The β-Amylase Polymorphism of Winter Common Wheat Grains

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Abstract—The polymorphism of winter common wheat with respect to β-amylase isoenzymes has been analyzed using electrophoresis in polyacrylamide gel (PAAG) buffered with a Tris–glycine system (pH 8.3). Seven β-amylase isoenzymes have been found in wheat varieties and the breeding stocks. Isoenzymes A, B, and C are the most frequent in Russian and Ukrainian varieties (51.7 ± 4.7, 30.7 ± 3.8, and 11.9 ± 2.5%, respectively). Two alleles of the β-*Amy-D1* locus of the long arm of chromosome 4D have been identified. The substrate–enzyme affine effect can be used to locate the zones of activity of this enzyme by means of staining for proteins. It has been determined that β-amylase isoenzymes may play a role in the aggregating capacity of the grain protein complex via the formation of S–S bonds.

Common wheat is known to be polymorphic with respect to grain β-amylases [1, 2]. For example, a study on more than 200 varieties of common wheat resulted in identification of six β-amylase zymotypes [1]. Rybalka (personal communication) used isoelectrofocusing of proteins of more than 400 winter and spring common wheat varieties to find 14 types of zymograms of this enzyme, whose number and frequency varied in different countries. Other researchers [2], on the basis of electrophoretic data, note that the number of allelic variants of wheat β-amylase is limited. On the other hand, it has been demonstrated [2–4] that these enzymes are capable of aggregating with endosperm storage proteins by forming S–S bonds.

The purposes of this study were to determine the β-amylase genetic polymorphism of winter common wheat grains by means of electrophoresis of β-amylases in polyacrylamide gel (PAAG) buffered with a Tris–glycine system (pH 8.3) and to estimate the number of disulfide bonds in the flour protein complexes that include different variants of this enzyme.

MATERIALS AND METHODS

The plant material consisted of winter common wheat varieties creaning in various breeding centers of Russia and Ukraine (Table 1) and breeding stocks intended for the competition and preliminary trials of the harvest of the year 2010 at the Belgorod State Research Institute of Agriculture (BelSRIA). To determine the number of genes controlling the synthesis of β-amylases, we used self-fertilizing populations of old generations of crosses (homozygous populations). With this taken into account, we estimated segregation in these populations according to the requirements for $F_{\to\infty}$ [5, 6]. To detect heterogeneity of the stocks of varieties, we analyzed at least three grains from each of them.

Vertical PAAG electrophoresis was performed on 190 × 105 × 1 mm plates. For this purpose, we used a device [7] manufactured in the Institute of Breeding and Genetics of the Ukrainian Academy of Agricultural Sciences (Odessa) allowing two gel plates of this size to be formed. β-Amylase was isolated from ripen grains preliminarily crushed with pliers. We added 250 μl of 3% Na_2SO_3 to each test tube containing crushed grains and left them for incubation over night. Then, the grains were ground with a stainless-steel rod, and the resultant suspension was centrifuged for 4 min at 10000 rpm. After that, 10 μl of the supernatant was transferred to a clean test tube, and 10 μl of a solution containing 2% β-mercaptoethanol, 40% sucrose, and 0.03% bromophenol blue was added. An extraction aliquot 5 μl in volume was placed to the starting gel. The conditions of electrophoresis and the composition of gel components have been described in detail for barley [8]. The separating gel contained 4.88 g of acrylamide, 130 mg of methylenebisacrylamide, 162.5 mg of Tris, 0.98 g of glycine, and 41 mg of ammonium persulfate dissolved in distilled water to a volume of 65 ml and 36 μl of TEMED. The electrode buffer solution (pH 8.3) contained 1.2 g of Tris and 5 g of glycine per liter. The electrophoresis was performed at a voltage of 300 V. The separation was terminated after the exit of 1.5 labels of the dye (1.5 h). Amylases were incubated in an acetate buffer solution (pH 5.7). The solution was made of 2.7 g of sodium acetate and 50.3 ml of 0.2 M acetic acid diluted with water to a volume of 300 ml,

