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# PROTEIN EXTRACTION OF MAIZE (*ZEA MAYS*) FOR PROTEOMIC 2 – DE ANALYSIS

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**ABSTRACT**

A strength of two-dimensional polyacrylamide gel electrophoresis (2D PAGE) is its ability to resolve and investigate the abundance of several thousand proteins in a single sample. Two different extraction procedures for two-dimensional electrophoresis of plant proteins are compared in this work. Phenol-based extraction methods have been mainly used to extract proteins from different organs or tissues on many species. We wanted to determine which of these protocols was optimal for starch plants in order to achieve both efficient protein extraction and high spot resolution on 2-D gels. The phenol-based protocol was superior to the sodium phosphatase methods, showing larger protein yields and greater spot resolution on 2-D gels.

**Keywords:** 2 – DE, maize, sweet corn, total soluble protein

## INTRODUCTION

The use of proteomics approaches is a powerful tool in food science in terms of process optimization and monitoring, quality, traceability, safety, and nutritional assessment (Pedreschi et al., 2010). To separate proteomes, scientists have used electrophoretic and chromatographic technologies, separately and in combination. 2D – PAGE is one of the most efficient methods to study complex patterns of gene expression of the level of proteins (Gygi et al., 2000). The introduction of 2D – PAGE in 1975 by O´Farrell for separating cellular proteins under denaturing conditions enabled the resolution of hundreds of proteins. The principle applied was very simple: proteins were resolved on a gel using isoelectric focusing (IEF), which separates proteins in the first dimension according to their isoelectric point, followed by electrophoresis in a second dimension in the presence of sodium dodecyl sulphate (SDS), which separates proteins according to their molecular mass. O´Farrell´s method is truly the basis of modern 2D – PAGE, which was quickly adapted and widely accepted by other researchers. The objective of separating proteins using 2D – PAGE is twofold: identifying new proteins and measuring their relative abundance between comparative samples. One advantage of 2D – PAGE as a separation technique is not only resolves large numbers of proteins, but staining these proteins enables the relative abundances of the proteins to be quantified (Issaq and Venstra, 2008). Sample preparation is one of the most crucial steps in obtaining high – quality resolution of proteins in proteomic analysis, yet it can be problematic (Görg et al., 2000). Proteins isolated from plant tissues are often difficult to resolve by 2D – PAGE due to the abundance of secondary metabolites. In particular, recalcitrant plant tissues such as aged evergreen leaves often contain high levels of materials that strongly interfere with 2D – PAGE, resulting in horizontal and vertical streaking, smearing and reduced numbers of distinctly resolved protein spots (Wang et al., 2003). Many sample preparation and protein separation methods to obtain well – resolved 2D maps have been reported (Hurkman and Tanaka, 1986; Mayer et al., 1988; Wang et al., 2004). These methods usually involve TCA/acetone wash or precipitation steps. High quality protein preparation from maize (*Zea mays* subsp. *mays* and *Zea mays* var. *saccharata*) is required for proteomics. The optimalisation was carried out as a suitable tool for breeding process in maize.

## MATERIAL AND METHODS

### Sample

Seeds were used for extraction of proteins from four inbreed lines of maize (*Zea mays* subsp. *mays*) and four inbreed lines of sweet corn (*Zea mays* var. *saccharata*), which were obtained from Zelseed of Slovak Republic.

### Extraction protocols

The first protocol used for extraction of the proteins was using phenol followed methanolic ammonium acetate precipitation (Hurkman and Tanaka, 1976 – modified). Plant tissue (100 mg) was homogenized well in the extraction buffer (0.1 M Tris – HCl pH 8.8, 10 mM EDTA, 0.4 % 2 – mercaptoethanol, 0.9 M sucrose) and the same volume of 0.4 M phenol buffer pH 8.8 was added. This mixture was shaken vigorously for 30 min at 4ºC and then centrifuged at 5000g for 10 min at 4ºC. The upper phenol phase containing the proteins was collected very carefully. Ammonium acetate (0.1 M) was added five times the volume of the phenol phase. Mixed well and kept for precipitation overnight at – 20ºC. Next day, the mixture was centrifuged at 5000 g for 20 min at 4ºC. The supernatant was discarded and precipitates were washed in 0.8 M acetone twice and once in 0.7 M ethanol.
The second protocol involved the extraction of proteins using sodium phosphate. Homogenization of 100 mg of tissue was done in 25 mM sodium phosphate (pH 7.5) and mixed vigorously for 60 min at 4ºC. After that was centrifuged at 10000 g 10 min at 4ºC. As described for phenol extraction, the upper sodium phosphate phase containing the proteins was collected carefully and ammonium acetate (0.1 M) was added five times the volume of the phase. Mixed and kept for precipitation overnight at – 20ºC. Next day, the mixture was centrifuged 14000 g 20 min at 4ºC. The supernatant was discarded and precipitates were washed in 0.8 M acetone twice and once in 0.7 M ethanol.

