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DIFFERENTIATION OF CROATIAN BARLEY VARIETIES BY GRADIENT GEL SDS-PAGE AND ISOELECTRIC FOCUSING OF DRY GRAINS AND GREEN MALT HORDEINS

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SUMMARY

Applicability of polyacrylamide gel electrophoresis and isoelectric focusing of hordeins for discrimination of six two-rowed winter barley varieties (Angora, Sladoran, Rodnik, Rex, Martin and Barun) has been investigated. Hordeins extracted from dry grains and green malt of barley varieties were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis in gradient gel of 8-18% T, and by isoelectric focusing in pH gradient of 3.5-9.5 and 5.5-8.5, respectively. In all separation experiments better resolution of proteins was achieved with dry grain extracts, than with malt extracts. Angora, Sladoran and Martin variety could be distinguished from other varieties by differences in hordein patterns obtained by gradient gel SDS-PAGE (8-18% T), and Angora, Sladoran, Martin and Rodnik by isoelectric focusing in pH gradient 5.5-8.5.

Key-words: barley variety discrimination, gradient gel SDS-PAGE, hordeins, isoelectric focusing

INTRODUCTION

Barley malting and brewing processing properties, as well as potential grain yield, morphology, physiological traits, and resistance to certain fungi and viral diseases are cultivar dependent. Therefore, reliable and simple method for variety recognition is of a great importance for the malting and brewing industries, as well as for barley breeders, growers and traders. Among various approaches, analysis of barley grain storage proteins, hordeins, by polyacrylamide gel electrophoresis at acidic pH or by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was recommended in year 1988 as the official method for barley variety discrimination (Cooke, 1995; Wrigley, 1995). However, according to the reports in the literature, hordein patterns obtained by the recommended methods, allow only varieties grouping and not their full differentiation (Cooke, 1995; Wrigley, 1995; White and Cooke, 1992). The main obstacle for discrimination of barley variety hordeins is their similarity, developed for two reasons: *(i)* the majority of modern barley varieties could be traced back to just a few ancestors making them closely related, and *(ii)* genes coding for the B and C hordeins are located on the same arm of chromosome 5, thus limiting the possibilities of recombination, and subsequent hordein polymorphism (Weiss et al., 1991). Additionally, the recommended method with applied separation conditions does not have high resolution power.

MATERIALS AND METHODS

Apparatus and chemicals

Equipment for SDS-PAGE and IEF, ExcelGel SDS Gradient 8-18, Ampholine PAGplate, Coomassie Brilliant Blue G-250, glycine, dithiothreitol (DTT), Tris(hydroxymethyl)aminomethane (Tris), glycerol, sodium dodecyl sulphate (SDS), protein standards for SDS-PAGE and IEF, were from Amersham Biosciences (Sweden), whereas N-(2-hydroxyethyl)piperazine-N'-(2-

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ethanesulfonic acid) (HEPES) and N-Tris(hydroxymethyl) methylglycine (TRICINE) were from Sigma (Germany), and other chemicals were of analytical grade from Merck or Sigma (Germany).

Barley samples and green malt preparation

Experiments were performed with six two-rowed winter barley (*Hordeum vulgare* L) varieties. Five of them (Sladoran, Rodnik, Rex, Martin and Barun) were domestic varieties created at the Agricultural Institute Osijek, Croatia, while one variety (Angora) was foreign one originated from Germany, bred by Saatzucht Josef Breun, GdbR-Herzogenaurach. According to previous studies conducted in Croatian agro-productive area, Angora variety is characterized by medium plant height moderately resistant to lodging with moderate number of tillers and seeds per plant, accompanied with higher thousand kernel weight, the later heading, lower hectoliter weight and higher grain protein content. The varieties Sladoran, Rodnik, Rex, Martin and Barun possess shorter elastic stem with emphasized lodging resistance and early heading dates. They have agro-economic characteristics favourable for Croatian and East European growing conditions. One of the main attributes of the Barun variety is its lower protein content preferred by beer and malt industries (Lalić et al., 2005; 2006).

Barley grains (50 g) were surface-sterilized with 1% sodium hypochlorite solution (20 minutes), rinsed several times with 0.5 M sodium chloride solution and then with distilled water. After steeping for 24 hrs at 24°C, the grains were germinated on a steel mesh for 3 days in the dark at 24°C. Developed green malt barley was collected and used for protein extraction.