Table 1. Winter common wheat varieties ($n = 88$) grouped according to the β-amylase zymotypes

β-Amylase zymotypes	List of winter common wheat varieties	Number of varieties	Frequency, %
A	BelNIISKh-1, Belgorodskaya 16, Belgorodskaya 19, Bogdanka, Vasilina, Vesnyanka, Vdala, Voyazh, Galina, Gubernator Dona, Dar Zernograda, Doka, Dolgushinka, Doskonala, Driada, Zimtra, Zolotokolosa, Zustrich, Kazanskaya 285, Kazanskaya 560, Kamyshanka 3, Kobra, Korochanka, Korotyshka, Malakhit, Moskovskaya 56, Nikolaevka, Odesskaya Krasnokolosaya, Ostistaya Belogor'ya,Patriarkh, Pisanka, Poshana, Prestizh, Svetoch, Severo-Donetskaya Yubileinaya, Skarbnitsa, Sluzhnitsa Odesskaya, Smuglyanka, Ukrainka Odesskaya, Kharus	40	45.5
A + *B*	Ariadna, BelNIISKh-2, Zarnitsa, List-25, Selyanka Odesskaya (tall), Sintetik	6	6.8
B	Avesta, Bezenchukskaya 380, Volzhskaya 100, Don 93, Donetskaya 48, Donskoi Mayak, Zernogradka 11, Kalach 60, Kuyal'nik, Konstantinovskaya, Liga-1, L'govskaya 4, Marafon, Mironovskaya 61, Moskovskaya 3251, Panna, Pervitsa, Rostovchanka 5, Selyanka odesskaya (nizkoros.), Stanichnaya, TM-04, Uni-1, Feya	23	26.2
C	Al'bidum 114, Al'borubrum (k-9051), Biryuza, Gospodynya, Zvonnitsa, Krastal, Monotip, Moskovskaya 2460, Odesskaya 200	9	10.2
A + *C*	Viktoriya Odesskaya, Kosovitsa	2	2.3
D	Vesta	1	1.1
A + *D*	Avgusta, Dashen'ka	2	2.3
A + *E*	Kristall	1	1.1
C + *E*	Krasota	1	1.1
B + *D*	Kamyshanka 2, Yuna	2	2.3
G + *H*	Snezhana	1	1.1

Note: *A* + *B* etc. are heterogeneous varieties carrying the corresponding β-amylase isozymes.

after which 3–5 g of hydrolyzed potato starch was added, and the suspension was heated to boiling while stirring. The incubation time was 30–40 min. All experiments were performed at room temperature. After the incubation, the gel plates were washed with running water and stained with iodine dissolved in potassium iodide solution, the final solution containing 2.5 g of KI, 1.3 g of crystal I, 25.2 g of trichloroacetic acid, and water to 500 ml.

The approach described earlier [9, 10] was used to estimate the degree of the protein complex aggregation via disulfide bonds.

The distribution of β-amylase isoenzymes among phenotypic classes was determined using the χ^2 test. The significance of the difference between the mean values was estimated using Student's *t* test [11, 12]. The frequency was evaluated according to Li [13].

RESULTS AND DISCUSSION

The analysis of 103 breeding stocks intended for the competition trial of the harvest of the year 2010 at the BelSRIA revealed seven β-amylase zymotypes (*A, B, C, D, F, G,* and *H*) (Fig. 1). Table 2 shows the frequencies of forms with these zymotypes. As evident from these data, the zymotypes considerably varied in frequency. The *A* zymotype was the most prevalent (about 68%). The next most prevalent was the *B* zymotype (more than 21%). The *C* zymotype ranked third in frequency (6%). Note that the same order of the frequencies of β-amylase variants was characteristic of Russian and Ukrainian varieties in general. The frequencies of other zymotypes were less than 4%. However, the frequency of the *A* zymotype in the Belgorod breeding stock was substantially higher than in varieties released in Russia and Ukraine (Table 2). This suggests a stronger selection in favor of the *A* variant of β-amylase in Belgorod compared to other regions of Russia and Ukraine.

