METHODOLOGICAL APPROACH TO EVALUATION OF BIOCHEMICAL MARKERS IN SPELT WHEAT (*TRITICUM SPELTA* L.)*

V. Dvořáček, V. Čurn, J. Moudrý

University of South Bohemia, Faculty of Agriculture, České Budějovice, Czech Republic

Esterase, aspartate aminotransferase, glutamate dehydrogenase, malate dehydrogenase, shikimate dehydrogenase isozymes, proteins extracted in 2% glutathione buffer (separated from leaves and seed in non-denaturing PAGE system) and seed proteins separated in SDS-PAGE were used as biochemical markers for evaluation of polymorphism level in three spelt wheat varieties (Hercule, Rouquin, Altgold), three breeders' spelt lines (H92.27, H92.28, M92.20 originated from hybridisation between spelt and common wheat) and reference common wheat variety (Brea). Only evaluation of three marker systems showed differences among common wheat variety 'Brea' and spelt varieties and spelt breeders' lines.

spelt wheat; biochemical markers; seed proteins; isozymes; electrophoresis

INTRODUCTION

In the development the spelt wheat (*Triticum spelta* L.) represents an older form of lemma hexaploid wheat forms with the genetic structure AA BB DD. Winzeler and Rügger (1990) reported that spelt wheat is an original independent form of cultural wheat varieties, but not hexaploid hulled form of *Triticum aestivum*.

The traditional distribution of spelt wheat is in south-western Germany and Switzerland. In recent years the interest in cultivation of spelt wheat has been growing even in the Czech Republic. It can be explained by the requirement of market for greater variegation of foods in rational nutrition together with the possibility to use wheat in the systems of ecological agriculture or in the system of "low input" management, respectively, for which spelt wheat is a

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suitable crop (Vlasák, 1995). Compared with common wheat, spelt wheat is less demanding for soil and weather conditions, it has better health conditions and is more valuable for its nutritional structure, particularly regarding essential amino acids (Grella, 1996; Ranhotra et al., 1996). On the contrary, lower yield, susceptibility to lodging, greater costs connected with grain hulling and worse technological quality of grain (Vlasák, 1995) in majority of traditional varieties are some disadvantage.

These disadvantages are removed mainly by purposeful hybridisation with common wheat (mostly of English provenance). Genetic determination between original common wheat and these spelt wheat hybrids is much difficult due to still mostly high percentage of common wheat genome in new hybrids. For these reasons, biochemical or molecular markers (Harsch et al., 1997), respectively, started to be used in identification of spelt wheat varieties.

Selection of suitable marker system has to be based on the following requirements:

1. Sufficient frequency of genetic variants.
2. Expression independent on environmental conditions.
3. Suitable electrophoretic and detection instruments (Nielsen, 1985).

Proteins may well fulfil the criteria for genetic markers, as a high degree of genetically fixed polymorphism, condominant heritability, differentiation of alleles in individuals and some extent of independence on external conditions mark them. Storage proteins or isozymes (molecular forms of enzymes) may represent such systems. Basically, all proteins, which demonstrate genetic polymorphism, may be used as differentiation markers (Sýkorová, Hadačová, 1992).

Four distinctly soluble protein fractions can be found in a wheat grain. These are as follows: albumins – soluble in water and weak salt solutions, globulins – insoluble in water, but soluble in salt solutions, prolamin fraction is then formed by gliadins – soluble in 70 to 90% of ethanol, and glutenins – soluble in weak acids and bases. The main differential factor of baking quality as well as a suitable genetic marker for determination of genotypes is a variability in the share of main storage protein in grain – in fraction of prolamins, which is in spelt wheat grain approximately 80% of the total protein (Peyne, 1987).

Methods based on detection of storage proteins (prolamins) separated in polyacryl amide gel in acid medium – Acide PAGE (Radic-Miehle et al., 1998; Harsch et al., 1997) can be mentioned of some examples of possible identification of spelt wheat varieties and their hybrids. They are followed by methods of detection of total proteins under the presence of amino active detergent sodium dodecyl sulphate – SDS (Radic-Miehle et al., 1997, 1998). Šašek and Černý (1997) were dealing with evaluation of spelt wheat genome among the Czech authors.

