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# Impact of homogenization and protein extraction conditions on the obtained tobacco pollen proteomic patterns

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## Abstract

Mature pollen grain represents a highly desiccated structure with an extremely tough cell wall. Thanks to it, it resists common proteomic protocols. Instead, a robust homogenization has to be performed since proteins are needed to burst out of the cell to be included in the extracted proteome fraction. Here, a novel way of pollen homogenization employing *Roche MagNA Lyser Instrument* is presented, sparing time and laborious work. However, plant proteomics does not rely solely on perfect homogenization; also the choice of the extraction protocol is of key importance. The composition of the extraction buffer has a decisive influence on which proteome fraction will be extracted. Therefore the second part of our study is dedicated to the comparison of different extraction protocols with respect to subsequent proteomic analyses.

*Additional key words:* proteomics, *Roche MagNA Lyser Instrument.*

## Introduction

Unlike animals, plants consist of cells enclosed in resistant cell walls and often loaded with a variety of interfering compounds, *e.g*. phenolic compounds, alkaloids and other secondary metabolites. Moreover, pollen grains have an even harder cell wall and are fully desiccated to withstand many environmental stresses and to protect the genetic information as well as stored compounds needed for all post-pollination processes including massive pollen tube growth leading to the subsequent successful fertilization.

Pollen represents a very interesting model for the studies of cell polarity (Hepler *et al.* 2001) and tip growth (Geitmann and Steer 2006). During germination, pollen grain rehydrates and develops into a metabolically extremely active, rapidly growing pollen tube. To achieve such an explosive growth, pollen grain has to store large amounts of RNA and protein reserves (Tupý 1982, Honys and Čapková 2000, Honys *et al.* 2000). During pollen maturation, both transcription and translation play an important role in the global and specific gene expression patterns. On the contrary, the germination of many pollen species has been shown to be largely independent of transcription but vitally dependent on translation (Twell 1994). In tobacco, transcription was detected only during the first several hours of the pollen tube growth, which led to the conclusion that pollen tube growth is mainly dependent on translation (Čapková *et al.* 1988). A significant fraction of mRNAs is stored in the form of translationally-inactive EDTA/puromycin-resistant RNP complexes (EPPs; Honys *et al.* 2000, 2009).

Proteome does not exactly correspond to the transcriptome (De Groot *et al.* 2007). Also in *Arabidopsis thaliana* pollen, mRNAs of some abundant proteins are present at very low concentrations or even not present at all and *vice versa*, mRNA abundance can be higher than that of corresponding proteins (Holmes-Davis *et al.*

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*Abbreviations*: BSA - bovine serum albumine; CHAPS - 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; DTT - dithiothreitol; IEF - isoelectric focusing; PMSF - phenylmethanesulphonyl fluoride; SB 3-12 - sulfobetaine-12; SDS-PAGE - sodium-dodecyl-sulfate polyacrylamide gel electrophoresis; TCA - trichloroacetic acid.
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2005). The former fact can be caused by a long-term storage of proteins. The latter situation is caused either by mRNA storage associated with a translation block, or by the undetectability of particular proteins under experimental conditions. All above-mentioned facts underline the importance of proteomic studies so that the data more accurately reflecting the reality are obtained. Moreover, proteins are often posttranslationally modified and such modifications are naturally undetectable at the transcriptomic level.

In order to perform genome-wide proteomic studies of the male gametophyte, it is necessary to overcome all already mentioned difficulties caused by pollen grains characteristics. A really substantial homogenization is necessary to break the rigid cell wall. This could be achieved manually by grinding the grains in a mortar with a pestle (Honys *et al.* 2000) or in several alternative ways. For instance, Holmes-Davis *et al.* (2005) used a ground-glass tissue grinder (*Kontes*, Vineland, USA). Alternatively, pollen cell wall was broken down by five rounds of 30 s vortexing in a tube with glass beads. Between the rounds of vortexing, the samples were cooled down on ice for 30 s (Noir *et al.* 2005). Sheoran *et al.* (2006) homogenized dry pollen by grinding the sample in a microcentrifuge tube with the extraction buffer using a glass rod. The nature of homogenization influences decisively the qualitative and quantitative characteristics of the obtained protein spectra and not all methods are optimal. For instance, the use of *Mini-Beadbeater 3110BX* (*Biospec*, *Product IHC*, Bartesville, USA) did not lead to the same quality of protein spectra (Čapková, unpublished data) as manual homogenization. Therefore, in the first part of our study we introduce a novel use of the *MagNA Lyser Instrument* (*Roche Applied Science*, Penzberg, Germany) which, according to the manufacturer's web site, was originally designed for nucleic acid extractions.

