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β-Amylase is not Involved in Degradation of Endosperm Starch During Seed Germination of Maize

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ABSTRACT: For species of Triticeae, e.g., barley, β-amylase was synthesized in endosperm during seed development and processed to active form during seed germination. Thus, β-amylase was suggested to be one of the enzymes to degrade endosperm starch for seed germination. β-Amylase expression in maize kernels apparently is different from that of barley. By immunolocalization, we found that maize β-amylase is expressed in the pericarp but not in the endosperm during seed development; and is synthesized in the aleurone cells but not in the scutellar epithelium cells during seed germination. The newly synthesized maize β-amylase is retained inside of the aleurone cells. On the contrary, maize α-amylase is synthesized and secreted from the aleurone cells to the starchy endosperm. By PAS and iodine stain, we showed that starch is present in the starchy endosperm and absent in the aleurone cells of maize seeds. Results of starch film assay and native activity gel analysis indicated that α-amylases are the enzymes for endosperm starch degradation during maize seed germination. Because maize β-amylase is synthesized and retained in the aleurone cells, it is unlikely that β-amylase acts on endosperm starch during seed germination.

KEY WORDS: Maize, β-amylase, Immunolocalization.

INTRODUCTION

Starch in the endosperm of cereals is the most abundant reserve synthesized during seed development. Degradation of starch into soluble sugars is important to support seedling growth during seed germination. Starch can be degraded either by hydrolysis with amylases or phospholysis with starch phosphorylase. In germinating cereal kernels, hydrolysis but not phospholysis is the major process to break down starch molecules. Because α- and β-amylases are the major amylolytic enzymes found during seed germination, it was suggested that both enzymes are involved in the degradation of endosperm starch (MacGregor, 1983; Kruger and Lineback, 1987; Beck and Ziegler, 1989). It is well known that majority of α-amylase is expressed and secreted out of cells of the aleurone layer and the scutellar epithelium in germinated cereal seeds (Okamoto and Akazawa, 1979; Higgins *et al*., 1982). Hormonal regulation of α-amylase expression in germinating cereal grains had been extensively studied (Ho *et al*., 1987). For barley, gibberellic acid (GA) induces *de novo* synthesis of α-amylases in the aleurone layer cells; abscisic acid (ABA) inhibits the effect of GA (Deikman and Jones, 1985; Nolan *et al*., 1987; Ho *et al*., 1987). In contrast to α-amylase, no β-amylase activity could be detected in barley aleurone layers during seed germination (Hardie, 1975). However, β-amylase is synthesized and accumulated as a latent form in the starchy endosperm during seed development of barley. (MacGregor *et al*., 1971; Laurière *et al*., 1986; Hara-Nichimura *et al*., 1986). A proteolytic mechanism activates the pre-existing

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β-amylase molecules in the starchy endosperm during seed germination (Shewry *et al*., 1988; Beck and Ziegler, 1989; Sopanen and Laurière, 1989). Because of their localization and enzymatic activities, the activated β-amylases in germinating barley endosperm and α-amylases secreted from the aleurone cells and the scutellar epithelium were regarded as the hydrolytic enzymes responsible for carbohydrate redistribution between storage endosperm tissues and growing seedlings. Similar expression patterns of α-amylases and β-amylases during seed development and germination were found in wheat and rye (Rorat *et al*., 1991). These plant species are members of the Triticeae tribe (Gould and Shaw, 1983).

