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Genetic polymorphism and quantitative variation of α-amylases from rye endosperm

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Abstract. α-Amylase isozymes from rye endosperm were analysed by means of isoelectric focusing, polyacrylamide gel electrophoresis, immunoelectrophoresis and colorimetric assay. α-AMY1 (high pI) group was separated into 13 IEF bands, whereas in group α-AMY2 (low pI) 2 intensive and 6-8 faint bands were found. Two linked (2±1 cM) polymorphic loci and a single locus with two alleles encoding for α-AMY1 and α-AMY2 groups, respectively, were identified after genetic analysis of the IEF patterns. All α-amylase isozymes developed on PAGE, were shown to belong to α-AMY1 group. It was demonstrated that a single PAGE isozyme corresponds to 2-4 separate IEF bands and that most of the IEF bands can be attributed to more than one PAGE isozyme. The activity of α-amylases from PAGE zone I was 2.3 times higher than the activity of zone II isozymes. A strong correlation between the activity and protein amount of particular α-AMY1 isozymes (r=0.94) was found.

Key words: α-amylase, immunoelectrophoresis, isozymes, *Secale cereale*

Isoelectric focusing (IEF) of α-amylase in germinating cereal grain revealed the existence of two groups of isozymes having high (α-AMY1) and low (α-AMY2) pIs (GALE et al. 1983, AINSWORTH et al. 1987, MACGREGOR et al. 1988). α-AMY1 isozymes, predominant in sprouting and germinating grain, were found to be encoded by a gene family on chromosomes of homoeologous group 6. α-AMY2 isozymes, present both in developing and germinating kernels, are controlled by genes from chromosomes of group 7 (LAZARUS et

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al. 1985). Recently, the third (α-AMY3) gene family has been detected on chromosomes of group 5, which produce isozymes during early stage of grain development (BAULCOMBE et al. 1987, DAUSSANT, RENARD 1987).

α-Amylase polymorphisms in wheat and barley were studied intensively by means of IEF (NISHIKAWA et al. 1981, GALE et al. 1983, MACGREGOR, BALLANCE 1980, MACGREGOR et al. 1988). In rye, two variants of polyacrylamide gel electrophoresis (PAGE) were applied to reveal α-amylase polymorphism. WAGENAAR, LUGTENBORG (1973) as well as KHOREVA et al. (1985) developed PAGE gels in starch solution. They found five α-amylase bands probably controlled by three structural genes. On the other hand, ŁAPIŃSKI and MASOJĆ (1983) resolved nineteen α-amylase bands using a modified method of MACKO et al. (1967), in which starch-containing PAGE gel was applied. This banding system was shown to be encoded by the three polymorphic structural genes and by a single independent gene producing endogenous α-amylase inhibitor (MASOJĆ 1987, MASOJĆ, GALE 1990). Relationship between the PAGE α-amylase patterns and the two groups of IEF isozymes is not clear. In addition, the IEF polymorphism of rye α-amylases has not yet been characterized.

Substantial α-amylase polymorphism raises the question about the existence of isozymes with significantly lower specific activities than the rest of the α-amylases. Detection of such forms would be important for the identification of sprouting resistant genotypes. This problem was approached by several authors, who compared catalytic properties of α-AMY1 and α-AMY2 groups (MACGREGOR 1980, MACGREGOR, MORGAN 1992). However, a comparison of the activities of individual α-AMY1 isozymes has not been done yet.

We report here on α-amylase polymorphism in rye identified by isoelectric focusing, its relationship with PAGE patterns and on the quantitative variation of individual α-AMY1 isozymes.

Material and methods

The studied material consisted of seven inbred lines of rye (S>10) and three inter-line F_2 crosses, developed at the Academy of Agriculture, Szczecin, Poland.

Crude α-amylase extracts were obtained from starchy endosperm of grain germinating for five days at a room temperature. Semi-liquid endosperm was ground in 0.1 ml of distilled water and centrifuged at 14 000 × g. PAGE in discontinuous buffer system was carried out in vertical gel slab apparatus,

according to the method described by MASOJĆ (1987). IEF separation was carried out in Multiphor II System (LKB) in pH range 5-8 for α-AMY1 and 3-6 for α-AMY2 (MASOJĆ, GALE 1990). β-Amylase isozymes were eliminated from the IEF patterns by heat treatment of the extracts (70°C for 10 min).

