Characteristics of α -Amylase Induced in Distal Half-grains of Wheat

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α -Amylase activity was measured in distal half-grains of four wheat lines, RL4137, OS21-5, Shirogane-komugi (Shirogane) and Hokkai250, after 72 h soaking at 20°C. The half-grains of RL4137 and Hokkai250 at the maturity stage (about 15% water content) showed a significantly ($P < 0.01$) higher α -amylase activity after 72 h soaking (3.95 mU/mg, and 2.14 mU/mg, respectively) than those of OS21-5 and Shirogane (1.05 mU/mg and 0.91 mU/mg). During grain development, 30 to 50 days postanthesis (DPA), soaking also induced the production of α -amylase in the half-grains of RL4137 after 72 h. The α -amylases induced consisted of heat-labile α -AMY2 (low pI) type of enzymes. They were similar to α -AMY2 which was reported to be induced in the developing pericarp in the absence of stimulus from gibberellic acid (GA), but different from heat-resistant α -AMY2 expressed in the aleurone tissue after stimulation by GA. It is suggested that the RL4137 aleurone tissue has a potential for the synthesis of heat-labile α -AMY2, although the level of induced α -amylase activity was very low compared with that of the enzyme synthesized in the embryo and endosperm at the time of germination.

Key Words: α -amylase, aleurone, heat-labile, wheat.

Introduction

Pre-harvest sprouting of wheat induces the production of α -amylase in grain and causes the deterioration of the end-use quality of flour. It is well known that α -amylase synthesis in grain is enhanced in the embryo and aleurone tissue by gibberellic acid (GA) and suppressed by abscisic acid (ABA). Wheat grains express three distinct α -amylase isozyme families during development and at the time of germination: α -AMY1 (Sargeant 1980), α -AMY2 (Sargeant 1980) and α -AMY3 (Daussant and Renard 1987). The α -AMY1 isozyme (malt/germination amylase) is mainly expressed in germinating grains, while α -AMY2 (green/pericarp amylase) is induced in the developing pericarp and at the later stages of germination, around 5 or 6 days after wetting (Marchylo *et al.* 1979, Marchylo *et al.* 1980, Nishikawa and Watanabe 1988). The α -AMY3 isozyme has been de-

tected only in the developing pericarp (Daussant and Renard 1987). However, Gale *et al.* (1983a) and Kettlewell *et al.* (1999) noticed that some wheat lines contained higher than usual levels of α -amylase in non-germinated grain. Gale *et al.* (1983a) suggested that the α -amylases in non-germinated grain belonged to the α -AMY1 family and were synthesized due to the enhanced sensitivity of the aleurone tissue to a stimulus like GA. It remains to be determined, however, where and how the stimulus is generated.

In barley, the production of low pI isozymes of α -amylase was induced in distal half-grains after 78 h soaking and the activity level reached about 35% of the activity in the GA₃-treated half-grains after 112 h (MacGregor 1976). Cornford *et al.* (1986) also observed increased levels of α -amylase activity in distal half-grains collected 25-28 days after anthesis when they were incubated in a simple buffer solution for 72 h. In a study of the wheat variety Spica, Mares and Gale (1990) observed that the production of α -amylase was induced uniformly in the entire aleurone tissue rather than gradually from the region adjacent to the embryo to the distal region of grain. This progression normally occurs in α -amylase production by GA stimulus from the embryo.

In this paper, we studied the α -amylase production in the distal half-grains of four wheat lines. Two out of the four lines showed a significantly higher α -amylase production than the others. The α -amylases synthesized consisted of heat-labile α -AMY2 and might be encoded by α -Amy2 genes different from the α -Amy2 genes expressed in the aleurone tissue after stimulation by GA.