Westermeier (1990) reports that methods based on presence of SDS detergent may be more suitable compared with other methods. The most of all wheat protein is solved in the presence of SDS. SDS is bound to protein in a constant ratio given by its size; moreover, it eliminates hydrogen bridges and other covalent forces, so linearization of protein molecule appears. Protein molecules modified in such a way are of similar both shape and size of charge per area unit (Králová, Rauch, 1995).

The aim of the contribution is a methodological evaluation of usability of selected biochemical markers for possible identification of spelt wheat varieties.

MATERIAL AND METHOD

Six spelt wheat varieties were used to verify methodology of analysis of biochemical markers: two Belgian varieties – Hercule and Rouquin, Swiss variety Altgold and three breeders'spelt lines originated from Switzerland – H92.28, H92.27 and M92.20. The Czech common wheat variety Brea was used as a reference variety. Different marker systems were evaluated in discontinuous vertical electrophoresis Hoefer SE 600 on polyacryl amide gel (PAGE).

The systems of isozymes (aspartate aminotransferase – AAT, esterase – EST, glutamate dehydrogenase – GDH, malate dehydrogenase – MDH, shikimate dehydrogenase – SDH), native protein (extraction in non-denaturing conditions and separation in PAGE medium) and total protein (extraction and separation in denaturation medium SDS-PAGE) were tested among biochemical markers.

Dry fat-extracted or one-day germinated grain or the first right leaf was used for analyses.

Extraction: complexes of enzymes and native protein: 2% glutathione buffer (2% glutathione-reduced form, titrated 2M Trizma up to pH = 7.6); SDS – total protein: 0.0625 M Tris-HCl, pH = 6.8, 5% BME, 2% SDS (see commentary in the part Results).

Applying buffers: native protein and isozymes – 60% glycerol, 10 mg BPB/100 ml; SDS – total protein – 50% glycerol, 5% BME, 2% SDS in 0.625 M TRIS-HCl, pH = 8.0, 10 mg BPB/100 ml of buffer.

Electrophoretic separation of isozymes and native protein

Electrophoresis proceeded in non-denaturing discontinuous PAGE system. 4% focusing gel of pH = 6.8, separation 7.5% gel and electrode buffer Tris-glycine of pH = 8.3 (EP-2), were used to separate isozyme markers.

Samples were applied into the cells in an amount 25–30 µl under the surface of electrode buffer. Electrophoresis proceeded under 25 mA/gel and 110–200 V at 6–8 °C for four hours. After finishing of electrophoresis gels were rinsed with distilled water, equilibrated 15 minutes in buffer used for detection of enzymatic activity (at 4.6 °C) and coloured.

SDS-electrophoresis of proteins

Division of proteins proceeded in discontinuous denaturing PAGE system – 4% focusing gel of pH = 6.8 + SDS and separation 10% gel of pH = 8.8 + SDS, electrode Tris-glycine-SDS buffer of pH = 8.3.

Samples were deposited in cells in an amount of approximately 25–30 µl under the surface of electrode buffer. Electrophoresis proceeded at 40 mA/gel and 150–200 V at 6–8 °C for about four hours. After finishing of electrophoresis gels were rinsed with distilled water, equilibrated 15 minutes in buffer used for detection of enzymatic activity (at 4.6 °C) and coloured by Coomassie Blue.

Detection of enzymatic activity on gel

Aspartate aminotransferase (AAT): 200 mg of aspartic acid, 100 mg of α-ketoglutar acid was solved in 100 ml of 0.1 M Tris-HCl of pH = 8.3; after dissolution 150 mg of Fast Blue BB salt is added together with 10 mg of pyridoxal-5-phosphate; coloration for 30–60 minutes at 28 °C in the dark.

Esterases (EST): 40 mg α- and β-acetate naphthyl (as 1% solution in 50% acetone), 100 mg Fast Blue BB salt, 100 ml 0.1M Tris-HCl buffer of pH = 7.2, coloration 2 times for 15 minutes at 37 °C in the dark.