For the identification by IEF of particular PAGE isozymes, the PAGE gels were cut into strips, each containing separate isozyme. α-Amylases were extracted from the gel according to the method described by MACGREGOR and BALLANCE (1980). Individual isozymes, recovered from the PAGE gel, were applied on the IEF gel by means of sample applicators and run parallely with the crude endosperm extracts of the respective line. They were also applied into wells punched in the 1.2% agarose gel containing 0.1% rabbit polyclonal antibodies raised against α-AMY1 group of isozymes. The rocket immunoelectrophoresis was performed in 25 mM veronal buffer pH 8.6, according to the method described by LAURELL (1966). Agarose gel was incubated for 1 h in 1% solution of soluble starch and stained with iodine. The amount of α-amylase protein was proportional to the peak area (XLARKE, FREEMAN 1967), which was assessed by weighing individual peaks cut out from the photographic pictures.

α-Amylase activity was assayed by means of colorimetric Phadebas method (BARNES, BLAKENEY 1974), in which one unit (U) was equivalent to the amount of enzyme hydrolysing 1 mmol of the glucosidic bonds during 1 min at 37°C. All quantitative assays were made in two replications of which the mean values were given.

Genetic linkage was determined according to ALLARD (1956).

Results

IEF patterns

In all, thirteen bands of α-AMY1 group of isozymes were detected among seven inbred lines of rye (Fig. 1), which span the range of 5.6-6.5 pH. Only RXL10 and C599 lines showed similar patterns. The other lines exhibited differences in respect to the composition and the number of bands, which ranged from five (Ot0-25) to eight (620/75). Bands no. 3, 5, 6 and 10 were present in the majority of lines.

Allelic segregation of α-AMY1 isozymes was found between bands 1 and 7 in the F_2 cross Ot1-3 × RXL10 and between bands 2 and 3 in the F_2 cross DS2 × RXL10 (Table 1). Additionally, in the latter cross, a two-loci segregation

Table 1. F_2 segregations of α-amylase isozymes detected by IEF

Cross	α Amylase group	Locus	F_2 phenotypes	No. of pheno-types	$\chi^2_{1:2:1 \text{ or } 3:1}$	$\chi^2_{Linkage}$	P	SE
DS2×RXL10	α-Amy1	1	2	27	3.41			
			2, 3	45				
			3	15				
		2	7	59	2.39			
			null	28				
		1 and 2	2, 7	1		131.96*	0.02	0.01
			2, null	26				
			2, 3, 7	43				
			2, 3, null	2				
			3, 7	15				
			3, null	0				
Ot1-3×RXL10	α-Amy1	2	1	16	0.33			
			1, 7	38				
			7	18				
DS2×Ot0-25	α-Amy2	1	1, 3, 5	7	0.60			
			1, 2, 3, 4, 5, 6	17				
			2, 4, 6	6				

* Significant at p=0.05

between bands 2 and 3 and the band 7 was observed. The map distance between these two loci, calculated according to the equations developed by ALLARD (1956), was 2 ± 1 centimorgans. The genetic interpretation of the remaining α-AMY1 bands was difficult to obtain, due to their irregular segregation, variation of intensity and overlapping.

The α-AMY2 group of amylases found in a single inbred line consisted of one intensive band (no. 1 or 2) and a number of faint bands located between 3.5 and 4.7 pH (Fig. 2). Line 620/75 and variety Dańkowskie Złote had both intensive bands, which suggested heterogeneity of these materials. The segregation ratio found in F_2 cross DS2 × Ot0-25 suggested that two alleles at a single locus underlie the observed polymorphism (Table 1).

