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GEL ELECTROPHORETIC METHOD FOR SEPARATING AMYLASE ISOENZYMES IN WHEAT

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SUMMARY

The amylase isoenzyme compositions of an early- and a mid-season-ripening variety of Hungarian wheat were investigated, during ripening and storage, by polyacrylamide disc gel electrophoresis. Of the two electrophoretic systems studied, the alkaline one (pH 7.9) gave a higher resolution and was used throughout the study. Amylase fractions detected on starch and β-limit-dextrin substrates, were studied in parallel with quantitative amylase activity determinations. Isoenzymes acting on β-limit dextrin (α-amylase) were detectable only during the first half of the ripening period. The number of the fractions detected on starch decreased during the first half of the ripening period and remained practically unchanged during the second half. Amylase activity varied in a similar way. The number of amylase isoenzymes increased during storage, while amylase activity showed but minor fluctuations. The increase in the number of isoenzymes during storage might be due to the dissociation of molecular aggregates of the enzyme. In the samples taken throughout ripening and storage these remained in the large-pore sample gel. The isoenzyme pattern of wheat seems to be primarily dependent on the stage of ripening and only to a lesser degree on the variety.

INTRODUCTION

Wheat amylases are known to occur in multiple molecular forms whose role in breadmaking technology might vary. As the first step of a study into this problem, amylase isoenzymes were separated by gel electrophoresis and their behaviour was studied during ripening and storage of two Hungarian varieties of wheat. Electrophoresis was carried out in parallel with quantitative determinations of amylase activity.

MATERIALS

The early ripening wheat variety Martonvásári 4 (hereafter referred to as Mv 4) and the mid-season wheat variety Bezostaya 1 (hereafter B 1), both of the genus *Triticum aestivum*, were used in the experiments. Wheat samples were supplied by courtesy of the National Institute for Agricultural Variety Testing, Budapest, Hun-

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gary. Samples of wheat kernels were homogenized in 10 volumes of distilled water, shaken for 60 min, and then the solution centrifuged and filtered. Solutions (0.5 or 1 %, w/v) of soluble starch (E. Merck, Darmstadt, G.F.R.) and of β-limit-dextrin (2 %, w/v) were used as substrates to detect (α + β)- and α-amylase, respectively. The β-limit-dextrin solution was prepared by hydrolysis of soluble starch with wheat β-amylase (Serva, Heidelberg, G.F.R.) (pH 4.8, 20 h, 5°C)[1]. Both substrates were dissolved in 0.1 *M* acetate buffer.

METHODS

Detection of multiple forms of amylases

The gel electrophoretic systems of Davis[2] and of Reisfeld *et al.*[3] were used at pH 7.9 and 4.8, respectively, to separate the multiple forms of the amylases. Runs were carried out at room temperature in the gel electrophoretic chamber (Reanal, Budapest, Hungary), using a current of 4 mA per gel tube. Bromophenol blue was used as marker dye in the alkaline system and runs were continued until the dye reached the lower (anodic) end of the gels. With the acidic system, runs took 3 h. The protein loads per gel tube corresponded to 20–80 mg and to 40–120 mg of wheat (wet-mass basis) for the samples taken during ripening and after harvest, respectively. The samples were polymerized into the sample gel[4].

In order to detect the amylase-active bands, the gels were first incubated for 5 min at 50°C and pH 4.8 in one of the substrate solutions, then immersed into an iodine (0.04 %, w/v)–potassium iodide (0.4 %, w/v) solution and, subsequently, stored in 5 % acetic acid. Different enzyme: substrate ratios were used and the most distinct patterns selected for evaluation. The active fractions appeared as colourless bands on a blue (starch) and a pink (β-limit-dextrin) background, respectively.

Measurement of amylase activity

Spectrophotometric amylase-activity determinations were carried out in water extracts of wheat at pH 7.0 and 50°C on Phadebas Amylase Test (Pharmacia, Uppsala, Sweden) substrate[5]. A change in optical density (620 nm) of 10^{-3} per min was adopted as enzyme activity unit and related to 1 g of fresh mass.

Determination of moisture content

Moisture content was determined as the weight loss after oven drying at 90 and 150°C, respectively, for the samples taken during the first 2 weeks and the later periods of ripening.

RESULTS AND DISCUSSION

The resolution of the wheat amylase isoenzymes in the two electrophoretic systems investigated is shown in Fig. 1.