Glutamate dehydrogenase (GDH): 30 mg NAD, 1 ml 1 M $CaCl_2$, 800 mg L-glutamate, Na-salt, 200 mg MTT, 4 mg PMS, 100 ml 50 mM Tris-HCl buffer, pH% 8.0; coloration up to 2 hours at 37 °C in the dark.

Malate dehydrogenase (MDH): 400 mg DL-malic acid (to modify pH to 7.5 using NaOH), 30 mg NAD, MTT 20 mg, 4 mg PMS, 100 ml 50 mM Tris-HCl buffer, pH = 8.5; coloration 1–2 hours at 37 °C in the dark.

Shikimate dehydrogenase (SDH): 50 mg shikimic acid, 5 mg NADP, 10 mg MTT, 2 mg PMS, 100 ml 50 mM Tris-HCl buffer, pH = 8.5; coloration at 37 °C in the dark.

Detection of proteins using Coomassie Blue

Using coloration Coomassie Blue (i.e. Coomassie Blue R-250, Brilliant Blue R, Coomassie Brilliant Blue R-250, *C.I. 42660*) gels are coloured during the night in the mixture methanol chilling acetic acid – water – in ratio 5 : 1 : 4 with 0.1% Coomassie Blue R-250 (colorant was solved in methanol, acetic acid was added and water, different affinity to protein is possible, when other method of preparation of solution was used). Discolouration proceeds in the mixture ethanol – acetic acid – water in ratio 2.5 : 1 : 6.5. After discolouration gels were fixed and dehydrated in the mixture 45% ethanol + 3% glycerol.

RESULTS AND DISCUSSION

Optimised methodologies of procedures for evaluation of the above-mentioned marker systems were elaborated.

Analysis of native protein

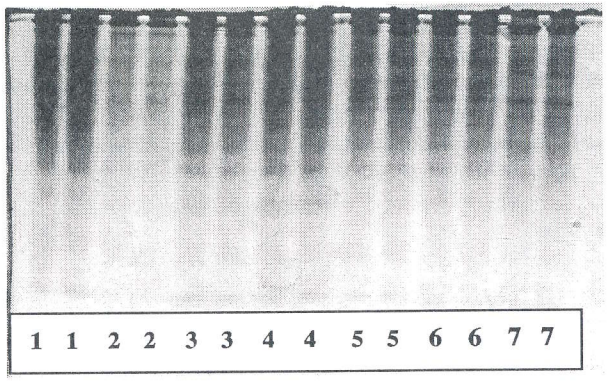
One-day germinated grain or 3 cm long segment of the first leaf was used for evaluation of the total native protein.

Analysis of native protein from seeds

The most suitable seemed to be 2% glutathione buffer for extraction of protein from grain. Extraction proceeded on ice at 4 °C. Amount 160–180 µl glutathione buffer per one ground grain was proved to be the best. Values above or below this limit, respectively, brought much worse results. After four-minute centrifugation at 10 000 g all supernatant (100–200 µl) was transferred into a new centrifugation test tube with 25 µl of applying buffer.

Samples prepared in this way can be stored in the freezer at –20 °C. Samples were applied on the gel in the amount 25 µl. Electrophoresis proceeded 4–5 hours at the voltage 200–220 V and current 25 mA per gel. Gels were coloured through night in the mixture with Coomassie Blue R-250. Discolouration lasted eight hours in discolouring mixture with 2–3 multiple exchange of solution. After sufficient discolouration gels were dipped into fixation solution. After fixation gels were dried in cellophane on the glass for minimum two days.