### Quantification

The proteins were quantified using Bradford reagent (Bradford, 1976), samples were analysed absorbance at 590 nm in triplicates.

### Two – dimensional gel electrophoresis

The samples dissolved in lysis buffer were taken such that their concentration reached to 0.1 – 2.5 mg.mL$^{-1}$ for 2 – DE. This concentration of the sample was dissolved in rehydration buffer (8 M urea, 2 % CHAPS, 5 mM DTT, 0,2 % 3/10

ampholyte, 0,001 % Bromophenol blue). This buffer was stored in small aliquots as per requirement at - 20ºC. The last two ingredients (DTT and ampholyte) were added fresh to the rehydration buffer just before use. A total of 315 µL of rehydration buffer containing the sample was evenly distributed in the rehydration strip holder. The ReadyStrip™ IPG Strip 17 cm (pH 3 – 10, Bio – Rad) was placed on it and this assembly was allowed to rehydrate passively overnight. Current of 50 mA strip as applied. The focusing conditions were: step 1 – 500V, step 2 – 1000V, step 3 – 4000V, step 4 – 8000V. The focused strips were first reduced in equilibration buffer (6 M urea, 50 mM Tris – HCl pH 8.8, 30 % glycerol and 2 % SDS) containing 50 mg DTT (added just prior to use) for 15 min on a gel rocker at room temperature. The reduced strips were then alkylated by adding fresh 1 g Iodoacetamide (IAA) at similar conditions. The reduced and alkylated strips were washed with 1x SDS buffer. These strips were then loaded onto 10 % SDS – PAGE without any stacking gel. This assembly was sealed using 1 % agarose sealing buffer. The gels were run, stained and destained just as for 1 – D electrophoresis. The gels were scanned using GS – 800™ Calibrated Imaging Densitometer (Bio – Rad).

Statistical analysis was performed using SPSS software 22 (SPSS, Chicago, Illinois, USA) and the statistical significance levels were set at 95 % ($p < 0.05$) and 99 % ($p < 0.01$).

## RESULTS AND DISCUSSION

Sample preparation is one of the most crucial, yet problematic, steps for high – quality resolution of proteins in 2 – DE. Most problems can be traced to coextraction of nonprotein cellular components that can affect protein migrations (Görg et al., 2000). Plant tissues are rich in compounds that interfere with 2 – DE. These interfering compounds, e.g. polyphenols, terpenes, and organic acids, mainly accumulate in vacuole in various soluble forms, and are more abundant in green tissues than in young seedlings or etiolated material (Granier, 1988). Usually, two main strategies exist for removing these contaminants: removal before protein extraction or removal after protein extraction. Conventional removal of nonprotein contaminants involves the use of organic solvents (e.g. acetone, 10 % TCA in acetone) to wash contaminants out of tissue powder (Damerval et al., 1986).

We extract proteins from dry power of maize seedlings with a mixture of phenol and mixture of sodium phosphate. Phenol dissolves proteins (including membrane proteins) and lipids leaving water – soluble substances in aqueous phase, thus proteins in phenol phase are purified and concentrated together with cold methanolic ammonium acetate precipitation. Another advantage of phenol extraction is that it minimizes protein degradation often encountered during sample preparation, due to endogenous proteolytic activity (Schuster and Davies, 1983). On the other hand, sodium phosphate dissolves only aqueous proteins, so we could extract only a special part of total protein and visual these differences. Because of that, higher protein yields were achieved by phenol extraction protocol (Table 1).

**Table 1** The protein content of the sample determined according to Bradford

| Sample no. | Phenol extraction [mg.mL$^{-1}$][a] | Sodium phosphate extraction [mg.mL$^{-1}$][b] |
|---|---|---|
| **Z43** | 1.8 ± 0.09 | 0.81 ± 0.09 |
| **Z44** | 1.7 ± 0.05 | 1.0 ± 0.04 |
| **Z77** | 1.4 ± 0.1 | 0.87 ± 0.07 |
| **Z80** | 1.86 ± 0.04 | 0.76 ± 0.15 |
| **Z1929** | 1.53 ± 0.1 | 0.72 ± 0.06 |
| **Z1930** | 2.01 ± 0.24 | 0.98 ± 0.3 |
| **Z1947** | 1.96 ± 0.32 | 1.26 ± 0.14 |
| **Z1948** | 1.79 ± 0.33 | 1.25 ±0.05 |
| **x ± σ[c]** | 1.76 ± 0.16 | 0.96 ± 0.12 |
| **v [%][d]** | 9.03 | 11.7 |

[a]protein content obtained by phenol extraction, [b] protein content obtained by sodium phosphate extraction, [c]arithmeticmean and standard deviation, [d]coefficient of variation

ANOVA of protein contents confirmed statistically significant differences between protein separation methods (phenol extraction, sodium phosphate extraction) and between maize and sweet corn (Table 2). In using these two extraction protocols we obtain average more proteins from samples of sweet corn inbreed lines (1.44 mg.mL$^{-1}$) than from grains of maize inbreed lines (1.28 mg.mL$^{-1}$). The highest protein yield was achieved from the sample of inbreed line Z1947 (sweet corn) (Figure 1).

