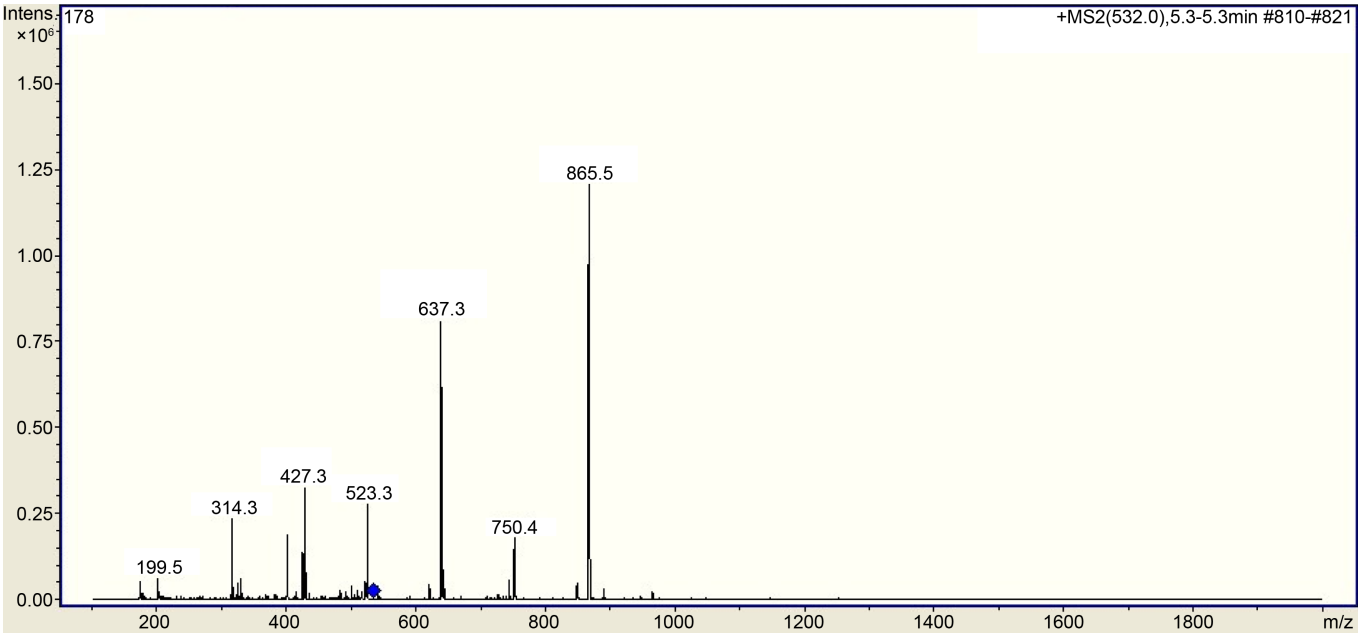

Figure 4. Peptide profile obtained by liquid chromatography-mass spectrometry in tandem or MS/MS of trypsin digestion of spot BS_1527_MS2 peptide 1.

presence of *Phaseolus vulgaris* proteins, but proteins functions could undoubtedly be identified by homology with proteins from other plant species.

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