Among the analyzed forms intended for the competition trial, we found 12 heterogeneous stocks representing homozygous populations with respect to genetic factors determining β-amylase isoenzymes. This accounted for 12% of all stocks and reflected the frequency of selection of heterozygotes for the genetic factors studied at early stages of breeding. Therefore, the original plants are mainly selected in F_4, because the expected frequency of heterozygotes in the F_4 self-fertilization generation is 12.5%. Note that there also

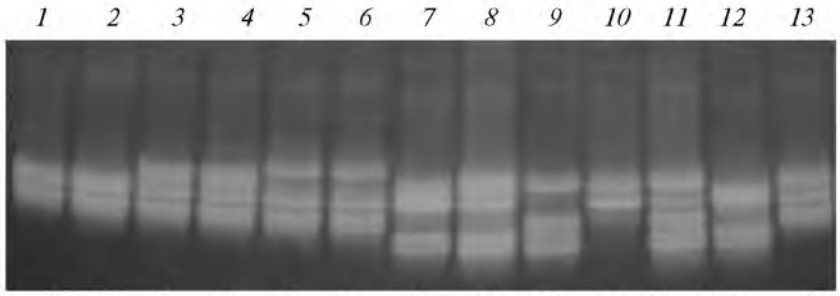

Fig. 1. Winter common wheat β-amylase zymotypes identified in varieties and breeding stock: *1*, Odesskaya 267; *2*, BelNIISKh 1; *3*, Feya; *4*, Volzhskaya 100; *5*, Zvonnitsa; *6*, Krastal; *7*, Kryzhinka; *8*, No. 45 KSI-10; *9–13*, No. 100 KSI-10 ($F_{\to\infty}$ No. 24/04 × Odesskaya Krasnokolosaya).

are heterogeneous forms (17%) among currently grown winter common wheat varieties (Table 1).

The analysis of the inheritance of the most common variants *A* and *B* (Fig. 2) showed that the differences between them were controlled by alleles of the same locus (Table 3). In the given case, we studied an $F_{\to\infty}$ homozygous self-fertilizing population represented by the BelNIISKh-2 cultivar. If the inheritance is monogenic, the expected segregation ratio in $F_{\to\infty}$ should be close to 1 : 1; if it is digenic, close to 1 : 3. The results shown in Table 3 confirm monogenic inheritance of the *A* and *B* zymotypes ($\chi^2_{1:1} = 2.04, p > 0.10$).

However, it remains unclear what chromosome is responsible for the synthesis of the *A*- and *B* variants of β-amylase. Studies on the chromosomal control of this enzyme in wheat employed other electrophoretic methods [1, 2, 14, 15]. For example, Rybalka and Sozinov [14] used isoelectrofocusing for separation of β-amylase to separate this enzyme in the Chinese Spring cultivar into two intense components with isoelectric points (pI) of 4.2 and 4.6. It was found that the pI 4.2 isozyme was controlled by the long arm of chromosome 4D; the pI 4.6 isozyme, by two independently inherited loci, one of which is located in the long arm of chromosome 4A. The long arm of chromosome 5A is also known to control β-amylase synthesis [2]. Therefore, the long arms of chromosomes 4A and 5A are responsible for the synthesis of the component with pI 4.6. The zymotype characteristic of Chinese Spring is the most prevalent among common wheat varieties of the former Soviet Union (Rybalka, personal communication). Judging by the frequencies (Tables 1 and 2), the *A* and *B* variants of β-amylase that we found in this study correspond to isoenzymes 1 (as in Chinese Spring) and 3 (with an additional component), respectively, detected using isoelectrofocusing. Genetic analysis demonstrated that the additional component was controlled by a gene allelic to one of the loci responsible for the synthesis of the pI 4.6 variant in Chinese Spring and/or Odesskaya 51 [14, 15]. These data may be interpreted in terms of our case. The lower double component indicated with the lower arrow on the left in Fig. 2 is invariant in both *A* and *B* zymotypes. Therefore, it should be controlled by the β-*Amy-D1* locus of chromosome 4DL in both of them. The upper double variant of enzyme *A* (Fig. 2, the

