Polymorphism for α -amylase in Germinating Seed and Malt of Barley Varieties Detected by Isoelectric-focusing Gel Electrophoresis

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The presence of polymorphism for α -amylase in germinating barley seeds and in malt in 130 barley varieties was examined by means of isoelectric-focusing gel electrophoresis. These varieties were classified into three types according to the banding patterns of the zymograms that were constantly obtained both in germinating seeds and in malt irrespective of pH of the buffer. Most of Japanese two-rowed varieties have specific banding patterns of the zymograms, i.e. they fall into type-1 and type-3, whereas almost all foreign two-rowed varieties fall into type-2 and type-3. This classification does not agree with the classification based on agar gel zone electrophoresis.

KEY WORDS : *Hordeum vulgare*, barley, α -amylase, germinating seed, malt, isoelectric-focusing, varietal differences.

Introduction

One important objective in the breeding of malting barley is to improve the α -amylase activity during germination.

Using agar gel zone electrophoresis, FRYDENBERG and NIELSEN (1966) revealed the polymorphic nature of α -amylase in barley. They showed that the co-dominant alleles for α -amylase are located on chromosome 6 (NIELSEN and FRYDENBERG 1971, 1974). With isoelectric-focusing on polyacrylamide disc gels, MOMOTANI and KATO (1970) obtained highly reproducible α -amylase zymograms containing 18 bands. Their result indicated the presence of varietal differences in zymographic patterns of barley α -amylase. MACGREGOR (1978) also reported varietal differences of α -amylase isozymes when applying isoelectric-focusing.

BROWN and JACOBSEN (1982), using wheat-barley addition lines, demonstrated that α -amylase isozymes detected by isoelectric-focusing were divided into two groups based on the isoelectric point and that the genes for these groups were located on chromosomes 1 and 6 respectively.

The aims of this study were firstly to clarify the differences of zymographic patterns for α -amylase between germinating seed and malt, and secondly to explain the varietal differences by means of isoelectric-focusing.

Materials and Methods

One hundred and twelve varieties of two-rowed and 18 varieties of six-rowed barley (*Hordeum vulgare* L.) were used.

Seeds were soaked in sodium hypochlorite solution (about 3.3%) for 30 minutes and then rinsed with sterile water. The seeds were germinated in darkness at 15°C in sterilized petri-dishes (9 cm diameter) on two pieces of filter paper that had been moistened with 5 ml of sterile distilled water. On the 6th day, the germinating seeds were used

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for analysis after plumules and rootlets were removed.

Malt and green malt of 42 varieties, produced by the equipment for micro-malting (TAKEDA *et al.* 1981), were also examined.

Two sorts of buffer (0.01 M Tris-HCl buffer, pH 8.5 and 0.01 M acetate buffer, pH 4.7) were used for enzyme extraction to investigate the effect of pH on the heat stability of α -amylase, since it had been reported that heat stability may differ because of differences of buffer (MOMOTANI 1970, MACGREGOR 1978).

Germinating seeds and green malt were ground in buffer (0.5 ml/kernel). After well stirred into buffer (10 ml/0.2 g of ground malt), the samples were incubated for 1 hour at 40°C. Then the vials with the samples were centrifuged at 25000 g

for 15 minutes. The aliquots of the supernatant were incubated at 70°C for 15 minutes and filtered. The filtrates were used for isoelectric-focusing.

Isoelectric-focusing was carried out according to the method of MOMOTANI (1970) using thin-layer polyacrylamide gel (60 mm $\times$ 100 mm $\times$ 1 mm) containing Ampholine (pH 5-8 LKB). Electrophoresis was performed at 200 V for the first 45 minutes, at 400 V for next 150 minutes, and at 500 V for the last 45 minutes.

The zymograms were detected by the method of MOMOTANI (1970). The electrophocused gel was brought in full contact with an acrylamide thin film containing 2% soluble starch attached to a glass plate. After incubation for 30 minutes at 37°C the acrylamide film was stained with I₂-KI solution. Zones containing α -amylase activity appeared as decolorized bands on a blue background.

Having been stained, the zymograms were scanned with a densitometer. The densities of the bands were divided into 7 classes based on the height of the densitometric peaks, with some modification (i.e. the band with class value $n+1$ has twice as much α -amylase activity as the band with class value n).