Four isoenzyme fractions were obtained from the acidic system while seven were obtained from the alkaline one. In the acidic system the migration distances covered by the fractions ranged from 1 to 3 cm from the cathodic end, while in the alkaline system the fractions were spread over the entire length of the gels. In both systems, part of the protein of amylase activity did not enter the small-pore frac-

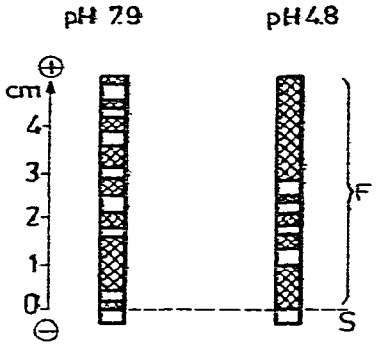

Fig. 1. Separation of wheat amylase isoenzymes by polyacrylamide disc gel electrophoresis at pH 7.9 and 4.8. Conditions of electrophoresis: the systems correspond to those of Davis[2] and Reisfeld *et al.*[3], respectively. Samples were polymerized into the sample gel (S). The arrows indicate the direction and the cm-scale the distance of migration in the fractionating gel (F) (room temperature, 4 mA per gel tube). Runs in the alkaline system were continued until the bromophenol blue marker reached the lower end of the gel; in the acidic system they took 3 h. Protein loads per gel correspond to 40 mg of wheat (wet-mass basis) of the variety Mv 4 as sampled after 9 months of storage. Preparation of the enzyme solution: wheat kernels were homogenized in a ten-fold amount of distilled water, shaken for 60 min, centrifuged and filtered. Detection of isoenzymes: incubation of the gels for 5 min at 50°C in 0.5% (w/v) soluble starch (Merck) dissolved in 0.1 *M* acetate buffer pH 4.8, then immersion into an iodine (0.04%, w/v)–potassium iodide (0.4%, w/v) solution and storage in 5% acetic acid.

tionating gel. As resolution was better in the alkaline system, this was the only system used in the remainder of the experiments.

The isoenzyme patterns of the two wheat varieties obtained with starch and β-limit-dextrin substrates at different stages of ripening are shown in Figs. 2 and 3. During the first 2 weeks of ripening the isoenzyme patterns of the Mv 4 variety showed great similarity on the two substrates, thus indicating the presence of α- and/or β-amylases. The patterns obtained on the third week are difficult to interpret. With β-limit-dextrin as substrate, the distribution of the isoenzymes showed but slight changes as compared to the sample taken the previous week, while on the starch substrate the characteristic triplet gave way to a broader active zone of somewhat higher mobility. This zone increased in intensity by the fourth week. From the fifth week onwards no α-amylase fraction migrating in the alkaline system could be detected. On the starch substrate, one or two β-amylase fractions of high and one of low mobility were detectable throughout the later phases of ripening. Part of the enzyme remained in the sample gel.

The results obtained with the B 1 variety were very similar to those described for Mv 4, with the exception that here the discrepancy between the patterns produced on the two substrates persisted until the fifth week.

The changes in amylase activity and moisture content during ripening of the two wheat varieties are shown in Fig. 4. During the first four (Mv 4) or five (B 1) weeks of ripening, an abrupt decrease in amylase activity took place. This coincided with the changes observed in the isoenzyme patterns and with the disappearance of the α-amylase fractions. During the second half-period of ripening, amylase activity showed only minor fluctuations for both varieties. This corresponds to the slight variations of the isoenzyme patterns obtained on the starch substrate.

