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Gel Isoelectric Focusing of Wheat Alcohol Dehydrogenase

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Summary. Alcohol dehydrogenase (ADH) of different hexaploid wheat subspecies and varieties was investigated by isoelectric focusing in polyacrylamide gels. With this technique six ADH isoenzymes can be separated, while by the standard electrophoretic technique only three are visible. The ADH pattern revealed by isoelectric focusing is in full accordance with the hypothesis that the active ADH isozymes in hexaploid wheat are dimers composed of six possible combinations of subunits coded by triplicate structural genes.

Key words: Alcohol dehydrogenase – Hexaploid wheat – Isoelectric focusing – pI values

Introduction

The development of the isoelectric focusing technique in polyacrylamide gel (Wrigley 1968, 1969; Fawcet 1968) led to the re-investigation of isozymes and proteins with known electrophoretic patterns because in several instances this technique yields a higher number of bands as a result of its improved resolving power compared to polyacrylamide gel electrophoresis (Dale and Latner 1968; Kohnert et al. 1973; Bozzini et al. 1973).

The alcohol dehydrogenase (ADH, EC.1.1.1.1.) isozymes are widely used in wheat genetics. After detailed analyses of wheat ADH by Hart (1969, 1970, 1971), it has been accepted that the ADH in wheat and related species is a dimer, and that random association of subunits into dimers results in the production of the active ADH enzymes. The genes involved in the production of ADH are located on the chromosomes of homologous group 4.

In accordance with these analyses, in diploid wheat species there is only one ADH enzyme, in tetraploids there are three and in the hexaploids, six ADH isozymes. However, with the PAGE (Polyacrylamide Gel Electrophoresis) technique it was possible to detect only three ADH bands in hexaploids because three (ADH-3 $\beta\beta,\vartheta\vartheta,\beta\vartheta$), and two (ADH-2,$\alpha\beta$, $\alpha\vartheta$) of them, respectively, have coincident electrophoretic mobility. To the best of our knowledge there is no data about the analytical isoelectrofocusing of ADH, except on the ADH of horse liver (Lutstorf et al. 1970).

In this study we describe in detail the developed method of isoelectric focusing of wheat ADH in polyacrylamide gels, and the results obtained by it. We believe that the method may represent some improvement in the isoelectric focusing of dehydrogenases in general.

Materials and Methods

Enzyme extracts were obtained from mature whole grains of *Triticum aestivum L.* (varieties 'Mironovskaja 808', 'Bezostaja 1', 'Fleischmann 481' and 'Penjamo 62'), *T. aestivum ssp. vavilovii* (Jakubz.), *ssp. macha* (Dek. et Men.), *ssp. spelta* (L. Thell.), *and ssp. sphaerococcum* (Perc., Mac Key). The extraction was performed by means of an MSE sonicator, in 20.0% sucrose which contained 0.01 M dithiothreitol. The slurry was centrifuged at 15,000 g for 30 min at 2° C, and the supernatant was placed on the upper part of the gel. The extracts were prepared daily and used immediately. From each sample we made at least three independent extracts, and from each extract there were a minimum of four parallel gels. This means that the results described here are obtained from at least 12 replicates.

Since neither the technique described by Wrigley (1968, 1969) (which is commonly used in our laboratory for separation of proteins and some enzymes, e.g. esterases, acid phosphatases, etc.), nor the method developed by Klose and Spielmann (1975) for isoelectric focusing of LDH appeared to be suitable for the separation of wheat ADH, we had to test a number of different conditions to find those best for our purposes. Finally we developed the following procedure.

The isoelectric focusing was carried out in vertical cylindrical polyacrylamide gels (height 9.0 and diameter 0.5 cm) in a refrigerator (+ 4° C). The aequeous gel solution contained 7% (w/v) acrylamide, 0.28% (w/v) bisacrylamide, 0,023% (w/v) ammonium persulfate, 3% Ampholine (LKB, pH 5-8), and 1,5 M urea. The

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crude enzyme extract (200 μl), was applied to the top of the gel and overlayered with a 10% sucrose solution containing 2,5% Ampholine. The lower (anode) compartment of the apparatus was filled with 5% (v/v) phosphoric acid; the upper (cathode) compartment with 5% (v/v) ethylendiamine (Klose and Spielmann 1975). The run was performed in a 'Shandon' apparatus connected to a 'Shandon-Vokam' power supply. The power was regulated as follows: 30 min 30 V, 60 min 60 V, 60 min 120 V, 90 min 200 V, 30 min 250 V. At the end of the run, the power was raised to 300 V for 2 min, and to 350 V for 2 min.