PAGE patterns

The PAGE zymograms of all seven lines had two intensive bands in zone I and one predominant band in zone II accompanied by faint diffuse bands (Fig. 3). They also revealed differences among the lines studied in the bands composition. All PAGE bands were earlier genetically identified as definite isozymes encoded by the three linked structural genes (MASOJĆ 1987, MASOJĆ, GALE 1991).

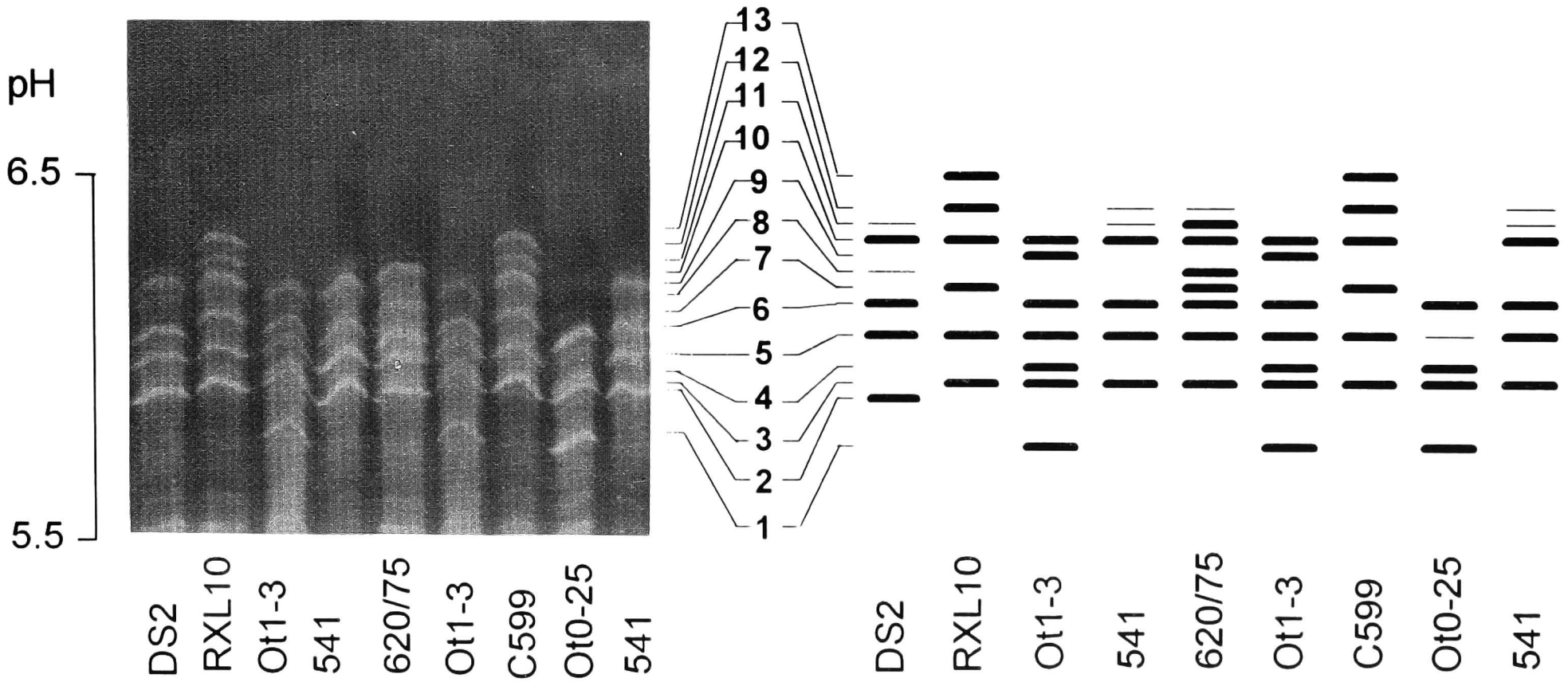

Fig. 1. High-pI (α-AMY1) group of isozymes. IEF patterns of the inbred lines of rye. Lines Ot1-3 and 541 are represented twice to facilitate bands identification.