Table 2. Frequencies of β-amylase zymotypes in the winter wheat varieties and KSI-10 breeding stock of the BelSRIA of the Russian Academy of Agricultural Sciences ($n = 103$) and the collection of varieties grown in Russia and Ukraine ($n = 88$)

β-Amylase zymotypes	Frequency, %		Significance of differences in frequency, *t/p*
	in the BelSRIA breeding stocks ($n = 103$)	in Russian and Ukrainian varieties ($n = 88$)	
A	67.9 ± 4.7	51.7 ± 4.7	2.44/>0.95
B	21.4 ± 3.1	30.7 ± 3.8	1.89/<0.95
C	6.3 ± 1.7	11.9 ± 2.5	1.85/<0.95
D	3.4 ± 1.3	3.4 ± 1.4	–
E	0 ± 0.94	1.1 ± 0.8	–
F	0.5 ± 0.5	0 ± 1.1	–
G	0.5 ± 0.5	0.6 ± 0.6	–
H	0 ± 0.94	0.6 ± 0.6	–

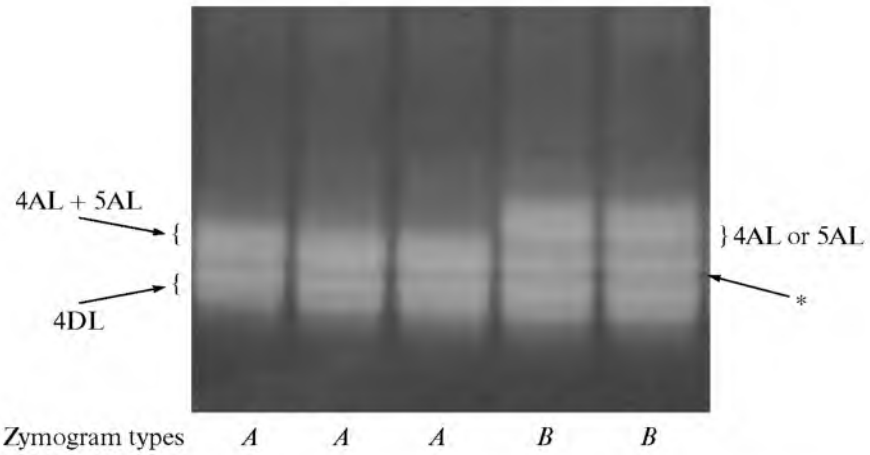

Fig. 2. β-Amylase zymotypes found in the self-fertilizing population $F_{\to\infty}$ BelNIISKh 2. The chromosomes responsible to the synthesis of these isoenzymes are indicated.

upper left arrow), as well as the pI 4.6 component [14, 15], is controlled by the alleles of loci of chromosomes 4AL and 5AL. The electrophoretogram of the *B* zymotype contains a single component in the middle zone instead of the double component characteristic of the *A* variant (Fig. 2, the arrow with an asterisk); instead of the other component, it contains a double component with a lower mobility (Fig. 2, indicated with a curly bracket on the right). Since the chromosomal localization of the gene encoding the two components near pI 4.6 in zymotypes of type 3 [14, 15] has not been determined, the localization of the gene(s) controlling the components of the *B* zymotype indicated with the curly bracket and the arrow with an asterisk remain unknown. Obviously, each of them is controlled by one of the loci of chromosome 4AL or 5AL.