It followed from electrophoreograms that the storage time of samples at –20 °C is rather limited and after 14-day freezing marked losses appeared in intensity of bands particularly in upper (high molecular) parts of spectrum. Immediate application of samples without their freezing seemed to be the best. Despite it, it has not been succeeded till now to fully optimise the system for better detection of narrow bands in upper and central parts of spectrum. To increase concentration of protein and hence to bring up these bans, lyophilization of germinated one-day grains was tested. Unfortunately, neither



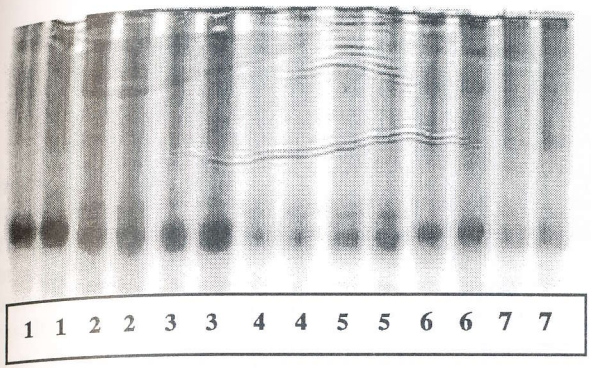
1. Seed proteins extracted in 2% glutathione buffer – leaf

For Figs. 1–6:
1 – HERCULE (*T. spelta*)
2 – ALTGOLD (*T. spelta*)
3 – ROUQUIN (*T. spelta*)
4 – H92.28 (*T. spelta*)
5 – M92.20 (*T. spelta*)
6 – H92.27 (*T. spelta*)
7 – BREA (*T. aestivum*)

this method brought more distinct shift in the character of spectrum. To detect genotypes only one to two distinct bands in the lowest parts of spectrum have sense (Fig. 1). Low variability in the spectrum of bands, their high coincidence in position and in narrower bands in the upper and central parts of spectrum are caused evidently to certain degree by extraction agent that can give us comparison of genotypes only on the basis of certain fraction of extracted proteins – albumins and globulins. These fractions are a part of the majority of enzymatic systems whose polymorphism is usually generally lower. This hypothesis should be also confirmed by results obtained for evaluation of enzymatic systems.

Analysis of native protein from leaves

Analogous procedure for extraction and detection, like in the case of evaluation of native protein from grain, was chosen. 3 cm long segment of the first upper leaf was used for extraction, which was extracted by 50 µl of glutathione buffer. Spectrum of bands was placed in the first third of gel and compared with outputs of spectra of proteins from seeds, the spectrum of



2. Seed proteins extracted in 2% glutathione buffer – seed

native protein was a little richer and was disintegrated into greater number of narrow bands. Despite the seed, a marked lower zone of one to two bands was missing (Fig. 2).

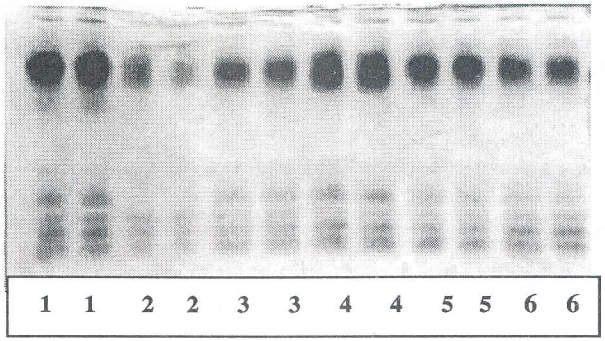
Analysis of isozymes

One-day germinated grain was used to evaluate isozymes and even leaves were used in the case of isozymes of esterases. Extraction proceeded analogously as in the above mentioned methods of analysis of native protein. Except esterases and complex of enzyme AAT, electrophoreograms of remaining enzymes (GDH, SDH, MDH) exhibited only one or two, respectively, distinct bands in the upper part of gel.

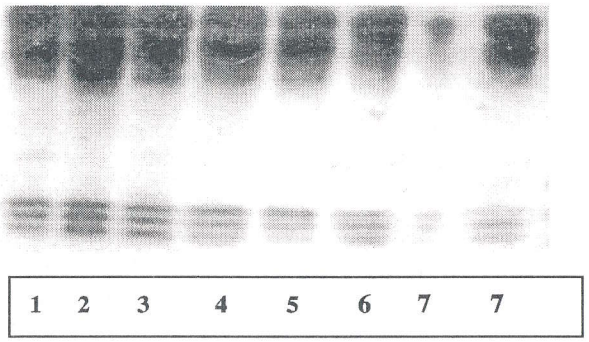
Two zones of bands were found in grains in esterases. Two to three bands were detected in the upper part corresponding to the position for substrate specificity to acetate α-naphthyl and three bands in the lower part corresponding to substratum specificity to acetate β-naphthyl (Fig. 3).