Not only the homogenization but also the chosen extraction protocol plays a key role in the quality of the resulting proteomic spectra. For *Arabidopsis thaliana* pollen, three independent proteomic datasets based on 2D SDS-PAGE approach were published (Holmes-Davis *et al.* 2005; Noir *et al.* 2005, Sheoran *et al.* 2006). The overlap between these three datasets was as low as 7 % (18 proteins out of 267). However, such a small overlap could be caused also by different experimental material. The ecotype of *Arabidopsis thaliana* used could contribute to it, since Columbia (Holmes-Davis *et al.* 2005, Noir *et al.* 2005) has a different phenotype than Landsberg erecta (Sheoran *et al.* 2006) so the phenotypic differences could be reflected also in their pollen proteomes. However, no such variation was observed at the transcriptomic level (Honys *et al.* 2006, Twell *et al.* 2006). So, the variations in the identified proteome fractions are likely to be caused by the homogenization procedure, extraction protocol, pI range of IEF strips, composition of IEF buffer, spot selection and, most probably, by a low coverage of traditional proteomic methods (Grobei *et al.* 2009). The recent study of Grobei *et al.* (2009) has also introduced the high-throughput shotgun proteomics to the tiny world of pollen. The gel-independent approach has extended *Arabidopsis* pollen proteome by the factor of 13 in comparison with the above-mentioned traditionally-obtained datasets. Moreover, it has identified 537 novel pollen proteins identification of which was not previously achieved. Although this approach has revolutionized the capability of covering the whole pollen proteome, it is still not widely available and so the traditional 2D SDS-PAGE-based approaches are still likely to play their role.

Finally, the composition of the protein extraction buffer has a qualitative and quantitative impact on the proteomic pattern obtained. To test the influence of the used protocol on the resulting proteomic spectra, several different extraction protocols followed by 2D SDS-PAGE under the same basic conditions (plant species and cultivar, the way of homogenization, pI range of IEF strips and composition of IEF buffer) were compared in the second part of our study.

## Materials and methods

**Tobacco plants** *(Nicotiana tabacum* cv. Samsun) were grown from seeds in a greenhouse. Flower buds were collected in the morning before anthesis. Anthers were excised and allowed to dehisce in open Petri dishes overnight at room temperature. Mature pollen was sieved and stored at -20 °C.

**Homogenization design:** Pollen samples were homogenized by *Green Beads* in *MagNA Lyser Instrument* (*Roche Applied Science*, Penzberg, Germany). Three homogenization protocols were applied: *1*) 7000 rpm for 60 s; *2*) 7000 rpm for 30 s and *3*) 5000 rpm for 25 s. 1 $cm^3$ extraction buffer [50 mM Tris-HCl, pH 6.8, 10 % (m/v) sucrose and inhibitory proteases (*Complete Protease Inhibitor Cocktail Tablets*; *Roche*)] was added prior to homogenization (buffered homogenization) or after it (dry homogenization). Manual homogenization was performed with a pestle in a mortar pre-chilled at -20 °C and twice cooled down by liquid nitrogen serving as a control. Soluble and insoluble proteins were separated by centrifugation (16 000 *g*, 20 min, 4 °C). Insoluble proteins were extracted from the pellet using 1.6 $cm^3$ of extraction buffer supplemented with 2 % (m/v) sodiumdodecyl sulphate (SDS). To both fractions, 5-fold volume of ice-cold acetone was added and proteins were let to precipitate at -20 °C overnight.

