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# Proteomic analysis of secreted proteins from *Arabidopsis thaliana* seedlings: improved recovery following removal of phenolic compounds

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**Abstract**

*Arabidopsis thaliana* seedlings grown in liquid culture were used to recover proteins secreted from the whole plant. The aim was to identify apoplastic proteins that may be lost during classical extraction procedures such as preparation of cell walls. The inclusion of polyvinyl-polypyrrolidone (PVPP) in the protocol of purification of secreted proteins allowed a more efficient identification of proteins after their separation by two-dimensional gel electrophoresis (2-DE) and mass spectrometry analyses. Improvement of identification was 4-fold. It is related to an increased number of detectable peaks on mass spectra increasing the percentage of sequence coverage, and the identification confidence. The role of PVPP was to trap phenolic compounds and to prevent their unspecific interactions with proteins. These experiments resulted in the identification of 44 secreted proteins, of which 70% were not identified in previous cell wall proteomic studies. This may be due to specific gene regulation in seedlings and/or to a better access to apoplastic proteins not bound to cell walls.


*Keywords:* *Arabidopsis thaliana*; Secreted proteins; Proteomics; Phenolic compounds; PVPP

## 1. Introduction

The ultimate goal of proteomic analysis of a cell compartment should be an exhaustive enumeration of resident proteins excluding proteins from other cell compartments. Such a goal is closely linked to the reliability of isolation and purification techniques for the cell compartment of interest. Many proteins may be lost during the purification procedure, especially in the case of cell wall preparations (Watson et al., 2004; Chivasa et al., 2002; Pitarch et al., 2002). These secreted proteins can be recovered from the culture medium when cell suspension cultures are used, but not in the case of plant tissues or organs (Pardo et al., 2000; Borderies et al., 2003). Until now, this problem could only be overcome in the case of *Arabidopsis thaliana* rosettes by adaptation of the vacuum-infiltration technique, allowing the identification of a great number of apoplastic proteins in a non-destructive manner (Boudart et al., 2005). An alternative is to grow seedlings in liquid culture medium and to analyze the proteins present in this medium (Bardy et al., 1998).

Plant tissues are rich in phenolic compounds. They accumulate into the vacuole, the largest cell compartment of plant cells. Phenolics are also secreted either towards the cell wall where they polymerize into lignins (Harborne, 1980), or into the rhizosphere by roots (Phillips, 1992). Procedures for protein extraction should not only allow solubilization of all the proteins of interest, whatever their physico-chemical properties, but also prevent protein modification including oxidation. Moreover, when 2-DE is used, the purification procedure

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should eliminate compounds known to interfere with the electrophoresis, i.e., salts, lipids, polysaccharides, nucleic acids and phenolic compounds (Rabilloud, 1996; Fichmann and Westermeier, 1999). It has been known for decades that proteins might bind phenolic compounds in aqueous media through different mechanisms such as hydrogen, ionic bonding, and hydrophobic interactions (Pierpoint, 2004). The resulting product is more hydrophobic and susceptible to protein aggregation and precipitation. Some structural features of proteins such as proline-rich regions predispose them to such complexing (Baxter et al., 1997). Phenolic compounds can also form irreversible covalent linkages with proteins as a consequence of their oxidation to quinones (Pierpoint, 2004). Even though that problem was constantly underlined for enzymology studies, it was not yet specifically addressed for proteomic analysis.

In this work we have used *A. thaliana* seedlings grown in liquid culture to recover the secreted proteins from the whole plant. Since culture media contain salts, organic compounds and secreted metabolites that may interfere with the separation and identification of proteins, we included water-insoluble polyvinyl-polypyrrolidone (PVPP) in the protein preparation protocol. Indeed, PVPP has been used for the removal of phenolic compounds including phenylpropanoid compounds and flavonoids from plant extracts, allowing the purification of proteins (Loomis, 1974; Pierpoint, 2004), RNAs (Salzman et al., 1999; Hu et al., 2002), and DNA (Young et al., 1993). We could show that the use of PVPP significantly improved the identification of proteins from culture medium by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) peptide mass fingerprinting, namely 4-fold. Forty-four secreted proteins could be identified among which 31 were not found in *A. thaliana* previous cell wall proteomic studies.

## 2. Results and discussion

### 2.1. Isolation and separation of secreted proteins from culture medium of A. thaliana etiolated seedlings

Two-week-old seedlings grown in liquid medium in the dark were filtered and the culture medium collected. The culture medium was either directly processed, or mixed with water-insoluble PVPP, before being treated as described in Experimental. Upon concentration, the sample non-treated with PVPP became brown suggesting that proteins and phenols were oxidized. Since our previous work on cell wall proteins (CWP) indicated that most of them are basic (Borderies et al., 2003), we divided each sample to separate the proteins on regular (pH 4–7) and basic (pH 6–11) 2-D gels. The images of the resulting 2-D gels did not show major differences between untreated (Fig. 1A and C) or PVPP-treated samples (Fig. 1B and D). In the same way, the amount of proteins in the two extracts were similar, i.e., 110 μg proteins per flask of culture. Besides, if the oxidized phenolic compounds modify proteins by combining to their reactive groups, they do not seem to cause protein aggregation and precipitation. *A. thaliana* seedlings are not as rich in phenolics as green leaves or fruits. The likelihood of precipitation and aggregation is increased in the presence of large polyphenols that can interact with more than one protein molecule (Pierpoint, 2004).

### 2.2. Efficient recovery and improvement of protein identification by PVPP

Phenolics, pigments and others compounds soluble in acetone are usually removed from the plant material of interest by a direct precipitation of proteins with TCA and acetone. However, the method leads to protein losses since not all the precipitated proteins can be resolubilized. When applied to our protein samples, only one fourth of the total proteins could be resolubilized. Many studies are presently devoted to improve that solubilization step in order to increase the number of proteins detectable by 2-DE (Jacobs et al., 2001; Giavalisco et al., 2003). In addition, when looking at a specific organelle proteome, sample preparation requires cell fractionation and purification in aqueous media, preventing direct protein precipitation from plant material. An alternative to the removal of phenolics from an aqueous medium is the addition of PVPP. Our results indicate that complexing phenolic compounds at a very early step of the procedure will be particularly useful for plant tissues rich in phenolic compounds and for plant cell compartments that accumulate them. In the case of the culture medium of *A. thaliana* etiolated seedlings, the adition of PVPP permits an efficient recovery of proteins for an improved identification by MALDI-TOF.

Overall, 112 protein spots were clearly resolved and reproducibly obtained after 2-DE. Trypsin-digested spots from both gels were submitted to MALDI-TOF mass spectrometry. Huge differences were found between the number of identified proteins through peptide mass fingerprinting in each case. For the PVPP-treated sample, 68–84% of the spots were identified compared to only 15–22% for the non-treated sample (Table 1). Comparison of MALDI-TOF spectra between non-treated (Fig. 2A) and treated samples (Fig. 2B) showed that the number of peptides above background is lower in the former case. In the treated sample most of the major peaks contribute to the identification of the protein. It was not possible to define clearly the physico-chemical properties of the peptides missing in non-treated samples, namely molecular mass or amino acid composition. Finally, the PVPP treatment enhanced the number of peptides matching a protein, rising significantly the