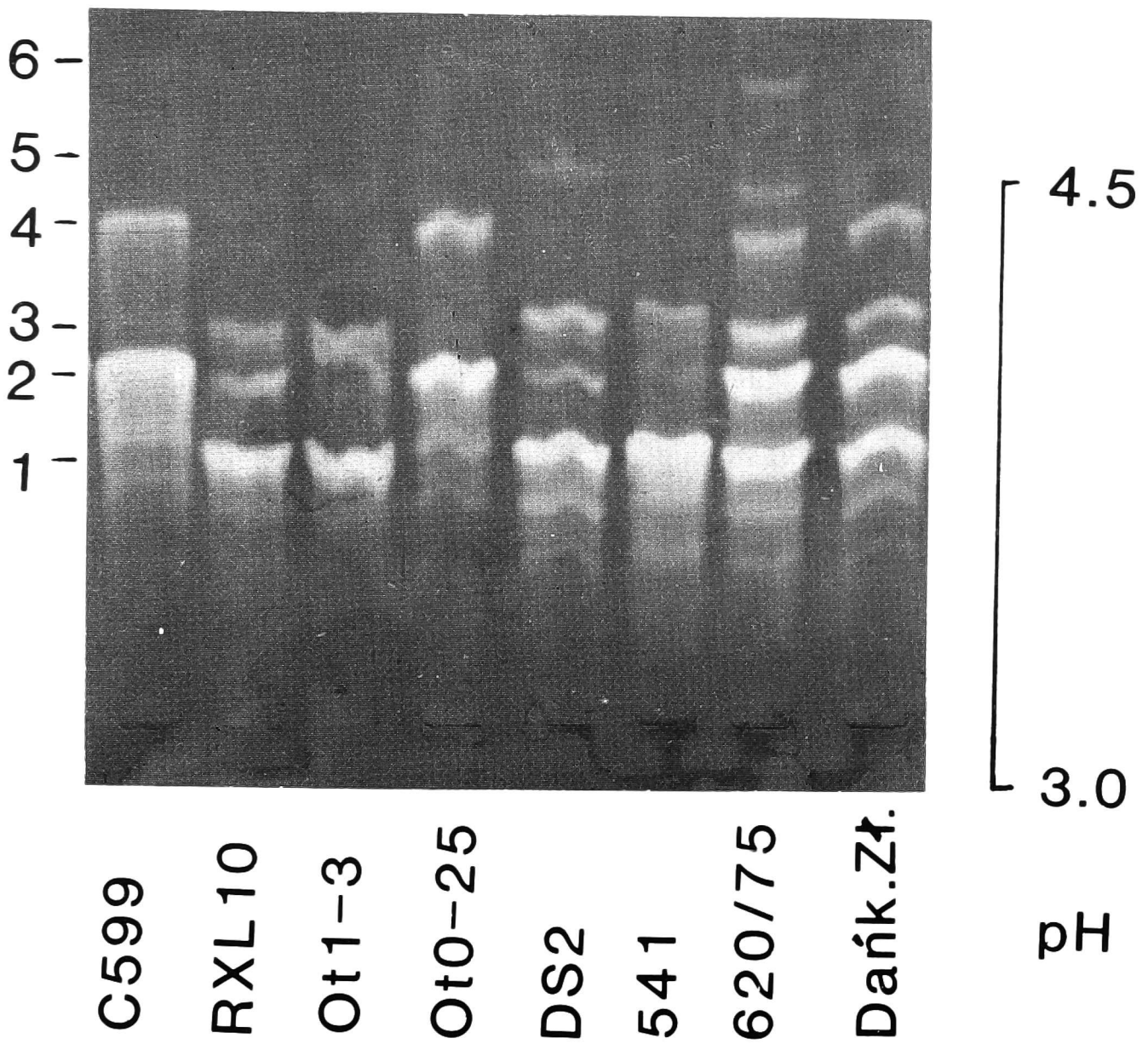

Fig. 2. Low-pI (α-AMY2) group of isozymes. IEF patterns of the inbred lines and cv. Dańkowskie Złote