Maize and rice are members of other tribes of Gramineae. Expression of α-amylases of maize and rice during seed germination are similar to those of barley, wheat and rye. However, β-amylases in germinating maize and rice are synthesized *de novo* in the aleurone layers but not in the developing endosperm (Wang *et al*., 1996; 1997). Maize β-amylase cDNA was cloned from germinating aleurone layers. Sequence analysis indicated that maize β-amylase has no signal sequence, suggesting that it is a cytosolic protein. RNA analysis showed that maize β-amylase mRNA is present in the aleurone cells, but could not be detected in the scutellum tissues during seed germination (Wang *et al*., 1997). These results are consistent with the observation that the aleurone layer cells of germinating maize kernels synthesize both α- and β-amylases, and the epithelium cells of scutellum synthesize only α-amylase (Dure, 1960; Wang *et al*., 1997). Analysis of the distribution of maize β-amylase in germinating seeds by measuring enzyme activity of dissected tissues indicated that it is mainly associated with the aleurone layer (Wang *et al*., 1992). Only a minute fraction of β-amylase activity could be detected in the starchy endosperm. However, it is difficult to separated the aleurone layers from endosperm, suggesting the β-amylase activity in the starchy endosperm may come from contamination of aleurone tissues. (Wang *et al*., 1992). Immunolocalization of proteins with antibody probes not only can prevent tissue contamination during dissection but also improve resolution to cellular level. If β-amylase is not present in the starchy endosperm and is expressed only inside of aleurone cells, it would imply that β-amylase does not participate in degrading endosperm starch for its spatial separation. In this study, we examined the distribution of β-amylase and α-amylase in germinating maize kernels by immunohistological method. We provided evidence that β-amylase is not located in the starchy endosperm of germinating maize seeds.

MATERIALS AND METHODS

Plant materials

Seeds of maize (*Zea mays* L. cv. TN351) were obtained from Taiwan Seed Service. Uniform seeds were imbibed in running water for 12 hours and allowed to germinate on moist vermiculite in the dark at 28℃. Developing kernels were harvested from greenhouse grown plants on 7-, 14- and 21-DAP (days after pollination).

Assay of amylolytic activity

Starch film assay was carried out to examine the occurrence and tissue location of amylolytic activity in germinating kernels. A starch film of 1 mm thickness was prepared by pouring melted 1% agar in 50 mM Na-acetate buffer (pH 5.4) containing 0.2% starch in a Petri dish. Maize kernels were collected at intervals after imbibition, and longitudinally cut

into two halves. The cut surface was placed downward to contact with starch containing agar film (Okamoto and Akazawa, 1979). After 10 minute-incubation, kernels were removed and iodine solution (1 mM I_2 and 10 mM KI in 0.1 M HCl solution) was added to the starch film. Clear zones against a dark blue background represented the amylolytic activity.

To examine the patterns of α- and β-amylase isoforms, germinating kernels were collected on day 1, 2, 3, 4 and 5 after imbibition (DAI), and dissected into the starchy endosperm, scutellum and aleurone layer. Tissues were homogenized in the extraction buffer containing 50 mM Tris-HCl (pH 7.0), 3 mM NaCl, 4 mM $CaCl_2$, 1 mg/L leupeptin and 10 mM β-mercaptoethanol (Wang *et al*., 1992), and cell debris was removed by centrifugation at 15,000 xg for 15 minutes. The supernatant was collected and subjected to electrophoresis on a polyacrylamide slab gel described as Kakefuda and Duke (1984). After electrophoresis, the gel was rinsed with 50 mM Na-acetate buffer (pH 5.4) trice, and soaked in 4% starch solution containing 50 mM Na-acetate (pH 5.4) and 5 mM $CaCl_2$ for 60 minutes. The incubated gel was briefly rinsed with distilled water and stained with iodine solution to visualize the amylolytic pattern.

Immunohistochemical detection

Developing kernels were collected on 7-, 14-, 21- and 28-DAP, and fixed with 2% paraformaldehyde in 50 mM Na-phosphate buffer (pH 7.0) at 4℃ for 8 hours. The fixed samples were washed with 50 mM Na-phosphate buffer (pH 7.0) for 6 hours, and then soaked in 10% sucrose. Prior to sectioning, samples were embedded in Tissue Tek II and frozen in a -26℃ freezer for 4 hours. Sections of 20 μm thickness were collected and mounted on gelatin-coated microscopic slides, and dried in an oven for overnight. Polysaccharides were stained with periodic acid Schiff's reagent (PAS) (Hansson and El-Ghazaly, 2000). Germinating kernels were collected on 1-, 3- and 5-DAI, fixed with 2% paraformaldehyde, passed through a series of t-butanol/ethanol dehydration steps, and infiltrated in paraffin (Muhitch *et al*., 1995). Paraffin embedded samples were sectioned to 10 μm, attached on slides, de-paraffinized with xylene, rehydrated, and subjected to immunohistochemical staining. For immunolocalization, sections were blocked with 3% gelatin for one hour and then incubated with rabbit antibodies against either maize α- or β-amylase in TBS buffer (20 mM Tris-HCl, pH 7.2, 0.5 M NaCl and 1% gelatin) for one hour. Slides were washed twice in TTBS (gelatin-free TBS plus 0.05% Tween 20) for 30 minutes. Biotinylated goat-anti-rabbit antibody (100 μL of 200x diluted with TTBS) was applied to samples, incubated for 30 minutes, and then washed for 30 minutes in two changes of TTBS. The ABC reagent (Vectastain Elite PK-6101) was applied to sections on the slide, incubated for 30 minutes, and then washed with TTBS. The slides were immerged in the color development solution till brown color developed. Pre-immune rabbit sera were used as controls to verify the specificity of α- and β-amylase antibodies, respectively.