After centrifugation (16 000 *g*, 20 min, 4 °C), the protein-containing pellet was vacuum-dried. Proteins were then resuspended in 1D-SDS-PAGE buffer [50 mM Tris-HCl, 10 % (v/v) glycerol, 2 % (m/v) SDS, 5 % (v/v) mercaptoethanol]. The concentration of proteins was estimated by amido black using bovine serum albumine (BSA) as a standard (Schaffner and Weissmann 1973) and proteins were separated according to Laemmli (1970) on stacking gel (T = 4, C = 2.6) and resolving gel (T = 12, C = 2.6). 25 µg proteins were loaded per slot. The run was performed on *Biometra Multigel Long* apparatus (*Whatman Biometra*, Göttingen, Germany) under constant voltage: 80 V/stacking gel and 180 V/resolving gel. Gels were conventionally stained by CBB R250.

**Protein extraction:** The phenol-based extraction applied *TRI-reagent* (*Sigma-Aldrich*, St. Louis, USA) according to the manufacturer's instructions. 10 mg mature tobacco pollen were frozen in liquid nitrogen and homogenized in a mortar. Parallelly, 10 mg mature tobacco pollen were homogenized in *MagNA Lyser Instrument* under both dry and buffered conditions at 5000 rpm for 25 s. In all cases, 1 $cm^3$ *TRI-reagent* was used for the extraction and 0.2 $cm^3$ chloroform was added. The sample was left for 15 min on ice and centrifuged (16 000 *g*, 20 min, 4 °C). The lower phase was supplemented with 0.3 $cm^3$ ethanol and centrifuged (2000 *g*, 5 min, 4 °C). Proteins in the supernatant were precipitated with 1.5-fold volume of isopropanol for 1 h at room temperature and centrifuged (16 000 *g*, 20 min, 15 °C). The pellet was washed three times with 1 $cm^3$ cold ethanol and centrifuged (16 000 *g*, 20 min, 15 °C). Finally, the pellet was vacuum-dried.

The trichloroacetic acid (TCA)-phenol-SDS extraction was carried out according to Wang *et al.* (2003) with several modifications: 50 mg mature tobacco pollen was homogenized in a mortar with a pestle. Obtained homogenate was transferred to a microtube with 1.5 $cm^3$ cold acetone, vortexed for 30 s at room temperature and centrifuged (10 000 *g*, 3 min, 4 °C). The pellet was subsequently washed with 1.5 $cm^3$ 10 % (m/v) TCA in acetone, or 1.5 $cm^3$ 10 % TCA in water, or twice with 1.5 $cm^3$ 80 % acetone. After each washing step, the sample was centrifuged (10 000 *g*, 3 min, 4 °C). The vacuum-dried pellet was resuspended in 0.8 $cm^3$ Tris-buffered phenol (pH 8.0) and 0.8 $cm^3$ SDS buffer (30 % sucrose, 2 % SDS, 0.1 M Tris-Cl, 5 % mercaptoethanol) and centrifuged again (10 000 *g*, 20 min, 4 °C). 1 $cm^3$ of 0.1 M ammonium acetate in methanol was added to the phenol phase and the proteins were precipitated for 1 h at -20 °C. The precipitate was centrifuged (10 000 *g*, 10 min, 4 °C) and subsequently washed twice with ice-cold 0.1 M ammonium acetate in methanol and three times with 80 % v/v acetone. After each washing, the samples were centrifuged under the same conditions as before.

The original two-phase extraction (Holmes-Davis *et al.* 2005) led to the isolation of two proteome fractions defined as salt soluble and salt insoluble. 35 mg mature tobacco pollen were homogenized in 1 $cm^3$ buffer A [70 mM KCl, 14 % (v/v) glycerol, 50 mM Tris-HCl (pH 7.1), 0.4 mM phenyl-methanesulphonyl fluoride (PMSF) and inhibitory proteases (*Complete Protease Inhibitor Cocktail Tablets*, *Roche*)]. The homogenate was centrifuged (150 000 *g*, 1 h, 5 °C) and salt-soluble (cytosolic) proteins in the supernatant (A-supernatant) were precipitated with methanol and chloroform according to Wessel and Flügge (1984). The pellet (A-pellet) was resuspended in buffer B consisted of 5 M urea, 2 M thiourea, 2 % (m/v) 3-[(3-cholamido-propyl)dimethylammonio]-2-hydroxy-1-propane-sulfonate (CHAPS), 2 % (m/v) SB 3-12, 1 % (m/v) DTT and 0.5 % (m/v) ampholytes. The homogenate was centrifuged (150 000 *g*, 1 h, 20 °C) and the supernatant containing salt-insoluble (membrane) proteins was precipitated in the same way as cytosolic proteins were.