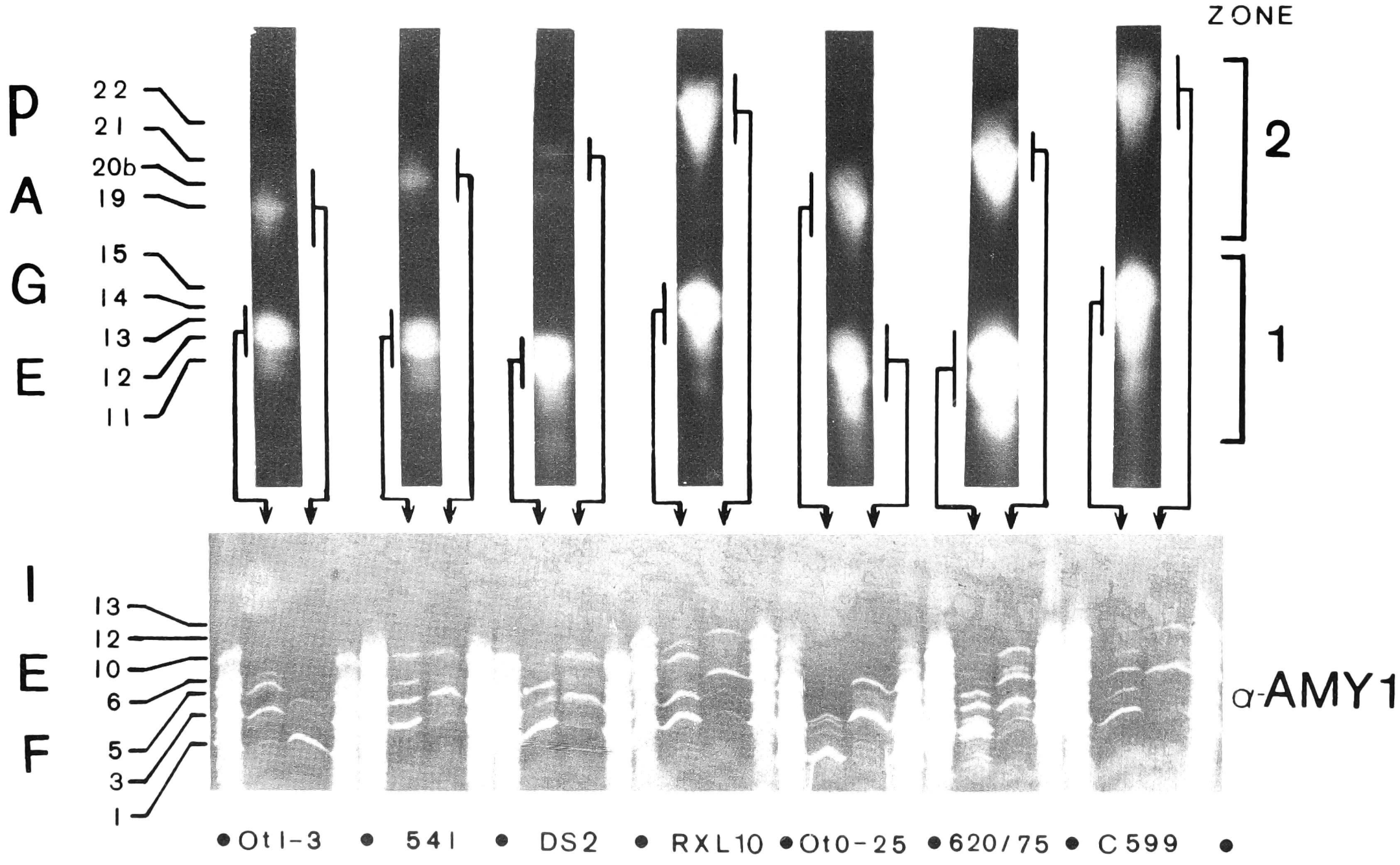

Fig. 3. Relationship between PAGE and IEF α-amylase forms determined by separation of individual PAGE isozymes on IEF gel. More intensive lanes are produced by crude endosperm extracts of each line