RESULTS AND DISCUSSION

Distribution of starch in developing maize kernels

To examine the distribution of starch in developing maize kernels, we stained samples with PAS. As shown in Figure 1, polysaccharide moiety was detected in young pericarp tissues but no starch was detected in the developing endosperm on 7-DAP. Starch began to accumulate in the endosperm at the crown region (CR) of kernel about 14-DAP. Along with

embryo development, the accumulation of starch in growing endosperm started from the crown region toward the basal region (BR). In 28 day-old kernels, the entire endosperm was almost filled with starch granules, whereas the outermost layer cells of endosperm, the well-differentiated aleurone cells, apparently had no starch response. When Coomassie Blue reagent and Sudan III were used to visualize the presence of proteins and lipid, respectively; the aleurone layer was heavily stained (data not shown). In addition, the sub-aleurone region accumulated much more proteins than the central portion of starchy endosperm where starch was the predominant reserve macromolecules.

Amylolytic activity in germinated kernels

Upon imbibition of maize kernels, reserve food in the endosperms and scutella is degraded to support the growth of seedlings. To observe changes of reserved starch in germinating seeds, longitudinal sections of kernels on 1- and 3-DAI were stained with iodine solution for starch (Fig. 2). The starchy endosperm on 1-DAI was closely contact with the epithelium of scutellum and there was no significant starch degradation. On 3-DAI, a faint staining zone appeared between the epithelium of scutellum and the dark-stained endosperm, indicating a significant amylolytic action occurred. There was no visible gap between the aleurone layer and the sub-aleurone region. The results clearly demonstrated that starch grains located adjacent to the epithelium of scutellum were rapidly degraded before those in the rest parts of endosperm. The phenomenon was consistent with the previous observation that the epithelium of scutellum was the initial site of amylase activity detected in imbibed cereal seeds (Okamoto and Akazawa, 1979; Gibbons, 1981; Karrer *et al*., 1991; Ranjhan *et al.*, 1992). Starch film technique was used to reveal the temporal pattern of amylolytic action in germinating maize kernels (Fig. 3). As clear zones shown on starch films, amylolysis took place first at the position of the scutellar epithelium 12 hours after the imbibition, prior to the observation of starch degradation *in situ* (Fig. 2). The next occurrence of amylolytic activity was found at the aleurone layer near the basal end of maize kernel after radicle protruding the seed coat (2-DAI). During post-germination growth period, the amylolytic activity increased rapidly and distributed throughout the starchy endosperm. After 5-DAI, the amylolytic activity in the endosperm became very high and the region of starch film contacted with the cut surface of a 5-DAI kernel turned clear in less than 5 min.

By native gel analysis, five major amylolytic bands were detected in germinating maize kernels (Wang *et al*., 1992). Amy-1, Amy-2, Amy-3 and Amy-4 were shown to be α-amylases and Amy-5 to be a β-amylase. The amylolytic activity increased along with the germination time. High level of α-amylase activity was found in the aleurone layer, scutellum and starchy endosperm on 5-DPI. β-Amylase was present in the aleurone fraction and absent in the scutellum (Fig. 4). With caution during tissue dissection, β-amylase activity could only be detected in the aleurone layer fraction but not associated with the starchy endosperm (data not shown). We had shown previously that protein molecules of β-amylase in maize germinating kernels were synthesized *de novo* in the aleurone layers (Wang *et al*., 1997). In addition, northern blot analysis indicated that maize β-amylase mRNA is present in the aleurone cells, but could not be detected in the scutellum tissues during seed germination (Wang *et al*., 1997). In order to degrade starch in germinating kernel, β-amylase synthesized in the aleurone cells must be secreted into the endosperm. DNA sequence of maize β-amylase cDNA and N-terminal peptide sequence of maize β-amylase protein indicates that maize β-amylase gene lacks a conventional signal peptide. It was shown that maize β-amylase was