The monogenic inheritance of the two polypeptides of the lower part of variant *A* and, hence, their control b an allele of the β-*Amy-D1* locus are confirmed by the results of analysis of the $F_{\to\infty}$ (Odesskaya 267 × Kryzhinka) (No. 74 KSI-10) population. Figure 3 shows the zymotypes of β-amylases found in this self-fertilizing population of winter common wheat. Odesskaya 267 has the *A* variant of the enzyme; Kryzhinka has the *H* variant. The material studied contained grains with the *A* and *H* variants and heterozygous grains containing both zymotypes (*A* + *H*) (Fig. 3). Statistical analysis has shown that the population studied is the F_4 offspring characterized by monogenic inheritance of polypeptides in the rapidly moving region of the zymogram (Table 4). This conclusion is confirmed by data shown in Table 5, where only homozygous classes are presented. It is conceivable that the higher χ^2 compared to the expected value indicates that natural selection favors the *H* isoenzyme. Thus, the double components in the lower part of the *A* and *H* zymograms are controlled by alleles of the same locus β-*Amy-D1*. The polypeptides in the upper part of the zymograms of grain β-amylases did not segregate, their pattern being identical in both parents.

The zymograms of β-amylases cannot be stored for long, because the gel fades as its loses iodine. However, if, after the zymogram is developed, the gel is put into the standard Coumassie dye for protein components [16], protein zones exhibiting the amylase activity are stained and are visible in the gel after washing (Fig. 4). As can be seen in Fig. 4, the protein spectrs (Fig. 4, the lower panel) entire correspond to the zymograms (Fig. 4, the upper panel). In the given case, the affine effect of enzyme–substrate binding is used. As a result, the enzyme is not washed off the gel and is visible as the corresponding protein components after staining. This technique employing the affine effect was earlier used to reveal the zones of peroxidase activity [17].

Table 3. Inheritance of β-amylase variants in the $F_{\to\infty}$ BelNIISKh 2 population

β-Amylase phenotypes		Expected segregation in $F_{\to\infty}$			
		monogenic inheritance 1 : 1		digenic inheritance 3 : 1	
A	*B*	χ^2	*p*	χ^2	*p*
55	41	2.04	>0.10	16.05	<0.01

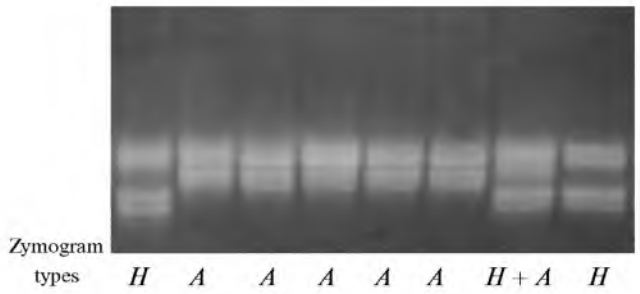

Fig. 3. Types of zymograms of endosperm β-amylases in population No. 74 KSI-10 ($F_{\to\infty}$ Odesskaya 267 × Kryzhinka).

The gel plate treated in this way and then dried can be stored for a long time.

It is known that β-amylases of a ripe grain are aggregated into the endosperm protein complex by means of S–S bonds [2–4]. Therefore, we estimated the number of intermolecular disulfide bonds in the flour protein complex as described earlier [9, 10]. Taking into account that this parameter depends on the weather in each particular year [18], we used plant material from the harvest of the year 2008, whose climatic parameters were close to those averaged over many years. In addition, we excluded from analysis the wheat varieties with the *1RS.1BL* and *1RS.1AL* translocations from rye, because the presence of the rye proteins controlled by the translocated rye material drastically decreases the aggregating capacity of the protein complex in the years when the weather was typical of Belgorod region. In hot years (2009 and 2010), the differences between varieties in the number of intermolecular disulfide bonds in the endosperm peptide complex leveled out. We studied two groups of stocks with the *A* and *B* zymotypes (Table 6) controlled by alleles of one of the loci of chromosome 4L or 5L. Calculations showed that, in the case of independent samples, the differences in the number of disulfide bonds of the flour peptide complex between the samples studied were significant. As evident from our data, the protein aggregating capacity in grains with the most common *A* zymotype of β-amylase was higher than in grains with the *B* variant. It is possible that this