Protein extraction

Ten dry seed or green malt grains of each variety were frozen and disintegrated in liquid nitrogen using mortar and pestle. The obtained powder (0.5 g) was mixed with 2 mL of 55% aqueous *iso*-propanol containing 0.5% dithiothreitol, and hordeins were extracted during 2 hrs at 24°C by vortexing for 30 seconds in intervals of 15 minutes. Extracts were clarified by centrifugation at 20000×g for 20 min at 24°C and *(i)* used immediately for isoelectric focusing, or *(ii)* mixed with equal volume of SDS sample buffer (100 mM Tris-acetate buffer, pH=7.5, containing 2% SDS, 0.02 M DTT and 0.02% Bromphenol Blue), heated in a boiling water bath for 5 min, cooled to room temperature and, after addition of 8 μL of aqueous DTT (γ=100 mg mL^{-1}), centrifuged at 20 000×g for 20 min at 24°C.

Electrophoretic analysis

The electrophoretic separations, SDS-PAGE in gradient gel and IEF, were performed on horizontal Multiphor II system (Amersham Biosciences, Sweden) using commercially available gels. All experiments were at least twice repeated.

Horizontal SDS-PAGE was performed at 15°C using commercial gradient gel plates (ExcelGel SDS Gradient 8-18% T), and paper electrode wicks soaked with 450 mM Tris-acetate, pH=6.6, containing 0.1% SDS for anode or with 800 mM TRICINE containing 80 mM Tris base and 0.1% SDS, pH=7.15, for cathode. Hordeins (1 μg) were applied as pipette droplets, then stacked at 200 V and 30 mA for 1 hour, and resolved at 600 V and 30 mA during 1.5 hr. After electrophoresis, separated hordeins were stained with silver-nitrate according to Hempelmann and Kaminsky (1986).

Isoelectric focusing was performed on horizontal system using commercially available IEF gels with carrier ampholytes (Ampholine PAGplate pH gradient 3.5-9.5 and 5.5-8.5) using electrode strips soaked *(i)* with 1 M phosphoric acid for anode and with 1 M sodium hydroxide for cathode, when working with pH gradient 3.5-9.5, and *(ii)* with 0.4 HEPES for anode and with 0.1 M sodium hydroxide for cathode, when using pH gradient of 5.5-8.5 (Westermeier, 2001). Prior to sample application, gels were placed onto cooling block (10°C) and prefocused 20 min at 700 V and 20 mA. Afterwards, using sample application pieces, hordeins (25 μg) were applied near anode. Separation of proteins in pH 3.5-9.5 gradient was carried out using the following routine: 20 minutes at 300 V and 16 mA; gradual increase of voltage in 20 min intervals first to 500 V and then to 800 V at 30 mA; voltage increase to 1200 during 30 min; final band sharpening at 1500 V for 30 min, all at constant current of 30 mA. Proteins separation in gels with pH 5.5-8.5 gradient started at 500 V and 16 mA, and continued by gradual voltage increase every 20 min, first to 800 V and then to 1200 V. Protein separation continued during next 120 min at 1500 V and 20 mA. After focusing the gels were immediately fixed in 10% trichloroacetic acid for 45 min and stained with Coomassie Brilliant Blue G-250 in phosphoric acid, according to Neuhoff et al. (1985).

RESULTS AND DISCUSSION

SDS-PAGE of barley hordeins

After SDS-PAGE of barley dry grain hordeins and protein silver staining, numerous bands could be discerned in molecular weight range from 30 to 97 kDa. Green malt analysis resulted in protein bands corresponding to 30 to 60 kDa size. The observed protein patterns were very similar for both examined barley extracts. However, differences between individual barley varieties could be observed among dry grain proteins ranging between 60 and 70 kDa (Fig. 1, numbers 2-7). Measurement of bands'

Rf values and subsequent calculation of molecular masses, revealed existence of 5 distinctive proteins with molecular mass of 69.3, 68.2, 67.1, 66.4 and 63.0 kDa, respectively. Angora barley (Fig. 1, No. 2) was distinguished from other varieties by protein bands with apparent molecular mass of 69.3 and 66.4 kDa. Sladoran variety (Fig. 1, No. 5) could be recognized by the presence of protein band at position of 68.2 kDa, and Martin (Fig. 1, No. 7) by protein of 67.1 kDa. The other varieties, Barun, Rodnik, and Rex, could not be distinguished between themselves.