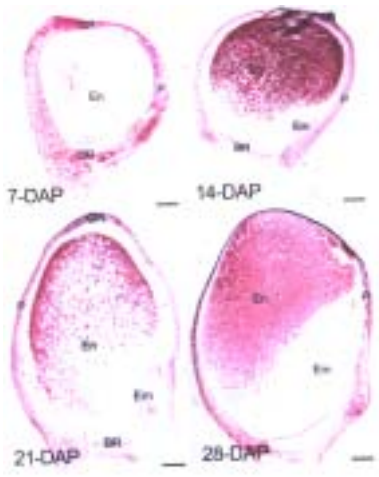

Fig. 1. PAS staining for polysaccharides in maize kernels at different developmental stages. Developing kernels were collected and fixed on 7-, 14- and 28-DAP for sectioning. Sample sections were subjected to PAS staining to visualize the presence of polysaccharides. Either starch grains and cell wall could be stained in red color. BR: basal region; CR: crown region; Em: embryo; En: endosperm; P: pericarp; Bar = 0.3 mm in 7-DPA; Bar = 0.5 mm in 14- and 28-DAP.

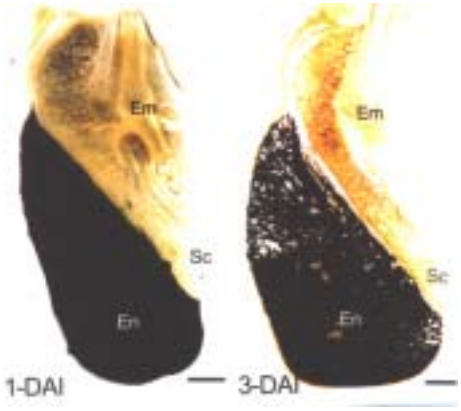

Fig. 2. Degradation of starch in the early stages of maize seed germination. Kernels were harvested on 1-DAP and 3-DAP for paraffin section. Iodine staining was conducted to identify the initial site of starch degradation upon germination. Em: embryo; En: endosperm; Sc: scutellum, Bar = 1 mm.