The SDS extraction was carried out according to Holmes-Davis *et al.* (2005) as follows: 35 mg mature tobacco pollen was homogenized in 1 $cm^3$ SDS buffer [200 mM Tris-HCl pH 8.8, 4 % (m/v) SDS, 20 % (v/v) glycerol and 80 mM DTT]. The homogenate was centrifuged (150 000 *g*, 1 h, 20 °C) and the supernatant was precipitated in the same way as salt-soluble cytosolic proteins were.

**2D SDS-PAGE:** All protein samples from the above-mentioned extractions were resuspended in 1 $cm^3$ isoelectric focusing (IEF) buffer (9.8 M urea, 4 % CHAPS, 50 mM DTT, 0.2 % ampholytes) and protein concentration was determined using amido black and BSA as a standard (Schaffner and Weissmann 1973). 100 µg proteins were isoelectrically focused on 11-cm strip, pI range 3 - 10 (*Bio-Rad*, Hercules, USA). The IEF run was performed on *Protean* IEF cell (*Bio-Rad*) under following conditions: active rehydration 14 h; 500 V, 1 h; 1000 V, 4 h; 8000 V, until 35 000 V h was reached. Equilibration of the strips was carried out for 15 min in equilibration buffer 1 (6 M urea, 2 % SDS, 0.375 M Tris-HCl pH 8.8, 20 % glycerol, 130 mM DTT) and 15 min in equilibration buffer 2 (same composition as buffer 1, only DTT was replaced with 135 mM iodoacetamide). Before the SDS-PAGE run, the strips were submersed briefly into electrode buffer. SDS-PAGE run was performed on *Biometra Multigel Long* apparatus under the same conditions as 1D SDS-PAGE. The gels were silver-stained according to Blum *et al.* (1987).

## Results and discussion

**Automatization of pollen homogenization:** Isolating proteins from such an extremely tough tissue as the mature pollen surely is, two requirements have to be considered. The homogenization must be robust and substantial enough to disrupt the resistant pollen cell wall. Simultaneously, the homogenization conditions have to be mild enough not to physically break down the isolated proteins into lower molecular mass ($M_r$) protein fragments or even peptides. Traditionally, manual grinding of pollen suspension was carried out. Especially when many samples are processed at a time, this time-consuming protocol represents a constraint and so an automatization of the homogenization process is desirable. Finding a compromise concerning the above-mentioned almost antagonistic demands is a factor of supreme importance for a successful protein extraction.