Materials and Methods

Treatments of embryos and distal half-grains

Wheat (*Triticum aestivum* L.) lines, RL4137 (red-grained, preharvest sprouting-resistant), OS21-5 (red-grained, preharvest sprouting-resistant), Shirogane-Komugi (Shirogane, red-grained, moderately susceptible to preharvest sprouting) and Hokkai250 (red-grained, preharvest sprouting-resistant) were selected out of 68 wheat lines which were subjected to a preliminary examination of the α -amylase induction in distal half-grains. Spikes of RL4137, OS21-5, Shirogane and Hokkai250 were tagged at anthesis and harvested at five-day intervals from 30 to 55 DPA. At around 55 DPA, the grains reached harvest maturity (water content, about 15%). Grains were sterilized by treatment with 70% EtOH for 1 min, 5% NaClO for 30 min and then rinsed five times with distilled water. Embryos were dissected out with a surgical knife on a clean bench (AIRTECH, Japan) after 1 h soaking. Distal half-grains (half-grain) were

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obtained by cutting off half part of the grains with embryos. Ten half-grains and twenty embryos were incubated in a Petri dish (9 cm in dia.) with 2 filter papers (No2. ToyoRoshi, Japan) moistened with 6 ml of distilled water. These samples were kept at -80°C for two month before the assay. To characterize the α -amylase synthesized in the embryos and half-grains, the half grains of RL4137 were incubated in the following solutions: distilled water, $1\text{ }\mu\text{M}$ GA_3 , 10, 20, 30 and $40\text{ }\mu\text{M}$ ABA, 20 mM and 80 mM glucose. Twenty embryos of RL4137 were also treated with the same solutions except for $1\text{ }\mu\text{M}$ GA_3 . Each Petri dish was kept at 20°C for 72 h. Embryos and half-grains were weighed before the incubation. All the experiments were performed in triplicate and analyzed statistically by Duncan's multiple range test.

Alpha-amylase assay

Twenty embryos and ten half-grains were homogenized with 1 ml of extraction buffer (50 mM Tris-HCl, 10 mM CaCl_2 , pH 7.5) in a glass homogenizer on ice. The homogenized solution was centrifuged at 12,000 rpm, 4°C for 10 min. The α -amylase activity of the supernatant fluid was measured using the NeoAmylase Test (blue starch tablet, Daiichi Pure Chemicals, Japan) according to the manufacturer's instructions, which were based on the method of Barnes and Blakeney (1974). The reaction mixture contained 100 μl of the supernatant fluid or an appropriate dilution, made up to 4.0 ml with assay buffer (20 mM $\text{NaHC}_4\text{H}_2\text{O}_2 \cdot 3\text{H}_2\text{O}$, 10 mM CaCl_2 , pH 6.2). This mixture was pre-incubated at 37°C for 5 min, after which one NeoAmylase Test tablet was added and the resulting mixture was vortexed. After further incubation at 37°C for 30 min, the reaction was stopped with 1 ml of 0.5 N NaOH and the mixture was centrifuged at 2,000 rpm for 5 min. The absorbance of the supernatant fluid was read at 620 nm and the reading was converted to enzyme activity units using a standard curve constructed with barley α -amylase activity (Type VIII-A, Sigma).

Heat stability

The crude extract solution of RL4137 was maintained at 50, 55, 60, 65 and 70°C for 15 min. The α -amylase activity was assayed using the NeoAmylase Test as described above.

Isozymes of α -amylase

Crude extracts of α -amylase were prepared from the embryos and half-grains incubated in water and $1\text{ }\mu\text{M}$ GA_3 , as described above. Half of the extract solution was treated at 70°C for 15 min. The α -amylase isozymes were separated by the isoelectric focusing method of Sargeant and Walker (1978). Filter paper wicks ($5 \times 5\text{ mm}$) onto which 100 μl of the extract solution had been adsorbed were applied to the cathode side of polyacrylamide gels ($210 \times 105\text{ mm}$) containing carrier Pharmalite (Pharmacia, Sweden) in the pH range of 4.5 to 8.0. A constant power of 1000 V/10 W was circulated through the gels at 4°C , with the paper wicks for 30 min

and then without the wicks for 250 min. The gel was immersed in 3% β -limit dextrin (Megazyme, Ireland) at 4°C for 10 min, taken out from the dextrin solution, and kept at 37°C for 30 min. Zymograms were obtained by immersion of the gel in the I_2 -KI solution (8 g/L and 1.27 g/L, respectively) for 30 min and rinsed with water for 5 min.