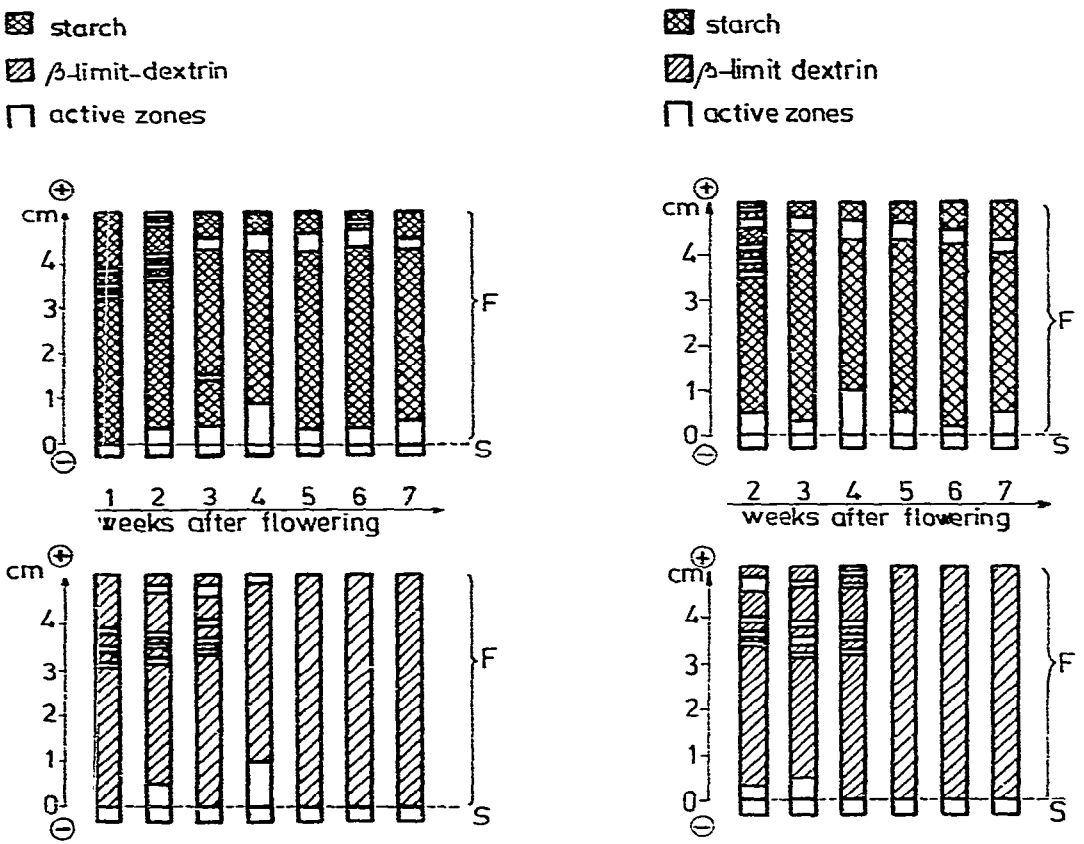

Fig. 2. Amylase isoenzyme patterns of the wheat variety Mv 4 as obtained with soluble starch and β-limit-dextrin substrates at different stages of ripening. Conditions of electrophoresis, symbols and abbreviations as in Fig. 1 (alkaline system), protein loads correspond to 20–80 mg of wheat (wet-mass basis) per gel tube. For the preparation of the enzyme solution and the detection of the isoenzymes on soluble starch substrate see Fig. 1. For the detection with β-limit-dextrin a 2% (w/v) solution of this compound in 0.1 *M* acetate buffer (pH 4.8) was used instead of soluble starch.

Fig. 3. Amylase isoenzyme patterns of the wheat variety B 1 as obtained with soluble starch and β-limit-dextrin substrates. Experimental conditions as in Fig. 2.

The results presented are partly borne out by data from the literature. Kruger[6] found, in several Canadian wheat varieties, three isoenzymes on β-limit-dextrin, detectable throughout the ripening period, which disappeared at the stage of maturity. These fractions might correspond to the triplet found in our experiments with β-limit-dextrin substrate. These, however, had disappeared before maturity was reached. Two major and five minor amylase active bands were detected with starch substrate in the ripening Canadian wheats, the band of the highest mobility being the major component. This latter finding is also in agreement with the results presented here. The difference in the number of isoenzymes detected in the Canadian and Hungarian wheat varieties might be due to differences in the pH of the electrophoretic systems or in the conditions of incubation. From our own findings as well as from those of Kruger[6] it seems that the influence of the variety on the amylase isoenzyme com-

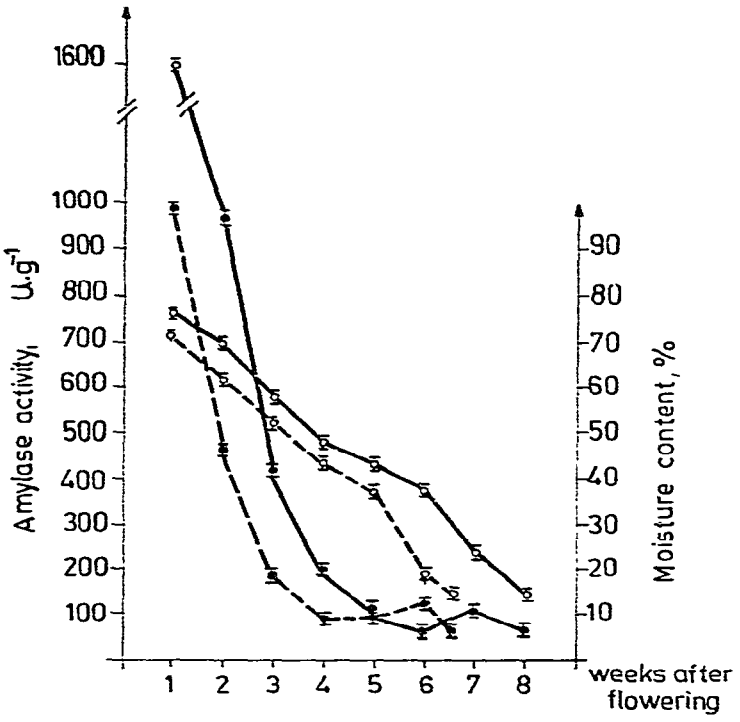

Fig. 4. Changes in amylase activities (wet-mass basis) (solid circles) and moisture contents (open circles) of the wheat varieties Mv 4 (broken line) and B 1 (full line) during ripening. Conditions of activity determination: Phadebas Amylase Test (Pharmacia) substrate, pH 7.0, 50°C. Unit enzyme activity (U): 10^{-3} A min^{-1} (A = absorbance at 620 nm)[5]. Vertical bars represent standard deviations.