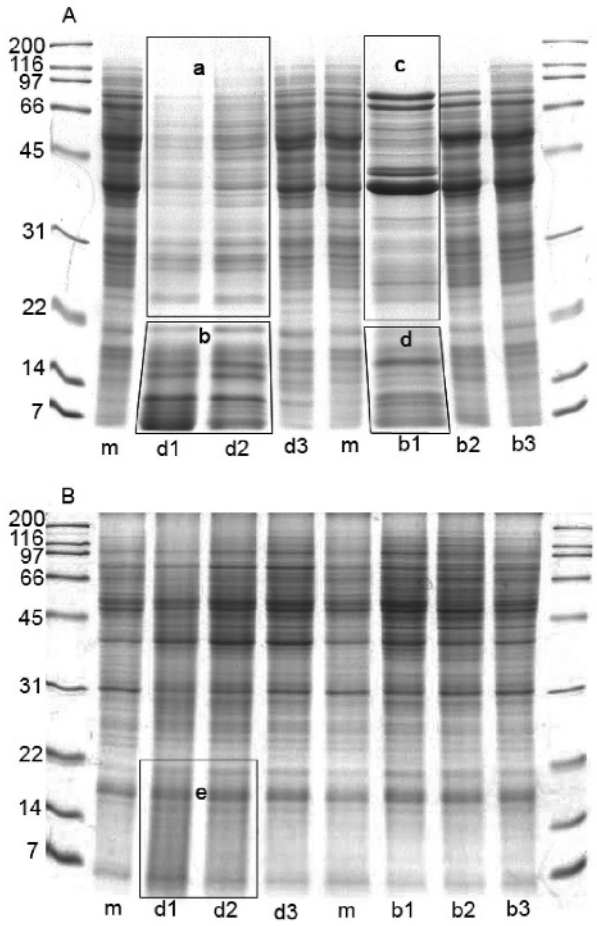


Fig. 1. Homogenization design. 1D SDS-PAGE proteomic spectra of cytosolic (*A*) and membrane (*B*) proteins, CBB R250 staining; m - manual homogenization, d - dry homogenization, b - buffered homogenization; automated homogenization conditions: d1/b1 - 7000 rpm, 60 s; d2/b2 - 7000 rpm, 30 s; d3/b3 - 5000 rpm, 25 s. Numbers *on the left* indicate the molecular mass in kDa. *Marked boxes*: a,c - spectra with underrepresented proteins with molecular mass higher than 25 kDa; b,d,e - spectra enriched with proteins and protein fragments ranging between 5 and 20 kDa.

In the first part of our study, three different pollen homogenization protocols were compared in order to identify the optimal homogenization conditions. The results were visualized by 1D SDS-PAGE spectra of proteins isolated. As concern cytosolic proteins, manual homogenization (representing the control sample) enabled the extraction of broad proteomic spectra including proteins of higher $M_r$. The application of more severe conditions on the *MagNA Lyser Instrument* changed the resulting protein spectra. Pollen grinding at 7000 rpm for 60 s led to the break-down proteins with $M_r$ over 25 kDa into fragments of $M_r$ 7 - 20 kDa (Fig. 1*A*). Very similar but less dramatic situation was observed when the homogenization time was shortened by half, showing that a shorter homogenization led to the reduction of cracking of proteins of high $M_r$. On the contrary, the mildest homogenization conditions (5000 rpm, 25 s) resulted in a protein pattern comparable to that obtained by the conventional manual homogenization. The tendency to protein breakage into fragments was apparently stronger under dry homogenization conditions compared to the buffered ones (Fig. 1*A*). We conclude that this is likely to be an effect of various proteases coming into contact with the released cellular proteins. The activity of these proteases is inhibited by protease inhibitor cocktail present in the extraction buffer, *i.e.* the buffered homogenization preserves the proteins immediately after the cell-wall break-down whilst the dry homogenization leaves the homogenate without any inhibitors until the buffer is added. Moreover, higher temperature achieved by faster movement of ceramic beads during dry homogenization favors the activity of proteases. The speed of moving beads itself provides an additional explanation as the motion of the beads is slower and milder in the buffer due to its density and viscosity.

Membrane proteins were less affected by the detrimental influence of fast-moving ceramic beads. Only in severe cases of dry homogenization at 7000 rpm for 60 s or 30 s the proportion of proteins in $M_r$ range of 7 - 20 kDa was higher (Fig. 1*B*). All other homogenization conditions including control manual grinding led to the same protein spectra. Membrane proteins were less affected because they were protected by the membrane persisting in the bilayer form even after the homogenization and so engulfing the membrane-binding domains of integral and/or associated proteins. The membrane lipid bilayer was preserved intact until the addition of SDS, the detergent playing key role in obtaining membrane proteins by disturbing the membrane bilayer. A supporting complementary explanation is that the membrane elasticity buffers the damaging effect of the hitting beads. On the contrary, soluble proteins came