Results

In an analysis of zymograms obtained by isoelectric-focusing, we detected 18 bands for germinating seeds and 13 bands for malt. Although the number of bands differed among the varieties, 21 discernible bands were found between pI 5.0 and 7.0. They were numbered from cathode to anode. Fig.1 shows the zymogram of α -amylase in germinating seeds of three varieties extracted with Tris-HCl buffer and acetate buffer. Bands 2, 3, 4 and 12 were common to the zymograms of all variety. It appeared that pH of the buffer

Fig. 1. Zymograms of α -amylase in germinating seeds of three varieties extracted with Tris-HCl buffer (pH 8.5) or acetate buffer (pH 4.7).

Plate 1, 2; Variety 'Haruna Nijo' (type 1),
3, 4; variety 'Klages' (type 2),
5, 6; variety 'Amagi Nijo' (type 3).
1, 3, 5; Extracated with Tris-HCl buffer,
2, 4, 6; extracted with acetate buffer.

¹⁾ Class value; see 'Materials and Methods'.

Fig. 2. Zymograms of α -amylase in the variety 'Amagi Nijo'.
 Plate 1, 5; Germinating seed extracted with Tris-HCl buffer,
 2, 6; germinating seed extracted with acetate buffer,
 3, 7; green malt extracted with Tris-HCl buffer,
 4, 8; malt extracted with Tris-HCl buffer.

for enzyme extraction affected the zymogram of germinating seed for each variety (Fig 1). That is, the high pI bands (band 17-21) and bands 1, 5, 8, which were detected in the zymograms of the Tris-HCl buffer extract, were absent in the zymogram of the acetate buffer extract.

Fig. 2 shows the zymograms of α -amylase in germinating seeds, green malt, and malt (variety 'Amagi Nijo') extracted with two sorts of buffer. The zymogram of green malt (before kilning) was identical with that of germinating seed. On the contrary, the zymogram of malt (after kilning) was quite different from that of germinating seeds. The high pI bands (above band 14) observed in the zymogram of germinating seed with Tris-HCl buffer could not be detected. In this case effect of buffers on zymograms could not be seen (data is not shown). On the other hand, band 1 of malt was clearer than that of germinating seed. Moreover, in the pI 6.0 region, several bands were detected in the zymogram of malt which were not detected in that of germinating seed.

Table 1. Comparison between germinating seed and malt in zymograms of α -amylase

	Type	Variety	Band															
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Germinating seed	1	Haruna Nijo			5	2						7	7	7	4	6		
	2	Klages			5	2								7		7	7	5
	3	Amagi Nijo			5	2							7	7	5	6		
	3	UM 570			5	2							7	7	5	6		
	3	Sandra			5	2							7	7	5	6		
Malt	1	Haruna Nijo	3		3		3		3	4	5	5	7	7				
	2	Klages	3		3		2	4		4	4			7		5	5	5
	3	Amagi Nijo	3		3		3	2		4	4		7	7				
	3	UM 570	3		3		2	3		4	4		7	7				
	3	Sandra	3		3		2	3		4	3		7	7	2			

Note : Numerals in table show the class values (see 'Materials and Methods') of each bands.

The classification of the varieties in this study by means of isoelectric-focusing

The classification in FRYDENBERG and NIELSEN (1966)

Fig. 3. Comparison between the classification of the varieties in this study by means of isoelectric-focusing and the classification in FRYDENBERG and NIELSEN (1966) by means of agar gel zone electrophoresis.

plate 1; Variety 'Golden Melon' (control),
2; variety 'Domen', 3; variety 'Kenia',
4; variety 'Zephyr', 5; variety 'Carlsberg II',
6; variety 'Spartan'.

The major bands (band 9-16) in the zymogram of germinating seed extracted with acetate buffer, showed varietal differences. As shown in Fig. 1, bands 10-14 were dominant in the zymogram of 'Haruna Nijo'. On the contrary, bands 12-15 were dominant in the zymogram of 'Klages'. Bands 11-14 were dominant in the zymogram of 'Amagi Nijo'. These three types were easily discernible by the presence or absence of band 10 and band 11. The varieties of type-1 had both band 10 and band 11, the varieties of type-2 had neither band, and the varieties of type-3 had only band 11.