position of wheat is of secondary importance as compared to the stage of ripening.

The changes in the amylase isoenzyme patterns of the two wheat varieties obtained on soluble starch substrate after various periods of storage are shown in Fig. 5. The number of isoenzymes was lowest at harvest and increased during storage. The upsurge of the new amylase-active fractions might be due to dissociation into smaller units of the part of the enzyme that had remained, in the earlier phases of development, in the sample gel.

The changes, during storage, in amylase ctivity and moisture content of the two wheat varieties are shown in Fig. 6. The moisture content of both varieties remained around 11.5% throughout the storage period. This value precluded sprouting. The amylase activity of B 1 varied only very slightly during the 10 months of storage, while with Mv 4 a slow decrease in activity during the first 6 months was followed by a slight increase during the rest of the storage period. Thus, in contrast to the ripening period, the changes in isoenzyme composition could not be related to the changes in amylase activity during storage. The relative invariability of the amylase activity during storage seems to corroborate the assumption that the simultaneous increase in the number of isoenzymes might be a consequence of molecular rearrangements of the enzyme and not the result of *de novo* protein synthesis.

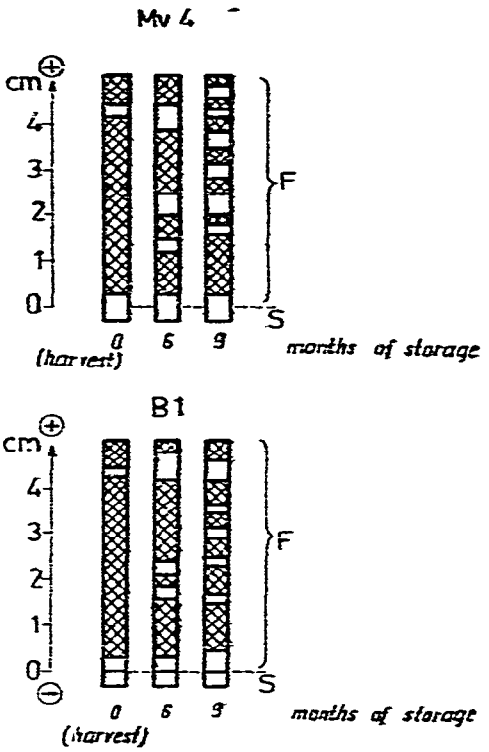

Fig. 5. Amylase isoenzyme patterns of the wheat varieties Mv 4 and B 1 after different storage times. Conditions of electrophoresis, symbols and abbreviations, preparation of enzyme extract and detection of isoenzymes as in Fig. 1. Protein loads per gel tube correspond to 40–120 mg wheat (wet-mass basis).

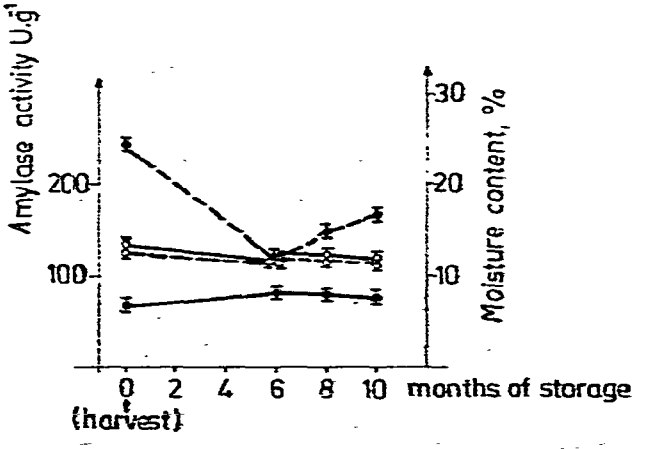

Fig. 6. Changes in amylase activities (wet-mass basis) (solid circles) and moisture contents (open circles) of the wheat varieties Mv 4 (broken line) and B 1 (full line) during storage. Conditions of activity measurements as for Fig. 4. Vertical bars represent standard deviations.

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