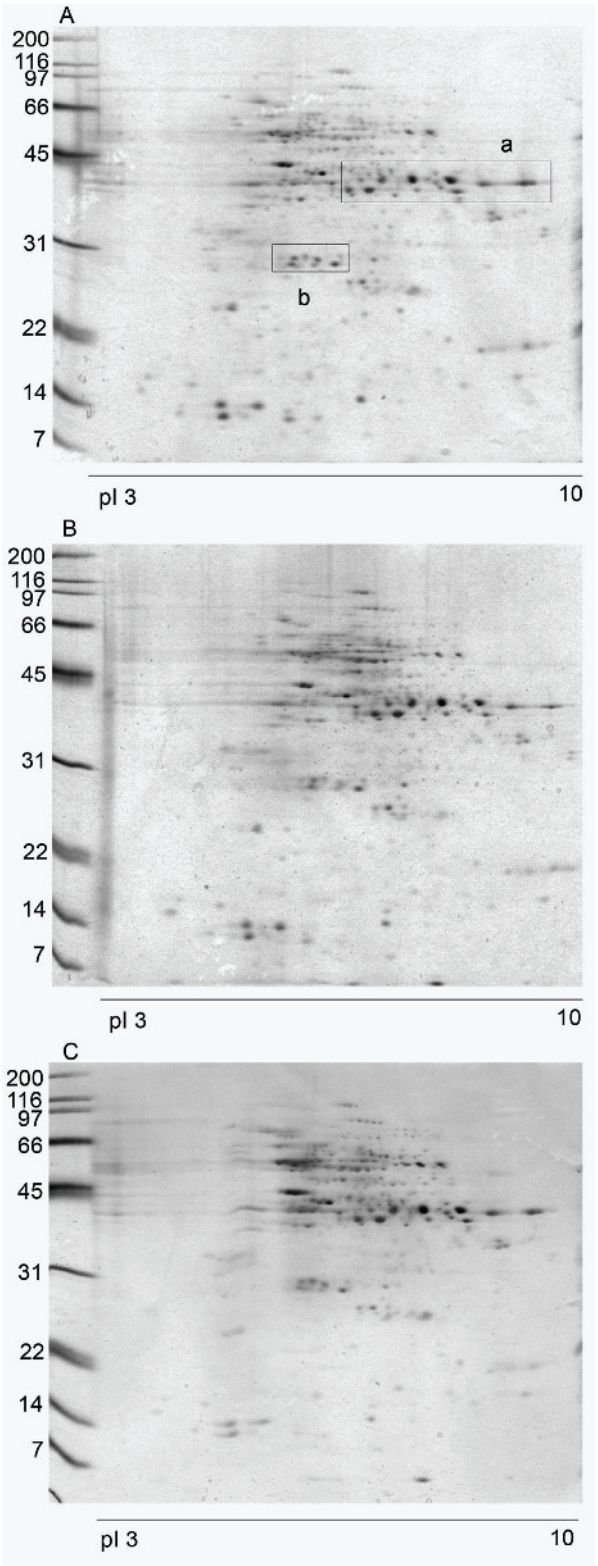


Fig. 2. Homogenization design. 2D SDS-PAGE protein pattern obtained by TRI-reagent protein extraction after manual (*A*), dry (*B*) and buffered (*C*) homogenization; pI 3 - 10, silver-staining. Numbers *on the left* indicate the molecular mass in kDa. *Marked boxes* a and b pinpoint representative areas for the comparison of the gels.

directly into contact with the proteases and were more affected by the beads movement.

Since it is necessary to preserve isolated proteins intact for the proteomic studies, it is necessary to choose the optimal homogenization procedure maintaining the identical protein pattern as the manual homogenization. The first experiment led to the identification of the optimal automated homogenization conditions for tobacco pollen: 5000 rpm for 25 s. After all, this stringency did not show on 1D gels any significant difference between dry and buffered alternative. Thus, we decided to compare both these alternative conditions followed by more complicated extraction protocol in order to see whether the application of more stringent dry or milder buffered homogenization was reflected in resulting protein spectra on 2D SDS-PAGE gels. Again, manual homogenization was used as a control and all samples were prepared in duplicate. Following all mentioned homogenization conditions, proteins were extracted by identical *TRI-reagent* protocol. Dry and buffered homogenization led to very similar proteomic spectra (Fig. 2) both compared to one another and to the ones from manually-homogenized samples.

Presented results clearly demonstrated that the *MagNA Lyser Instrument* in combination with *Green Beads* is capable of carrying out the homogenization at desirable efficiency. Moreover, it spares time and avoids laborious manual homogenization. All data demonstrated the key importance of the stringency and duration of the homogenization procedure. As a result, both dry and buffered homogenization protocols carried out at 5000 rpm for 25 s were considered optimal for tobacco pollen disruption preceding the subsequent protein extraction.

**Protein extraction:** In each of the three published 2D-based proteomic datasets of *Arabidopsis*, different protein extraction protocol was employed. Holmes-Davis *et al*. (2005) used the combination of two-phase protein extraction with one-step SDS extraction, Noir *et al*. (2005) applied Tris-Cl buffer supplemented with CHAPS and Sheoran *et al*. (2006) extracted the proteins with TCA. It is likely that, among other possible reasons, the chosen extraction protocol played an important role in the resulting proteomic spectra. Moreover, together with the low coverage of 2D-based methods, it contributed to the limited overlap of the identified proteome fractions.