Results

α -Amylase activity in the distal half-grains

The half-grains of the four wheat lines, harvested at 55 DPA, showed a very weak α -amylase activity in the range of 0.53 mU/mg (OS21-5) to 1.39 mU/mg (Shirogane) before soaking (Fig. 1A). After 72 h, the α -amylase activity increased significantly ($P < 0.01$) in Hokkai250 (1.89 mU/mg at 48 h and 2.14 mU/mg at 72 h from the initial value of 1.0 mU/mg) and in RL4137 (3.95 mU/mg at 72 h, up from 0.72 mU/mg at the initial stage). On the other hand, OS21-5 and Shirogane did not show an increase in the α -amylase activity after 72 h. The α -amylase induction was also evaluated in the half-grains of RL4137 and Shirogane during grain development (Fig. 1B). More α -amylase was induced in the half-grains of RL4137 than in those of Shirogane from 30 to 55 DPA, especially at 50 and 55 DPA. On the other hand, Shirogane did not show any significant increase during grain

Fig. 1. α -Amylase induction in the distal half-grains of wheat lines. (A) α -Amylase activity in the distal half-grains of the wheat lines OS21-5, RL4137, Shirogane and Hokkai250 incubated with distilled water for 72 hours. Grains were collected at 55 days postanthesis (DPA). (B) α -Amylase activity in the distal half-grains of the wheat lines RL4137 and Shirogane which were collected from 30 to 60 DPA at intervals of 5 days. The grains were incubated with distilled water for 72 hours. Bars: S.E.

development. These results indicated that the wheat lines varied in their potential for α -amylase induction in the soaked distal half-grains. Wheat lines such as RL4137 appeared to display this potential during grain development. The α -amylase synthesis may take place in the aleurone tissue, since the pericarp, which has been reported to be responsible for α -amylase synthesis during the early stages of grain development (Daussant and Renard 1987), is no longer active at later stages of grain development.

Isozyme analysis

The mature embryos of RL4137, incubated in water for 72 h, showed 9 bands in the high pI region and 9 bands in the low pI region (Fig. 2). The same isozyme pattern with a much higher activity was observed in the half-grains incubated in 1 μ M GA₃ for 72 h. The activity of these isozymes did not appear to be reduced by heat treatment at 70°C for 15 min. On the other hand, the half-grains soaked in water for 72 h showed 7 isozymes only in the low pI region; bands 12 and 15 were missing compared with the low pI isozyme pattern in the half-grains treated with GA₃. These isozymes were not detected after the heat treatment (Fig. 2). They appeared to correspond to α -amylase, since they were active on a β -limit dextrin substrate. These results suggest that the low pI isozymes induced in the aleurone tissue by soaking in water were different from the low pI α -amylase induced in the aleurone tissue by GA₃, even though the majority of the

enzymes showed the same pI.

Heat stability

The heat stability of α -amylase that was induced in half-grains incubated with either GA₃ or water and in the embryos was compared by heating the crude extracts at temperatures of 50 to 70°C for 15 min (Fig. 3). Most of the activity of α -amylase of the embryos and half-grains treated with GA₃ persisted even after treatment at 70°C (67.5% activity in the embryos and 72.8% in the half-grains), while the activity of the enzyme induced in the half-grains by water alone decreased to 6.7% of the initial activity observed before soaking (Fig. 3C). The reduction of the α -amylase activity was not due to the low activity in the half-grains, since the α -amylase activity of the embryos and half-grains treated with GA₃ was measured in an extract diluted to give similar absorbance at 620 nm to that of the extract from the water-soaked half-grains. These results are in agreement with previous findings revealing the heat-labile nature of the α -amylases induced in the half-grains.