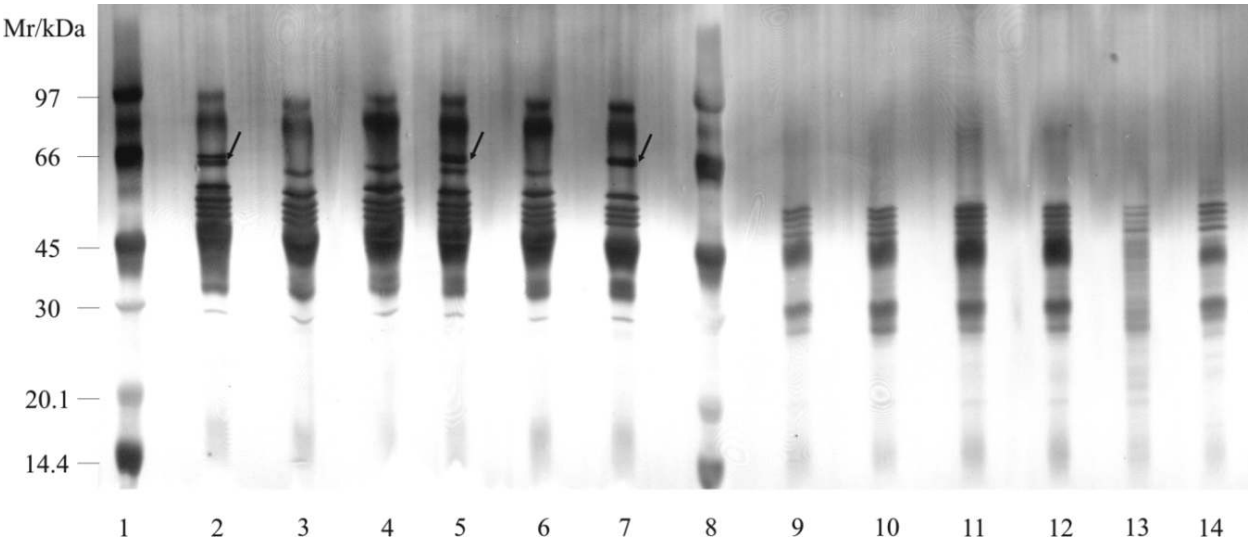

Figure 1. SDS-PAGE in gradient gel of 8-18% T of barley hordeins extracted with 55% aqueous *iso*-propanol from dry grains and green malt. Vertical lanes present: low molecular weight protein standards (1, 8); dry grains (2-7) and green malt (9-14) of varieties: Angora (2, 9); Barun (3, 10); Rodnik (4, 11); Sladoran (5, 12); Rex (6, 13); Martin (7, 14). Arrows indicate differences between varieties

Slika 1. SDS-PAGE u gradijent gelu (8-18% T) hordeina ekstrahiranih iz suhoga zrnja i zelenoga slada ječma pomoću 55% vodene otopine izo-propanola. Staze predstavljaju: niskomolekularne standarde proteina (1,8); suho zrno (2-7) i zeleni slad (9-14) sorti: Angora (2,9); Barun (3,10); Rodnik (4,11); Sladoran (5,12); Rex (6,13); Martin (7,14). Strelice pokazuju razlike među sortama

The observed difference between varieties disappeared in green malts (Fig. 1, numbers 9-14) due to the lack of high molecular weight hordeins (97-66 kDa). In addition, reduced intensity of protein bands in molecular mass ranged from 55 to 45 kDa, and increased number of protein bands between 45 and 25 kDa, were observed. (Fig. 1, numbers 9-14). However, different relative intensities of hordein bands of individual varieties could be detected. They most probably originate from differences in degradation intensity of barley seed storage proteins during germination process, observed by Šimić et al. (2007).

On the basis of silver stained hordein patterns after SDS-PAGE in gradient gel (8-18% T), it could be concluded that this type of electrophoretic analysis might be applicable for Croatian barley variety discrimination, using dry grains. Similar finding on dry grain barley variety discrimination by SDS-PAGE in gradient gel (12-15% T) has been reported by Weiss et al. (1991), who found differences in hordein patterns in the range of molecular mass from 49 to 72 kDa and 30-45 kDa.