The gels were stained for ADH activity with the following solution: 6 ml 0,1 M $MgCl_2$, 6 ml 0,03 M NaCN, 12 ml 44% ethanol, 36 mg NAD, 45 mg Nitro Blue Tetrazolium, 6 mg phenazine methosulfate in 126 ml 0,05 M tris-HCl buffer, pH 8,0. All chemicals were reagent grade and used without further purification.

In every run a 'blank' gel without stain was cut into 0,5 cm segments; each segment was placed in 3 ml distilled water for 12 hours, and the relation of pH values to gel length was determined with a 'Radelkis Digital OP-206' pH meter.

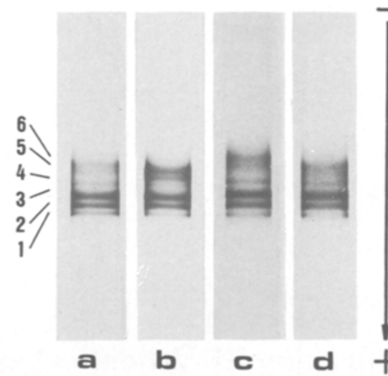

Fig. 1. pI-ADH zymograms of (a) *T. aestivum* 'Bezostaja 1' (winter wheat), (b) 'Penjamo 62' (spring wheat), (c) *ssp. vavilovii*, (d) *ssp. spelta*

Results and Discussion

Six distinct ADH bands were observed in all the tested hexaploid wheat species (subspecies) and varieties (Fig. 1), while by the PAGE technique it was possible to detect only three. The ADH isozymes separated by the present method are characterized by their isoelectric pH values (= pI values), therefore, they may be called pI-isozymes: pI-ADH-1, ... pI-ADH-6, (e.g. pI-isoesterases, Yasuo Nakai et al. 1971).

The detection of six ADH isozymes by isoelectric focusing is consistent with the accepted assumption (Hart 1970) that in wheat strains in which each of the 4th homologous chromosome is present (hexaploid wheats with the genome *AABBDD*), random association of the α,β and ϑ subunits results in the production of six possible dimers, namely, $\alpha\alpha$, $\beta\beta$, $\vartheta\vartheta$, $\alpha\beta$, $\alpha\vartheta$ and $\beta\vartheta$. The results of this study clearly demonstrate that by isoelectric focusing we have been able to separate and to detect these six dimers.

The phenotypes of the obtained pI-ADH zymograms are similar in each tested sample, but they differ with respect to the relative staining intensity of individual bands. In most cases the most intensive are the pI-ADH-3 and pI-ADH-2, but in the 'Penjamo 62' (spring wheat) the pI-ADH-6 and pI-ADH-5 are nearly as intensive. In the ssp. *sphaerococcum*, on the contrary, the pI-ADH-3 and pI-ADH-2 stain less intensively than pI-ADH-6 and pI-ADH-5 (Fig. 2).

The unequal intensity distribution of the six pI-ADH isozymes within every tested hexaploid wheat does not agree with the accepted hypothesis that each of the ADH structural genes (Adh) produces an approximately equal amount of subunits. This contradiction may be explained by one – or more – of the following assumptions: 1/in spite of the above mentioned theory, unequal quantities of subunits may be synthesized; 2/ the activities of either