Suppression by glucose and ABA

The activity of α -amylase synthesized in the embryos soaked for 72 h was 53.5 ± 4.3 mU/mg (Fig. 4). When the embryos were incubated with glucose, the application of 20 mM and 80 mM glucose decreased the α -amylase activity to 42.9 ± 8.4 mU/mg and 22.5 ± 1.1 mU/mg, respectively. The reduc-

Fig. 2. Zymogram of α -amylase induced in the embryos (Lanes 1, 2) and distal half-grains of the wheat line RL4137 incubated in the presence (Lanes 3, 4) or absence (Lanes 5, 6) of GA₃ (1 μ M) for 72 hours at 20°C in darkness. Lanes 2, 4 and 6: heat treatment at 70°C for 15 min. Bars: S.E.

Fig. 3. α -Amylase activity of the embryos incubated in water and of the distal half-grains of RL4137 in the presence or absence of GA₃ (1 μ M) for 72 hours. Crude extracts of α -amylase were kept at room temperature or were subjected to heat treatment at 50, 55, 60, 65 and 70°C for 15 min before the assay.

(A) Embryos in water and distal half-grains incubated with GA₃ at 20°C.

(B) Distal half-grains incubated with distilled water at 20°C for 72 hours.

(C) Relative activity of α -amylase in the embryos and distal half-grains incubated in the presence or absence of GA₃.

Bars: S.E.

tion by the treatment with 80 mM glucose was significant ($P < 0.05$). A similar reduction was observed in α -amylase synthesized in the half-grains incubated in the presence of glucose (Fig. 4B). The α -amylase activities of the half-grains incubated in water, 20 mM and 80 mM glucose were 1.2 ± 0.2 mU/mg, 0.7 ± 0.03 mU/mg and 0.6 ± 0.02 mU/mg, respectively. The reduction by treatment with 20 mM and 80 mM glucose to 63.3% and 45.5% was significant ($P < 0.05$), respectively (Fig. 4C). On the other hand, glucose did not exert any significant effect ($P > 0.05$) in the distal half-grains treated with 1 μ M GA₃; 94.8 ± 1.8 mU/mg in GA₃, 92.6 ± 5.4 mU/mg in GA₃ plus 20 mM glucose, 112.6 ± 12.3 mU/mg in GA₃ plus 80 mM glucose (Fig. 4A).

In the embryos incubated with 10, 20 and 40 μ M ABA, the α -amylase activity was significantly lower ($P < 0.01$), at 6.5 ± 1.3 mU/mg, 6.6 ± 1.6 mU/mg and 5.1 ± 0.6 mU/mg, respectively, compared to the value of 43.7 ± 1.6 mU/mg

Fig. 4. α -Amylase activity of the embryos incubated in water or glucose solution and of the distal half-grains of RL4137 in the presence or absence of GA₃ with glucose solution for 72 hours. (A) Embryos in 0, 20 or 80 mM glucose and distal half-grains in GA₃ (1 μ M) plus 0, 20 or 80 mM glucose.

(B) Distal half-grains in 0, 20 or 80 mM glucose.

(C) Relative activity of α -amylase in the embryos and distal half-grains incubated in the presence or absence of GA₃ (1 μ M) with 0, 20 and 80 mM glucose.

Bars: S.E.

recorded in the embryos that had not been treated with ABA (Fig. 5A). ABA reduced the α -amylase activity to a level of about 15% of that without ABA treatment on the average (Fig. 5C). A significant ($P < 0.01$) reduction of the α -amylase activity by ABA was also observed in the half-grains in the presence or absence of GA₃ (Fig. 5A, B). The average reduction of the α -amylase activity in the half-grains by ABA was about 50% (Fig. 5C).