In Table 1 the zymograms for malt of five varieties are compared with those for ger-

Table 2. Varieties classified into three types according to the banding pattern of α -amylase zymograms by means of isoelectric-focusing

Varieties (two-rowed)			
TYPE-1			
Aichi Wase 13 ¹⁾	Hakata 2 ¹⁾	Kanto Nakate Gol ¹⁾	Kou A ¹⁾
Asahi 19 ¹⁾	Haruna Nijo ¹⁾	Kawa Honami ¹⁾	Kou 1-18 ¹⁾
Azuma Golden ¹⁾	Hatakaze ¹⁾	Kawa Mizuki ¹⁾	New Golden ¹⁾
Ebisu ¹⁾	Hokuiku 18 ²⁾	Kawa Saigoku ¹⁾	Nitta Nijo ¹⁾
Golden Melon	K 3 ¹⁾	Kirin Choku 1 ¹⁾	
TYPE-2			
Ametyst	Hector	Mazurka	Ris ϕ 1508
Betzes	Hoshimasari ²⁾	Nackta	Roxane
Carmen	Imber	Pallas	Shabet
Clipper	Kinia	Porthos	Shunsei ²⁾
Emir	Kimberly	Proctor	Waxy Betzes
Ershabet	Klages	Rapid	
Hassan	Mala	Rika	
TYPE-3			
Akagi Nijo ¹⁾	Elgina	Midas	Sundance
Amagi Nijo ¹⁾	Elsa	Miho Golden ¹⁾	Svanhals
Ant-13	Favorit	Moravian	Tern
Aramir	Fuji Nijo ¹⁾	Morex	Topas
Ark Royal	Foma	Nijo Mochimugi ¹⁾	Triumph
Aura	Hanna	Nissei ²⁾	Tyra
Birgitta	Hannchen	Nordal	Una
Breuns Wisa	Hiproly	Oriol	Union
Canova	Hydra	Pirolina	Vanguard
Carina	Igri	Prior	Villa
Carlsberg II	Ingrid	Research	Volla
Columba	Julia	Sandra	Weeah
Copelia	Keg	Satsuki Nijo ¹⁾	Wing
Dampier	Koral	Seijo 1 ¹⁾	Wisa
Dana	Lara	Seijo 17 ¹⁾	Ymer
Denar	Malta	Sonja	Zephyr
Domen	Maris Otter	Spartan	
Varieties (six rowed)			
TYPE-1			
Texan			
TYPE-3			
Beacon	Hashiri Hadaka ¹⁾	Manriki ¹⁾	UM 570
Beecher	Hoppel	Musashino Mugi ¹⁾	Yuunagi Hadaka ¹⁾
Benkei Mugi ¹⁾	Houten Kuro	Nanpuu Hadaka ¹⁾	
Dickson	Iwata Santoku ¹⁾	Suifu ¹⁾	
Doriru Mugi ¹⁾	Darl	Svan	

¹⁾ Winter varieties bred in Japan²⁾ Spring varieties bred in Japan

minating seeds. In the zymograms of malt, the bands moved toward the region of low pI. However, if the varieties were classified into three types by the presence or absence of band 10 and 11 just as for germinating seed, the type of each variety is the same as that for germinating seed. This similarity between the zymograms of germinating seed and malt was confirmed by examining 42 varieties. This indicates that these classification can be utilized as a standard of identification of barley varieties with the zymograms both of germinating seed and of malt.

All the varieties used in this study were classified into the three types (Table 2). Two-rowed barley varieties in Japan were classified into type-1 and type-3 except 'Hoshimasari' and 'Shunsei' which belonged to type-2. Two-rowed barley varieties bred in the other countries were classified into type-2 and type-3, except 'Golden Melon' which belonged to type-1. On the contrary, all six-rowed barley varieties were type-3 with the exception of 'Texan' from U. S. A. which belonged to type-1.

The zymogram of a part of the varieties that had been investigated before with agar-gel electrophoresis by FRYDENBERG and NIELSEN (1966, 1969), are shown in Fig 3. 'Domen' and 'Kenia' were classified in the first group, 'Zephyr' and 'Carlsberg II' in the second, and 'Spartan' in the third. The materials used in this study were classified in the same way explained above, which was reconfirmed by using the method of FRYDENBERG and NIELSEN (1966). However, by means of isoelectric-focusing in this study only 'Kenia' was classified in type-2, whereas the other varieties were all classified in type-3.

In bands 1-4, varietal differences were not found in this study. Bands 5-7 in malt and bands 17-21 in germinating seeds have not been investigated yet in detail.