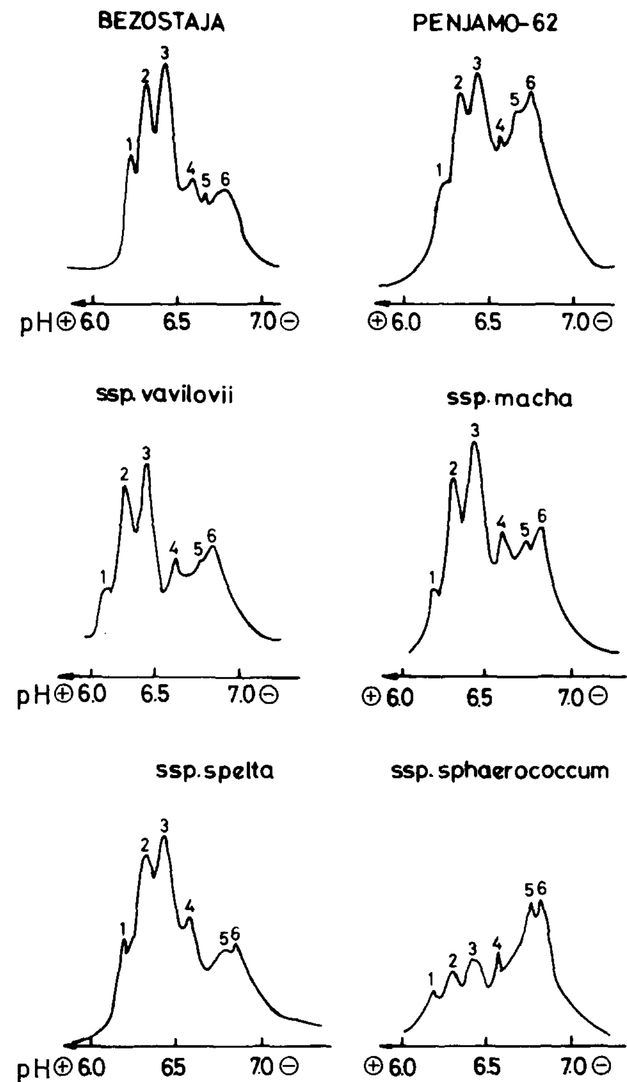

Fig. 2. Densitometric tracings of pI-ADH isozymes of different species (subspecies) and varieties of *T. aestivum*

Table 1. The pI values of the pI-ADH isozymes of tested hexaploid wheat subspecies and varieties

	Mironov-skaja 808	Fleisch-mann 481	Bezos-taja 1	Penja-mo 62	ssp. *vavi-lovii*	ssp. *macha*	ssp. *spelta*	ssp. *sphae-rococcum*	Mean ± S_X
pI-ADH-1	6.18	6.20	6.20	6.20	6.20	6.18	6.18	6.18	6.19 ± 0.01
pI-ADH-2	6.28	6.32	6.30	6.33	6.30	6.30	6.30	6.28	6.30 ± 0.02
pI-ADH-3	6.38	6.40	6.40	6.43	6.45	6.43	6.40	6.40	6.41 ± 0.02
pI-ADH-4	6.58	6.60	6.58	6.58	6.63	6.58	6.55	6.55	6.58 ± 0.03
pI-ADH-5	6.73	6.70	6.65	6.65	6.75	6.73	6.75	6.73	6.71 ± 0.04
pI-ADH-6	6.80	6.80	6.78	6.75	6.78	6.80	6.80	6.80	6.79 ± 0.02

the enzymes or the genes involved (which have hitherto been accepted to be equally expressed) may be different; 3/ modifier genes may alter Adh gene activities; 4/ as a result of the isoelectric focusing process the dimers may exhibit different enzymatic activities or unequal stability. Further investigations are needed for the clarification of the above mentioned factor(s) which is (are) expressed in this case. Similar explanations are given by Leibenguth (1977) for the unequal intensity distribution of the PAGE ADH isozymes in *Triticum aestivum* and in hexaploid winter *Triticale*.

The six pI-ADH isozymes are located in the gel column between 2,9 and 3,9 cm distance from the top (cathodal), namely, approximately in the 6th, 7th and 8th 0.5 cm segment of the gel, respectively. The pH values of the three bottom pieces (No. 12, 13 and 14) strongly deviated from linear regression, indicating that the pH gradient produced by ampholine was disturbed by the 5% phosphoric acid in the anode compartment. (To a much lesser degree this is valid also for the top pieces of the gel, where apparently the 5% ethylene diamine has a disturbing effect on the pH gradient). Excluding the three bottom pieces, the measured pH values give a pH gradient which is practically linear. On the basis of the pH gradient obtained and densitometric tracings of the pI-ADH zymograms, it was possible to estimate approximately the pI values of each pI-ADH enzyme by extrapolation (Table 1).

The data for all tested subspecies and varieties are very close. As is seen, the pI-ADH isozymes of hexaploid wheat are located in a rather narrow pH range; they occupy only 0.6 pH units in the pH gradient. It seems that the subunits synthesized by each homologous chromosome of group 4 (4A, 4B and 4D) are very similar in their composition, and therefore the active dimers differ only slightly with respect to their isoelectric point values.

In the review of isoelectric points of 400 different proteins (Malamud and Drysdale 1978) there is a single datum on the pI values of the ADH enzymes from horse liver, which is between pH 8, 7-9, 3. To the best of our knowledge, no other ADH enzyme has been tested for its pI value. We therefore believe that data presented here will be of interest.

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