Discussion

The production of α -amylase was induced in the half-grains of RL4137 and Hokkai250, but not in those of OS21-5 and Shirogane. There was a variation in the potential of the aleurone tissue to synthesize α -amylase among the wheat lines by soaking. This ability of the aleurone tissue to induce the production of α -amylase did not appear to be related to the pre-harvest sprouting resistance or to the level of grain dormancy since RL4137 is known to be a line with pre-harvest sprouting resistance and a high level of dormancy. During grain development, RL4137 showed an α -amylase

Fig. 5. α -Amylase activity of the embryos of RL4137 incubated in water or ABA and of the distal half-grains in the presence or absence of GA_3 plus ABA.

(A) Embryos incubated in water and ABA (10, 20 or 40 μM) and distal half-grains in 1 μM GA_3 plus ABA (10, 20 or 40 μM).

(B) Distal half-grains incubated in ABA (10, 20 or 40 μM).

(C) Relative activity of α -amylase in the embryos and distal half-grains incubated in the presence or absence of GA_3 with ABA (10, 20 or 40 μM).

Bars: S.E.

activity of about 2 mU/mg (45 mU/half-grain on the average) after soaking. It is possible that under wet conditions, the aleurone tissue of the developing grains of RL4137 might have contributed to the accumulation of α -amylase in mature grains. Gale *et al.* (1983a) indicated that two wheat lines, MarisHuntsman and Bezostaya, showed a high α -amylase activity (more than 10 mU/grain) in field-grown mature grains, compared to two other wheat lines, KogaII and Bersee, which did not display any α -amylase activity. RL4137 stood midway between these lines with high and low α -amylase activity. They also suggested that α -amylase produced during grain development in MarisHuntsman might represent the α -AMY1 family and contribute to an increase in the α -amylase activity at the time of germination. Thus, although the level of the α -amylase activity induced in soaked aleurone tissue was very low, the potential of the aleurone tissue to produce α -amylase without GA signal from the embryo might also be one of the factors that reduce flour quality.

Wheat harbors 12-14 α -Amy1 genes for high pI isozymes (α -AMY1) on the chromosomes of the homoeologous group 6, while 10 or 11 α -Amy2 genes for low pI isozymes (α -AMY2) on the chromosomes of group 7, and three α -Amy3 genes for unusually high pI isozymes (pI > 10, α -AMY3) on the chromosomes of group 5 (Baulcombe *et al.* 1987, Gale *et al.* 1983b, Huang *et al.* 1992). The α -AMY2 isozymes were detected during early grain development and after five or six days throughout grain germination (Marchylo *et al.* 1979, 1980, Nishikawa and Watanabe 1988). Gale and Ainsworth (1984) and Baulcombe *et al.* (1986) suggested that the α -Amy2 expression was not controlled by GA in developing grains and that α -AMY2 with the same pI might be encoded by a different α -Amy2 gene. Lunn *et al.* (1999) examined the heat-labile nature of α -AMY2 induced in the pericarp during grain development. Recent studies have showed that the expression of the α -amylase genes decreased by treatment with sugars (Morita *et al.* 1998, Sheu *et al.* 1996, Loreti *et al.* 2000).

The present results indicated that the isozymes of α -amylase induced in the aleurone tissue were heat-labile and lacked two low pI isozymes (bands 12 and 15), compared with the α -amylase isozyme pattern in the aleurone tissue treated with GA_3 . Glucose also suppressed the expression of α -amylase in the aleurone tissue soaked with water alone, but not in the aleurone tissue treated with GA_3 , although ABA suppressed both α -amylases similarly. These results suggest that the induction of α -amylase in the aleurone tissue may be similar to that of α -AMY2 in the pericarp and might be encoded by α -Amy2 genes that are different from the α -Amy2 genes expressed in the aleurone tissue and embryos at the time of germination. Some wheat lines may maintain a potential to synthesize 'green' α -AMY2 in the aleurone tissue during the middle to late stages of seed development.

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