The esterase complex in leaves showed two zones of bands similarly to grains. Nevertheless, a part of bands corresponding to specificity of acetate α-naphthyl appeared in the second zone of three bands characterising substrate specificity to acetate β-naphthyl (Fig. 4).

Two zones of bands were also found in the complex of AAT enzymes. Owing to great uniformity of tested isozyme spectra (with some exception of esterases tested isozyme complexes do not seem to be suitable for identification of different genotypes).



3. EST seed



4. EST leaf

Analysis of the total protein in the SDS medium

Three starting types of material were used for analyses:

1. crushed defatted grain (whole-grain grout)
2. grain or grout, respectively, that was extracted before the analysis alone in 1M NaCl in 0.1 M Na-phosphate buffer at pH = 7 (in the amount 300 µl per grain) and centrifuged 15 minutes at 6000 g; solid residue was used for extraction in extraction buffer SDS
3. grain or grout, respectively, that was extracted before the analysis alone in 1M NaCl in 0.1 M Na-phosphate buffer at pH = 7 and centrifuged 15 minutes at 6000 g; remaining sediment was extracted by 70% ethanol (in both extraction solutions in the amount 300 µl per grain); solid residue was in extraction buffer SDS.

Residues of extraction agents from sediments were removed by lyophilization.

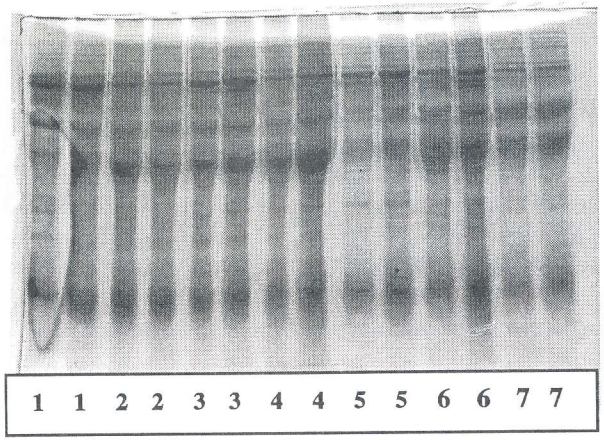
The crushed grain or pre-extracted grout, respectively, was homogenized in microcentrifuge test tube with 170 µl of extraction buffer SDS. After four-hour extraction at the temperature 4 °C samples were centrifuged 10 minutes at 10 000 g. All clear supernatant was transferred into a new microcentrifuge test tube (100–200 µl) with 20 µl of applying buffer. Samples were stored at the temperature –20 °C. Despite it, in this case, too, better results were obtained when samples were applied immediately after extraction. Before the proper application samples were denatured in a hot water bath (100 °C) and then applied on gel in the amount 25–30 µl. Electrophoresis proceeded approximately 2–3 hours at the voltage 280 V and the current 45 mA per gel.

Ad 1) Electrophoreograms obtained of evaluated grains without previous extraction in phosphate buffer manifest relatively rich spectrum of bands along the whole length of a gel. With decreasing molecular weight their sharpness is falling too and their distinction is rather complicated in the central and lower parts of the gel (from molecular weight 45 kDa). Bands were coalesced and shifted in samples stored for a longer period of time.

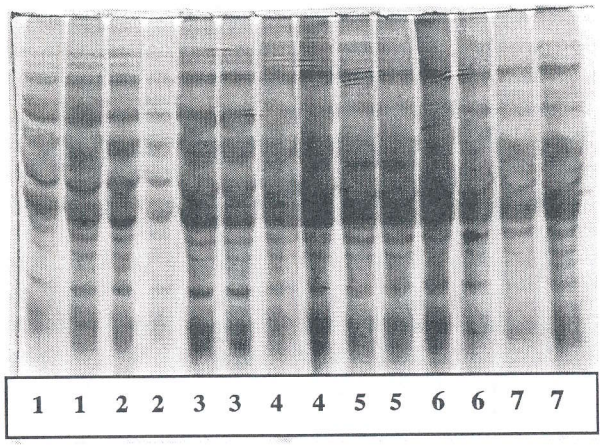
Ad 2) Fig. 5 shows the gel after extraction by Na-phosphate buffer. Electrophoreogram is marked by relatively rich spectrum of somewhat narrower, nevertheless well bounded bands which did not show any tendency to degradation even after longer storage.