Isoelectric focusing of barley hordeins

When the same protein fractions (hordeins) were separated by IEF in pH gradient of 3.5-9.5, differences between individual varieties were observed, although the protein patterns were similar. Distinction of varieties was greater in protein patterns of dry grains, than of green malt. Barley Angora (Fig. 2, No. 2, marked by arrow) could be clearly distinguished from other varieties by significantly lower intensity of protein bands in the range of isoelectric points (pI) between 6.0-7.0, while Sladoran variety (Fig. 2, No. 5) was recognized by the lack of protein band with isoelectric point of 7.35. By their protein pattern, other varieties could be divided in two groups, one including Barun (Fig 2, No. 3) and Rex (Fig. 2, No. 6) and the other one Rodnik and Martin (Fig. 2, numbers 4 and 7). In the case of green malt, only Rodnik and Sladoran variety (Fig. 2, numbers 11 and 12, marked by arrows) could be distinguished from the others by the absence of protein band with apparent isoelectric point of 7.35.

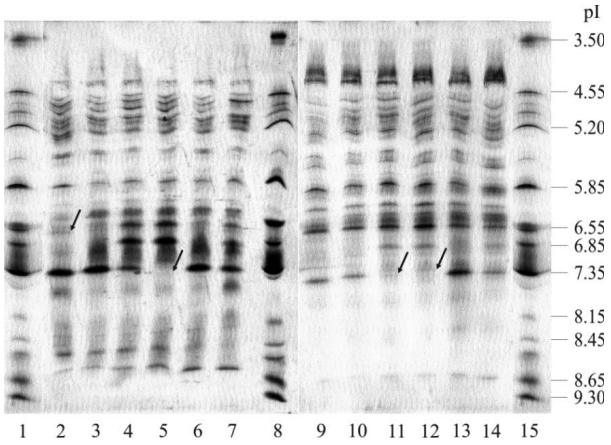

Figure 2. IEF in pH 3.5-9.5 gradient of barley hordeins extracted from dry grain and green malt with 55% aqueous *iso*-propanol. Vertical lanes present: protein pI standards, pH=3-10 (1, 8, 15); dry grains (2-7) and green malt (9-14) of varieties: Angora (2, 9); Barun (3, 10); Rodnik (4, 11); Sladoran (5, 12); Rex (6, 13); Martin (7, 14). Arrows indicate differences between varieties

Slika 2. Izoelektrično fokusiranje u pH gradijentu 3,5-9,5 hordeina ekstrahiranih iz suhoga zrnja i zelenoga slada ječma pomoću 55% vodene otopine izo-propanola. Staze predstavljaju: pI standarde, pH=3-10 (1, 8,15); suho zrno (2-7) i zeleni slad (9-14) sorti: Angora (2, 9); Barun (3, 10); Rodnik (4, 11); Sladoran (5, 12); Rex (6, 13); Martin (7, 14). Strelice pokazuju razlike među sortama

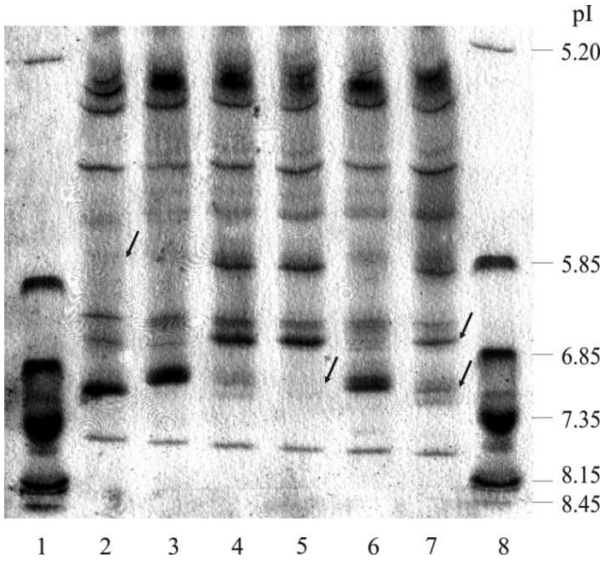

Figure 3. IEF in pH 5.5-8.5 gradient of barley hordeins extracted from dry grains with 55% aqueous *iso*-propanol. Vertical lanes present: protein pI standards, pH=5-10 (1, 8); varieties: Angora (2); Barun (3); Rodnik (4); Sladoran (5); Rex (6); Martin (7). Arrows indicate differences between varieties