Discussion

MACGREGOR and DAUSSANT (1979) reported that α -amylase separated with isoelectric-focusing in germinating barley seeds consisted of three groups, α -amylase I, II and III. Furthermore, MACGREGOR and BALLANCE (1980) and MACGREGOR and DAUSSANT (1981) showed that α -amylase III converted into α -amylase II during heat treatment and that α -amylase II was relatively heat stable and was hardly influenced by pH during enzyme extraction. Comparing their results with ours, we can conclude that α -amylase I, II and III roughly correspond to bands 1-4, bands 5-16 and bands 17-21, respectively. In the present experiments, there were no varietal differences in α -amylase I. The main bands which were regarded as members of α -amylase II and used for classification of the varieties in this study are present also in the zymograms both of germinating seed and malt. Therefore it is argued that both of the zymograms for germinating seeds and for malt are utilized to classify varieties irrespective of the methods for enzyme extraction. This result may be useful for the breeding of malting barley. According to the result of BROWN and JACOBSEN (1982), the bands that were used for classification of the varieties in this study may be controlled by the gene located on chromosome 6.

The result of our classification does not agree with that of FRYDENBERG and NIELSEN (1966), possibly because the method of electrophoresis was different. They revealed that their classification was based on the allelic difference of co-dominant genes on a locus located on chromosome 6 (FRYDENBERG and NIELSEN 1969, NIELSEN and FRYDENBERG 1971,

1974). The relationship between the results of agar gel zone electrophoresis and that of isoelectric-focusing should be examined.

In our classification the major two-rowed barley varieties bred in Japan were separated into type-1 and type-3. It is known that two-rowed barley was introduced into Japan in the 1870's in the first place for malting purposes. Since then the breeding of two-rowed barley for malting has been done by selecting the favorable varieties, by carrying out hybridization between two-rowed barley varieties and sometimes crossing with the Japanese indigenous six-rowed barley varieties which had desirable characteristics for cultivation (MEGURO 1971). It has been said that among the varieties introduced from abroad, 'Golden Melon' whose origin is not clear (TAKAHASHI¹⁾ 1980) and 'Australian Chevalier' were frequently used as breeding materials and that many Japanese varieties were derived from them. Our experiment revealed that 'Golden Melon' has the type-1 zymogram, and 'Australian Chevalier' ('Prior') has the type-3 zymogram. We may say that Japanese two-rowed winter barley varieties were divided into 'Golden Melon type' and 'Australian Chevalier type' from the feature of the zymograms. In this study, it is not yet clear whether there are any relation between the zymogram types and the malting qualities. There were no type-2 varieties in Japanese two-rowed winter varieties. It is however well known that type-2 varieties as 'Klages', 'Imber' and 'Clipper' have good malting qualities. On the other hand, there were no type-1 foreign varieties except two varieties, although type-1 Japanese variety as 'Haruna Nijo' has generally excellent malting qualities (AIDA 1979). Therefore it will be necessary to clarify the genotypic features of the varieties in three types and their relation with the other characters.

Six-rowed barley varieties used as materials were only 18 varieties among which 17 varieties showed type-3 zymograms. It will be necessary to examine more varieties for clarifying the general feature of the zymograms in six-rowed varieties.

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等電点焦点法による、オオムギ発芽種子および麦芽の
 α -アミラーゼの酵素多型

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オオムギ α -アミラーゼ活性の遺伝的制御を明らかにする目的の1つとして、発芽種子および麦芽抽出液の α -アミラーゼを等電点焦点法によって分離し、酵素多型の品種間差異を比較検討した。発芽種子 130 品種 (うち、二条大麦品種 118)、麦芽 60 品種のザイモグラムから総計 21 本のバンドを検出した。発芽種子では酵素抽出用緩衝液の pH によってザイモグラムに変化が見られ、pH 8.5 の時に存在した等電点の高いバンド群を、pH 4.7 の場合には検出できなかった。一方、麦芽では緩衝液 pH によるザイモグラムの変化は見られず、等電点の高いバンド群は常に存在しなかった。また麦芽では等電点 6.0 附近に発芽種子では存在しないバンドが検出された。

発芽種子および麦芽のザイモグラムには明らかな品種間差異があった。ザイモグラムは主要なバンドの有無によって3種類のタイプに類別することができ、それによって供試品種をそれぞれ3群に分けることができた。発芽種子と麦芽のザイモグラムの類別は両者で一致していた。しかし、FRYDENBERG ら (1966) の寒天ゲル電気泳動による類別の結果とは一致していなかった。

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