Ad 3) Fig. 6 shows electrophoreogram after extraction by Na-phosphate buffer and 70% ethanol. It is characterised by greater sharpness of bands and absence of initial two basic bands of molecular weight ranging from 116 and 84 kDa. Absence of these bands can be explained by extraction capacity of ethanol into the part of prolamin proteins (gliadins). The reason of not fuzziness and partial decomposition of bands should consist in denaturing effect of ethanol on residual not extracted protein (glutenins and residual insoluble fractions).

Methods 1 and 2 seemed to be the best for resulting identification of different genotypes. For better focusing of bands in the above mentioned methods and 2 the output on 20% polyacryl amide gel was used. However, bands got sharp on 20% gel, nevertheless, high density of gel prevented better movement of high-molecular protein fractions and these then remained close to each other, what worsens their mutual determination possibilities. Though 200 gel was used, certain sharpness of bands can be seen despite it during extraction by alcohol. High density of gel caused also its uneven drying out (formation of maps) embarrassing more perfect digitalisation of gel.



5. SDS seed protein preextracted in Na-phosphate buffer (10% gel)



6. SDS seed protein preextracted in Na-phosphate buffer and in 70% ethanol (10% gel)

CONCLUSION

Three basic marker systems were evaluated in total: complexes of isozymes, native protein and total SDS protein. All marker systems were proved on eletrophoreogram, nevertheless, their usability for identification of varieties of spelt wheat due to their polymorphism is different. Enzymatic systems, except some variability of complex of esterases, showed a high uniformity and are not very suitable for the proper identification. The situation is a little better in evaluation of native protein extracted from grain and leaf.

The method based on extraction of protein under the presence of SDS seemed to be the best for resulting identification of genotypes, from which it was possible to read certain differences among some genotypes. Neither this method itself should be certain to distinct different genotypes for 100 per cent. For reliable detection it will be necessary to use more marker systems, starting by traditional phenotypic determination (if it is possible) and finishing by combination of methods of different biochemical or molecular markers, respectively.

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DVOŘÁČEK, V. – ČURN, V. – MOUDRÝ, J. (Jihočeská univerzita, Zemědělská fakulta, České Budějovice, Česká republika):

Metodologický přístup k hodnocení biochemických markerů u pšenice špaldy (*Triticum spelta* L.).

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Izoenzymy, tj. esterázy, aspartát aminotransferáza, glutamát dehydrogenáza, malát dehydrogenáza a shikimát dehydrogenáza, nativní protein extrahovaný v 2% gluthathionovém pufru, separované z listu a semene v prostředí nedenaturačního PAGE systému a SDS zásobní protein v SDS-PAGE byly použity jako biochemické markery pro vyhodnocení polymorfismu u tří odrůd pšenice špaldy (Hercule, Rouquin, Altgold) a u tří novošlechtění pšenice špaldy (H92.27, H92.28, M92.20) vytvořených křížením mezi pšenicí setou a referenční odrůdou pšenice seté (Brea). Pouze tři markerovací systémy (SDS zásobní protein, EST a nativní protein extrahovaný v 2% gluthathionovém pufru ze semene) vykazovaly polymorfismus umožňující zjištění diferencí mezi referenční odrůdou pšenice seté Brea a odrůdami pšenice špaldy.

pšenice špalda; biochemické markery; zásobní proteiny; isoenzymy; elektroforéza

Contact Address:

Ing. Václav Dvořáček, Jihočeská univerzita, Zemědělská fakulta, katedra rostlinné výroby, Studentská 13, 370 05 České Budějovice, Česká republika, tel.: 038/777 24 30, e-mail: dvoracek@zf.jcu.cz