We studied to what extent the application of different extraction protocols following an identical homogenization affects the resulting protein spectra. 2D SDS-PAGE patterns of proteins extracted using one two-phase protein extraction protocol and three one-phase protocols were compared to each other (Fig. 2*A* and 3). The one-phase protocols comprised phenol-based extraction (*TRI-reagent*), simple SDS extraction (Holmes-Davis *et al.* 2005) and complex TCA-phenol-SDS extraction (Wang *et al.* 2003).

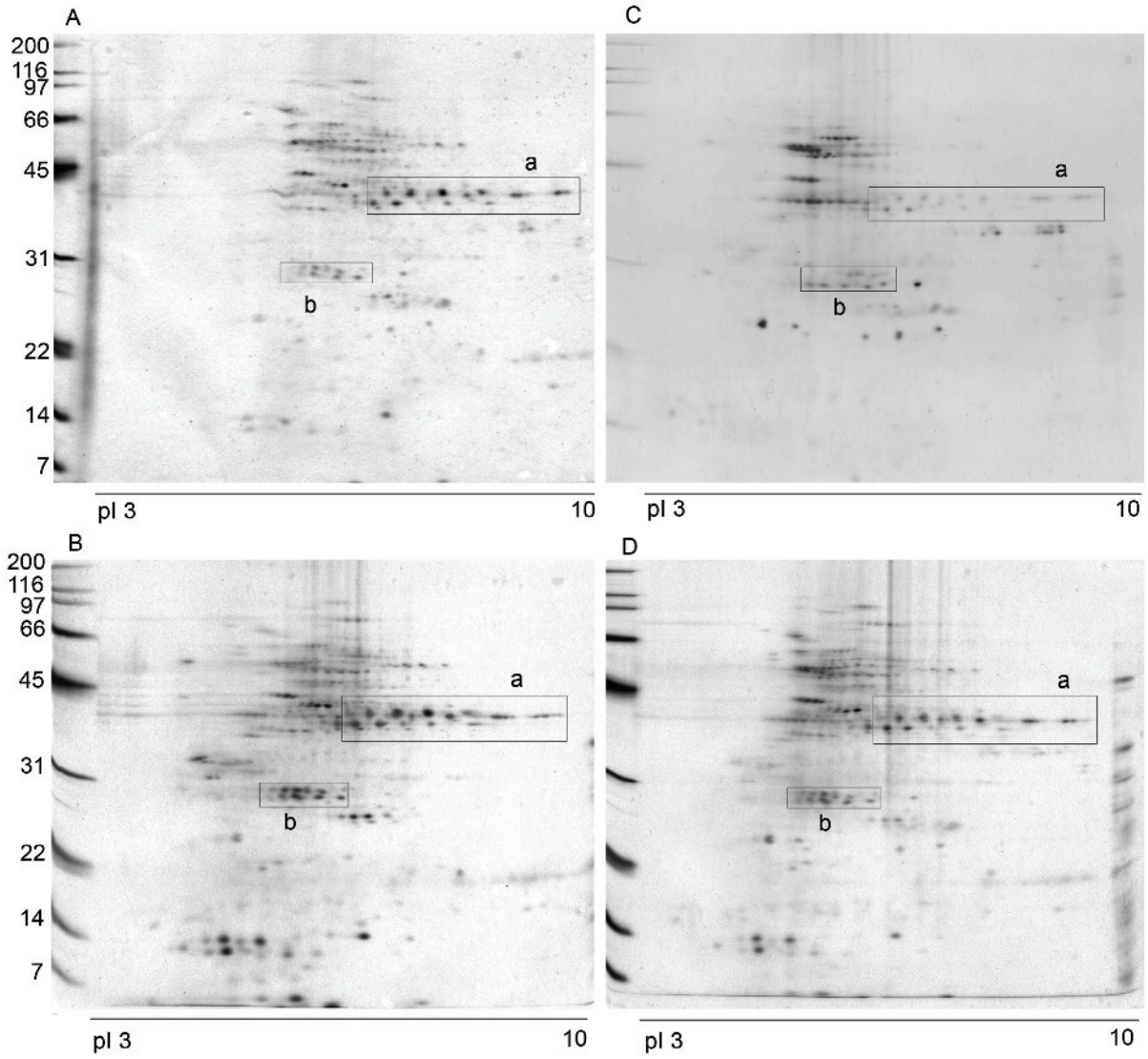


Fig. 3. 2D SDS-PAGE pattern of TCA-phenol-SDS-extracted proteins (*A*), cytosolic proteins from two-phase protocol (*B*), membrane proteins from two-phase protocol (*C*) and SDS-extracted proteins (*D*); pI 3 - 10, silver-staining. Numbers *on the left* indicate the molecular mass in kDa. *Marked boxes* a and b pinpoint representative areas for the comparison of the gels.