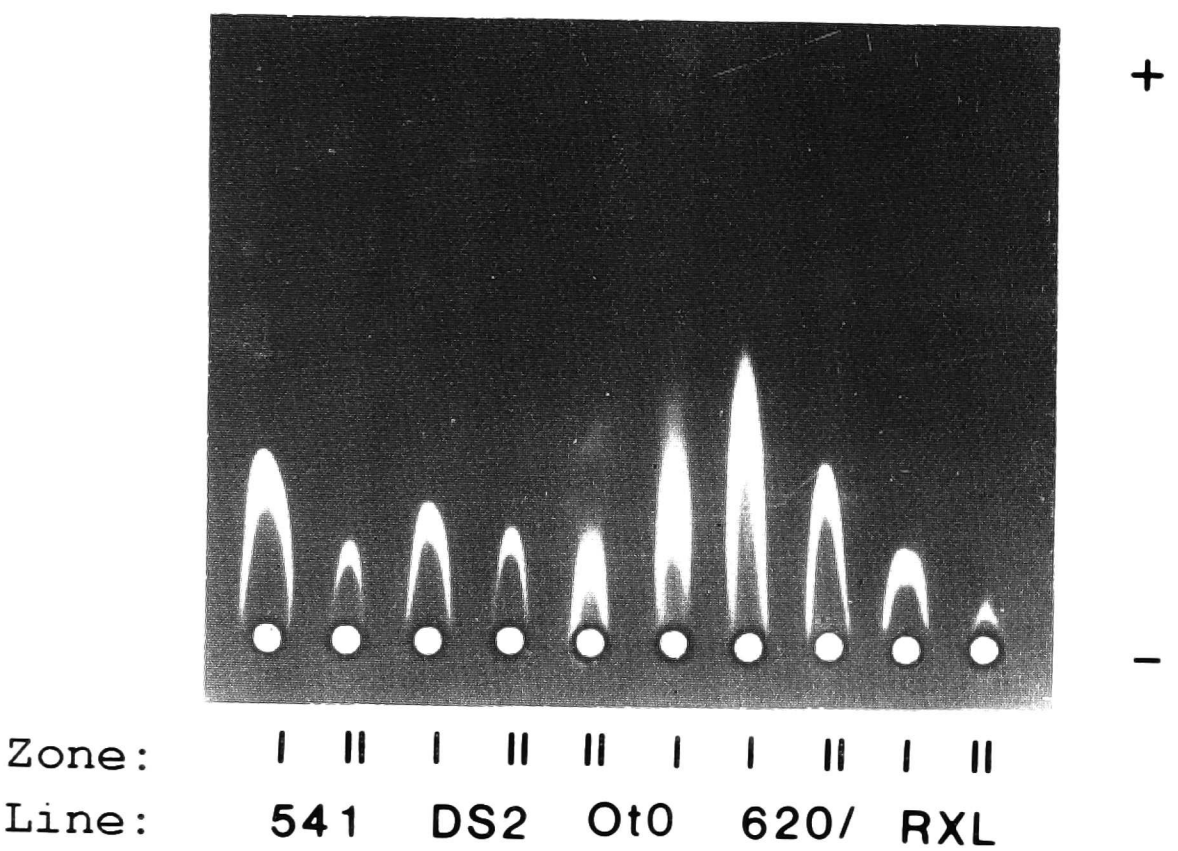

Fig. 4. A quantitative assessment of individual PAGE α-amylase isozymes performed by Laurell's rocket immunoelectrophoresis

The relationship between PAGE and IEF α-amylase isozymes

Each of the isozymes extracted from the PAGE gels was found to generate a part of the α-AMY1 group characteristic of the IEF pattern of a given line (Fig. 3). None of the PAGE isozymes generated α-AMY2 bands when separated on IEF. It was, therefore, concluded that all isozymes detected by means