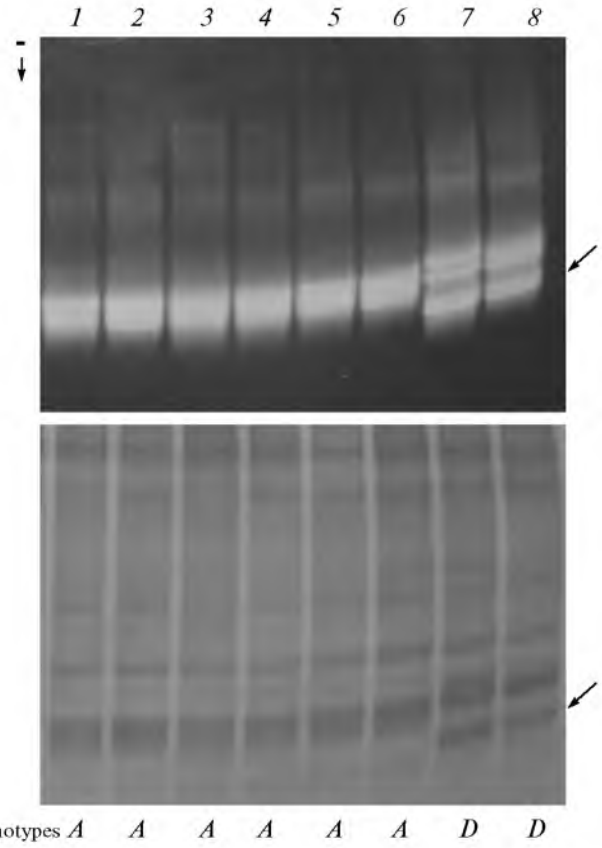

Fig. 4. Zymograms and electrophoretograms of common wheat β-amylases: *1–3*, No. 43 KSI-10; *4–6*, No. 44 KSI-10; *7, 8*, No. 45 KSI-10.

is mainly related to the artificial selection during wheat breeding for baking purposes.

Thus, we found β-amylase polymorphism of winter common wheat grains. It has been shown that different variants of the enzyme are unevenly distributed among varieties bred in Russia and Ukraine, as well as in the breeding stock. We identified two alleles of the β-*Amy-D1* locus of the long arm of chromosome 4D. The use of the substrate–enzyme affine effect allows the zones of activity of this enzyme to be detected by means of staining for proteins. It has been found that β-amylase isoenzymes may play a role in the aggregat-

Table 4. Segregation of β-amylase isoenzymes in population No. 74 KSI-10 (=$F_{\to\infty}$ Odesskaya 267 × Kryzhinka)

β-Amylase phenotypes			Self-fertilization generation and expected segregation in the case of monogenic inheritance		
H	*H* + *A*	*A*	$F_3\ \chi^2_{3:2:3}$	$F_4\ \chi^2_{7:2:7}$	$F_5\ \chi^2_{15:2:15}$
133	28	94	36.10	7.35	16.10
			$p < 0.01$	$0.05 > p > 0.025$	$p < 0.01$

Table 5. Inheritance of β-amylase variants in population No. 74 KSI-10 ($F_{\to\infty}$ Odesskaya 267 × Kryzhinka)

Homozygous phenotypic classes		Expected segregation in $F_{\to\infty}$			
		monogenic inheritance 1 : 1		digenic inheritance 3 : 1	
H	*A*	χ^2	*p*	χ^2	*p*
133	94	6.70	<0.01	32.6	<0.01

Table 6. The degree of aggregation of the flour protein complex of winter common wheat via disulfide bonds in stocks with different β-amylase zymotypes (KSI-10)

β-Amylase zymotypes	Number of samples	Number of disulfide bonds of the protein complex, ml/% dry gluten (ΔSDS)	t_{exp}	$t_{0.05}$
A	25	4.26 ± 0.20	1.42	1.03
B	7	3.56 ± 0.45		

ing capacity of the grain protein complex via the formation of S–S bonds.

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