All the three one-phase protocols resulted in very similar protein spectra. It is noteworthy that relatively simple extraction protocols (*TRI-reagent* and one-phase SDS extraction) and quite complicated protocol using more than one extracting agent (TCA-phenol-SDS protocol) led to comparable protein spectra (Fig. 2*A*, 3*A*,*D*). We can assume that SDS or phenol by itself and both these chemicals supplemented with TCA are substitutable for each other. Although the TCA-phenol-SDS protocol is very useful for protein extraction from tough olive leaves (Wang *et al.* 2003), for tobacco pollen it was found suboptimal. Its extremely high time requirements are not compensated by any significant improvement of either protein quantity or the quality of obtained proteomic spectra. SDS extraction procedure requires ultracentrifugation. It is impossible to perform automated homogenization before this protocol since the ultracentrifugation of the sample containing beads is hazardous due to the very high speed (150 000 *g*). It has to be mentioned that the one-phase SDS extraction is not comparable with the extraction of membrane proteins in the respective homogenization design. One-phase SDS extraction released a complete set of non-covalently bound proteins while SDS-containing buffer in the homogenization design extracted membrane proteins from the insoluble pellet after the first round of extraction. From these three protocols, *TRI-reagent* seems to be the most effective because of the possibility of automated homogenization and minimal technical and time requirements.

Until now, only single-phase protocols were discussed. However, they were compared to an alternative two-phase protocol (Fig. 3*B*,*C*). Interestingly, similarity between the protein spectra after one-phase SDS extraction and after the extraction of the cytosolic fraction were observed (Fig. 3*B*,*D*). It seems to be quite noteworthy, since the buffer for cytosolic protein extraction is free of detergents and/or organic solvents and thus is not to be expected to extract particular proteome fractions (*e.g.* membrane proteins). The cytosolic fraction spectra were also comparable to those obtained by phenol-based extraction and complex TCA-phenol-SDS extraction. On the other hand, the membrane fraction spectrum showed the most distinct pattern from all other protocols (Fig. 3*C*). This was probably caused by the depletion of cytosolic proteins during the first step of extraction. Taken together, the two-phase protocol does not seem to add any significant value in the case of the tobacco pollen treatment because both phases are very similar to each other. Thus, acquiring two so similar phases is worthless for obtaining information about protein localization since many proteins are included in both phases.

In agreement with our results, tomato pollen proteomic spectra also showed similarities when several different one-phase protocols were compared (Sheoran *et al.* 2009). On the other hand, *Arabidopsis* pollen, unlike tobacco, showed significant differences between the SDS, cytosolic and membrane fractions (Holmes-Davis *et al.* 2005). The differences among the phases observed in *Arabidopsis* on the one hand, and the relative similarities observed in tobacco pollen on the other hand could be caused by different mature pollen morphology in the respective species. The more advanced *Arabidopsis* three-cellular pollen could show more precise protein localization than the bicellular tobacco pollen. Accordingly, many tobacco pollen proteins may be present in more than just one fraction.

Taken together, the use of several chemicals (namely SDS, phenol and both of these supplemented by TCA) led to comparable results, so they can substitute each other in the extraction buffer. The two-phase protocol resulted in more similar protein fractions than in *Arabidopsis* pollen. SDS-phenol-TCA extraction resulted in the identical proteomic spectra as SDS and phenol itself but the protocol is more complicated. After all, *TRI-reagent* protocol was found to be the best extraction procedure since it has the lowest demands on laboratory equipment, is quite simple, leads to the isolation of the same qualitative and quantitative proteomic spectra as other more complicated protocols and can follow the automated homogenization using the *Roche MagNA Lyser Instrument*.

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