Table 2. Relationship between α-AMY1 isozymes revealed by PAGE and IEF

Line	PAGE isozyme*	IEF isozyme
Ot1-3	12, 13 19	3, 5, 6, 10 1, 4
541	12, 13 20b	3, 5, 6, 10 5, 10
DS2	11, 12 21	2, 5, 6 5, 10
RXL10	14, 15 22	3, 5, 10, 12 7, 13
Ot0-25	11, 13 19	3, 5, 6 1, 3, 4
620/75	11, 13 21	2, 3, 5, 6, 8 5, 7, 8, 11
C599	14, 15 22	3, 5, 7, 10, 13 7, 13

* Bands are numbered according to MASOJĆ (1987)

Table 3. Activity and protein quantity of α-amylases separated by PAGE

Line	Zymogram zone	Isozymes	Activity (mU/ml)	Quantity (arbitr. units)
541	I II	8, 10b, 12a, 13 17, 20b	3019 825	460 170
DS2	I II	7, 10a, 11, 12b 18,21	1737 723	297 206
RXL10	I II	8, 10b, 14, 15 17, 22	637 324	225 98
Ot0-25	I II	7, 10b, 11, 13 16, 19	1033 896	343 234
620/75	I II	7, 8, 10b, 11, 12a, 13 18, 21	3440 1578	534 343
Correlation coefficient			r = 0.94*	

* Significant at p=0.05

of PAGE belong to the α-AMY1 group. The relationship between PAGE isozymes and IEF bands is given in Table 2. It is apparent that a single allele represented by one PAGE band gives a multiple-banded IEF pattern. In addition, most of the IEF bands can be attributed to different isozymes found on PAGE gel.

Activity of separate α-amylase isozymes revealed on PAGE gel

As assessed by Laurell's rocket immunoelectrophoresis (Fig. 4), the amount of zone I isozymes was 1.4-2.7 times higher than that found in zone II. The activity and quantity of particular isozymes were strongly correlated (Table 3), which indicates that possible differences in their specific activities were negligible.

Discussion

The results of this study showed that α-amylase resolved by PAGE and identified by MASOJĆ (1987) belongs to α-AMY1 group, which is predominant in germinating grain (MACGREGOR et al. 1988). The absence of α-AMY2 isozymes on PAGE zymograms was probably due to their low activity. Polymorphism of rye α-AMY2 isozymes, identified by means of IEF, was apparently under control of a single locus with two codominant alleles.

Unlike products of α-Amy 2 alleles, α-AMY1 isozymes were better resolved by PAGE in starch-containing gels. As explained by ROZHKOV (1983), the migration rate of α-amylase isozymes in the presence of starch is related to their affinity towards the substrate. It is thus possible that the observed differences in rye α-amylases positions on the PAGE zymogram reflect the affinity polymorphism of isozymes, which could account for their functional heterogeneity. This suggestion is supported by results of KHOREVA et al. (1985) who obtained only 5 closely migrating rye α-amylase bands after PAGE performed without starch.

Resolution of a single PAGE isozyme into several IEF bands, demonstrated in this study, might suggest the existence of more than three genes that encode for α-AMY1 group. However, the RFLP analysis of α-Amy1 sequences (MASOJĆ, GALE 1991) strongly supports a three-genes model. It, therefore, is more probable, that a substantial part of multiple α-amylase bands, revealed by IEF, are either artefacts or post-translational modifications, originating similarly as the majority of β-amylase multiple forms (SHARP et al. 1988).

As shown earlier (MASOJĆ 1987), α-amylase PAGE isozymes from zone I are governed by two tightly linked genes, whereas those from zone II are

products of alleles at a single locus. It can be assumed that, if the transcription rate in each gene is similar, the amount of zone I α-amylase should be two-fold higher than that of isozymes from zone II. A quantitative analysis carried out by Phadebas (activity) and rocket immunoelectrophoretic (protein amount) methods seems to support this hypothesis, as the average zone I : zone II α-amylase ratio was 2.3 and 1.8, respectively. A strong correlation between protein amount and activity of α-AMY1 isozymes found in this study shows, that α-amylase activity in rye grain is predominantly affected by the rate of its synthesis.

REFERENCES

AINSWORTH C.C., MILLER T.E., GALE M.D. (1987). α-Amylase and β-amylase homoeoloci in species related to wheat. Genet. Res., Camb. 49: 93-103.