Slika 3. Izoelektrično fokusiranje u pH gradijentu 5,5-8,5 hordeina ekstrahiranih iz suhoga zrnja ječma pomoću 55% vodene otopine izo-propanola. Staze predstavljaju: pI standarde, pH=5-10 (1, 8); sorte: Angora (2); Barun (3); Rodnik (4); Sladoran (5); Rex (6); Martin (7). Strelice pokazuju razlike među sortama

Since differences between varieties based on electrophoretic protein pattern were observed in the range of isoelectric points between 6.0 and 8.15, hordeins of dry grains were separated in narrower pH gradient of 5.5-8.5. With this pH gradient almost identical protein pattern differences between individual varieties could be observed as before (Fig. 3, marked by arrows), but with an improvement of Martin and Rodnik separation. Martin variety could be distinguished from Rodnik by intensified protein band of isoelectric point 7.0, and lower intensity of protein band of pI=6.6. (Fig. 3, numbers 7 and 4, respectively).

According to the protein patterns obtained by IEF in pH gradients 3.5-9.5 and 5.5-8.5, and Coomassie Brilliant Blue G-250 staining, it can be concluded that isoelectric focusing in pH 5.5-8.5 gradient, of hordeins from barley dry grains, might be applicable for Croatian barley variety discrimination. Similar results were reported by Weiss et al. (1991), who have found that among 55 barley varieties 22 could be identified, whereas the others formed 10 groups according to their hordeins pattern obtained by IEF in immobilized pH gradient of 4.0-8.0. Considering the data of Šimić et al. (2007), such analyses of hordeins might be useful in brewing quality assessment of Croatian barley varieties.

CONCLUSION

The applicability of gradient gel SDS-PAGE and IEF of hordeins for differentiation of six two-rowed winter barley varieties was examined. By the all experiments, dry grain extracts appeared to be better starting material for the analysis, than green malt extracts. Both methods discriminate Angora barley from other examined varieties, which could not be completely discerned. By SDS-PAGE in gradient gel of 8-18% T, recognition of additional two varieties, Sladoran and Martin, was achieved, as others could be set in one group. IEF in pH 5.5-8.5 gradient could differentiate four varieties, Angora, Sladoran, Martin and Rodnik. Thus both methods could be used for partial discrimination of Croatian barley varieties. Analysis of greater number of domestic barley varieties is desirable.

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RAZLIKOVANJE HRVATSKIH SORTI JEČMA SDS-POLIAKRILAMID ELEKTROFOREZOM U GRADIJENT GELU I ELEKTROFORETSKIM FOKUSIRANJEM HORDEINA EKSTRAHIRANIH IZ SUHOGA ZRNJA I ZELENOGA SLADA

SAŽETAK

Ispitana je mogućnost primjene gel elektroforeze i izoelektričnoga fokusiranja hordeina za razlikovanje šest sorti ozimoga dvorednoga ječma (Angora, Sladoran, Rodnik, Rex, Martin, Barun). Hordeini ekstrahirani iz suhoga zrnja i zelenoga slada ječma razdvajani su pomoću SDS-poliakrilamid-gel elektroforeze u gradijentu od 8-18% T i pomoću izoelektričnoga fokusiranja u gradijentima pH 3,5-9,5 i pH 5,5-8,5. U svim pokusima elektroforetskoga razdvajanja ekstrakti proteina iz suhoga zrna davali su bolje rezultate od onih iz zelenoga slada. Sorte Angora, Sladoran i Martin mogle su se razlikovati međusobno i od drugih sorti po elektroforetskome profilu svojih hordeina dobivenih SDS-PAGE u gradijent gelu (8-18% T), a sorte Angora, Sladoran, Martin i Rodnik pomoću IEF u pH gradijentu 5,5-8,5.

Ključne riječi: razlikovanje sorti ječma, SDS-PAGE u gradijent gelu, hordeini, izoelektrično fokusiranje

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