**Table 2** ANOVA for protein contents obtained by phenol and sodium phosphate extractions from grains of maize and sweet corn

| Parameter | Source of variation | DF | MS | F-value | P-value |
|---|---|---|---|---|---|
| Genotype | 0.309 | 1 | 0.309 | 5.053 | 0.031* |
| Extraction | 7.618 | 1 | 7.618 | 124.485 | 0.000** |
| Repetition | 0.017 | 2 | 0.009 | 0.141 | 0.869 |
| Genotype x Extraction | 0.013 | 1 | 0.013 | 0.210 | 0.650 |
| Residuum | 2.203 | 36 | 0.061 | | |

DF – degree of freedom; MS – mean square; Significant on level * P < 0.05 or ** P < 0.01

Based visualizations of proteins for 2 – DE extracted samples is show in Fig. 2 and Fig. 3 we evaluated phenolic extraction as the most efficient extraction of proteins from maize/sweet corn grain. Carpentier et al.,2005 have found that by means of phenol extraction, it is possible to obtain a high number of different proteins in a sample. These findings suggest that the use of phenol extraction for separation of proteins by 2-DE is suitable for the separation of proteins from the fruit, as well as dry grains, which are described above in our results.

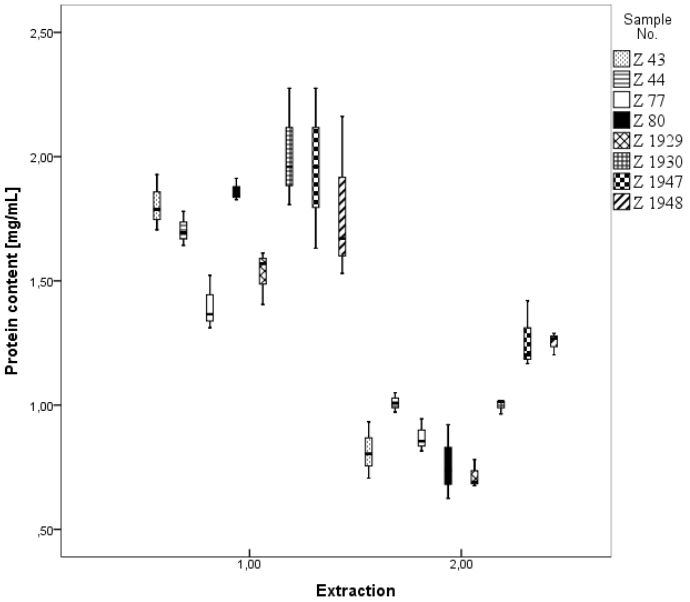


**Figure 1** Boxplots of the protein contents from grains obtained by phenol (1) and sodium phosphate (2) extractions

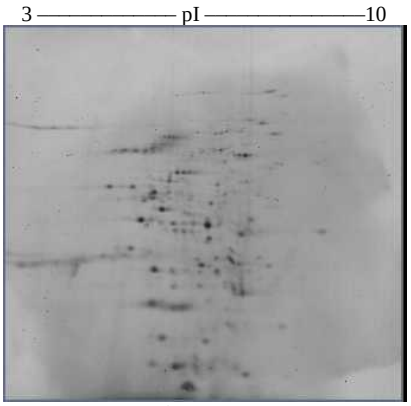


**Figure 2** Visualization of total protein in maize (smp. Z43)

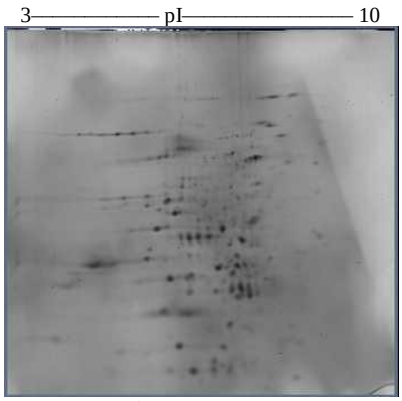


**Figure 3** Visualization of total protein in sweetcorn (smp.Z1930)

## CONCLUSION

For qualitative identification of proteins in the seeds of maize/sweet corn is the most important preparation and the amount of sample alone. We found out that the most appropriate protocol phenol extraction of proteins for the grains of maize/sweet corn in terms of quantity as well as quality visualization of total proteins for 2-DE analysis is according to Hurkman and Tanaka.

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