ALLARD R.W. (1956). Formulas and tables to facilitate the calculation of recombination values in heredity. Hilgardia 24: 235-278.

BARNES W.C., BLAKENEY A.B. (1974). Determination of cereal alpha-amylase using a commercially available dye-labelled substrate. Die Starke 26: 193-197.

BAULCOMBE D.C., HUTTLY A.K., MARTIENSEN R.A., BARKER R.F., JARVIS M.G. (1987). A novel wheat α-amylase gene (α-Amy3). Mol. Gen. Genet. 209: 33-40.

CLARKE H.G.M., FREEMAN T. (1967). A quantitative immunoelectrophoresis method (H. Peeters ed.). Prot. Biol. Fluids 14, Elsevier, Amsterdam, pp. 503-509.

DAUSSANT J., RENARD H.A. (1987). Development of different α-amylase isozymes, having high and low isoelectric points during early stages of kernel development in wheat. J. Cereal Sci. 5: 13-21.

GALE M.D., LAW C.N., CHOJECKI A.J., KEMPTON R.A. (1983). Genetic control of α-amylase production in wheat. Theor. Appl. Genet. 64: 309-316.

KHOREVA V.I., KUDRYAKOVA N.N., KOBYLYANSKY V.D., LAPIKOV N.S. (1985). Genotypical variability of α-amylase in rye clones with various resistance to seed germination in ear. Agric. Biol. (Russ.) 2: 43-45.

LAZARUS C.M., BAULCOMBE D.C., MARTIENSSEN R.A. (1985). α-Amylase genes of wheat are two multigene families which are differentially expressed. Plant Mol. Biol. 5: 13-24.

LAURELL C.B. 1966. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal. Biochem. 15: 45-52.

ŁAPIŃSKI M., MASOJĆ P. (1983). Polymorphism of amylases from rye endosperm. 1. Variability of the electrophoretic patterns of amylases from inbred lines. Genet. Pol. 24: 131-137.

MACGREGOR A.W. (1980). Action of malt α-amylases on barley starch granules. MBAA Technical Quaterly 17: 215-221.

MACGREGOR A.W., BALLANCE D. (1980). Quantitative determination of α-amylase enzymes in germinated barley after separation by isoelectric focusing. J. Inst. Brew., London 86: 131-133.

MACGREGOR A.W., MARCHYLO B.A., KRUGER J.E. (1988). Multiple α-amylase components in germinated cereal grains determined by isoelectric focusing and chromatofocusing. Cereal Chem. 65: 326-333.

MACGREGOR A.W., MORGAN J.E. (1992). Determination of specific activities of malt α-amylases. J. Cereal Sci. 16: 267-277.

MACKO V., HONOLD G.R., STAHMANN M.A. (1967). Soluble proteins and multiple enzyme forms in early growth of wheat. Phytochem. 6: 465-471.

MASOJĆ P. (1987). Genetics of α-amylases from rye endosperm. Theor. Appl. Genet. 73: 440-444.

MASOJĆ P., GALE M.D. (1990). The factor modifying α-amylase isozyme pattern from rye endosperm is an endogenous α-amylase inhibitor. Hereditas 113: 151-155.

MASOJĆ P., GALE M.D. (1991). α-Amylase structural genes in rye. Theor. Appl. Genet. 82: 771-776.

NISHIKAWA K., FURUTA Y., HINA Y., YAMADA T. (1981). Genetic studies of α-amylase isozymes in wheat. 4. Genetic analyses in hexaploid wheat. Jpn. J. Genet. 56: 385-395.

ROZHKOV Y.I. (1983). Electrophoretic division of multiple enzyme forms in substrate-containing media. Agric. Biol. (Russ.) 1: 107-115.

SHARP P.J., KREIS M., SHEWRY P.R., GALE M.D. (1988). Location of β-amylase sequences in wheat and its relatives. Theor. Appl. Genet. 75: 286-290.

WAGENAAR S., LUGTENBORG T.F. (1973). α-Isoamylases of rye seeds. Phytochem. 12: 1243-1247.