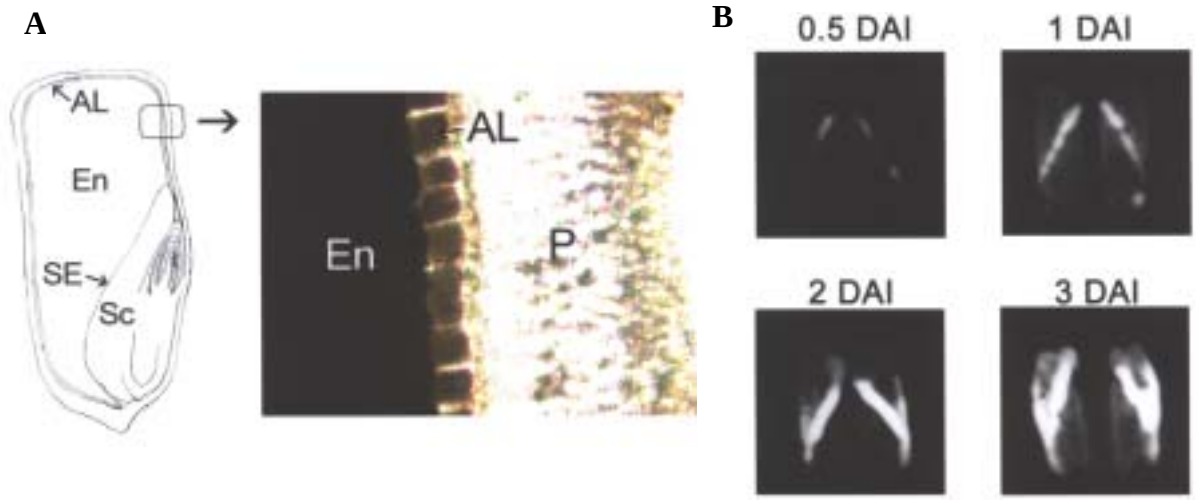

Fig. 3. Occurrence of starch and amylolytic activities in germinating maize kernels. A: Schematic picture to show various parts of a mature maize kernel. A piece of endosperm of 12-h imbibed kernel obtained by free-hand section technique was stained with iodine solution to identify the presence of starch grains. As revealed in the amplified sector that starch grains were heavily accumulated in the starchy endosperm and the aleurone cells were free of starch. B: Starch film assay for the occurrence of amylolytic activity in germinating maize kernels. Germinating kernels were cut longitudinally into two halves, and the cut surface were placed to contact with starch-containing agar film. With 10-minute incubation, iodine solution visualized the zone of amylolytic action. AL: aleurone layer; En: starchy endosperm; P: degenerated pericarp; Sc: scutellum; SE: scutellar epithelium.

secreted from the aleurone layers of 6-day-old germinating kernels upon the stimulation by calcium ions *in vitro* (Laurière *et al*., 1992); however, this result may be due to the leakage from certain senescing aleurone cells. To resolve this issue, immunohistochemical assay was conducted to clarify the destination of β-amylase in developing and germinating kernels.

Immunolocalization of β-amylase in developing and germinating maize kernels

To immunolocalize the distribution of β-amylase and α-amylases in maize kernels, we raised antibodies reacted specifically with α- or β-amylases. Two rabbit polyclonal antibody preparations used in this study could antigenically react with α-amylases (both Amy-3 and Amy-4) and β-amylase (Amy-5), respectively. To exclude the non-specific background signals in the immunolocalization, we also used preimmune sera as controls in all experiments described. In developing maize kernels, β-amylase was immunolocalized to the crown region of pericarp on 7- and 14-DAP (Fig. 5). At the same developmental stage α-amylase was not detected. On 21-DAP, a gradual degeneration of ovary wall became evident at the crown region where the β-amylase signal was significantly reduced. On 28-DAP, the collapse of pericarp tissues progressed from the crown region towards the basal region, and β-amylase signal was only present in the lower half of pericarp (Fig. 5). These results are similar to that the ubiquitous type β-amylase appeared in rye pericarp and disappeared during seed ripening (Daussant *et al*., 1991). It was shown that the endosperm type β-amylase is present in the endosperm of barley and rye during seed development (Shewry *et al*., 1988; Daussant *et al*., 1991). However, our immunolocalization data verified that neither α- nor β-amylase was present in the starchy endosperms during seed development.

In germinating kernels, α-amylases were immunologically detected in the epithelium of scutellum, the starchy endosperm and the aleurone layer on 3- and 5-DAI (Fig. 6). In contrast, β-amylase was found only inside the aleurone cells but not in the scutellum or the starchy endosperm. It is clear that β-amylase was not secreted from the aleurone cells into the endosperm either. The results were consistent with the zymogram patterns. Our results showed that the scutellar epithelium of the germinating maize kernel could synthesize α-amylase but not β-amylase, and the aleurone cells could produce both α- and β-amylases. Newly synthesized α-amylases were secreted to the starchy endosperm from the scutellar epithelium and the aleurone layer for starch degradation. However, β-amylase is not secreted from the aleurone cells, thus it would not directly participate the amylolytic process in the endosperm.

Fig. 4. Zymogram of amylolytic enzymes in the post-germination period. Scutella and aleurone layers were isolated from various germinating kernels. Enzyme extracts were subjected to electrophoresis and followed by activity staining.

In conclusion, in spite of high homology in β-amylase protein sequences between maize and barley, the gene expression patterns in kernels of these two species are completely different in terms of developmental stages and the tissue specificity. Phylogenetic analysis of β-amylase genes suggested that a ubiquitous type of β-amylase gene is present in plants. For species in the Triticeae tribe, an additional endosperm type of β-amylase branched from the ubiquitous β-amylase is present in their genome. According to the expression pattern, activation process and tissue location, the physiological function of barley endosperm β-amylase could be considered as not only a storage protein but also a hydrolytic enzyme.

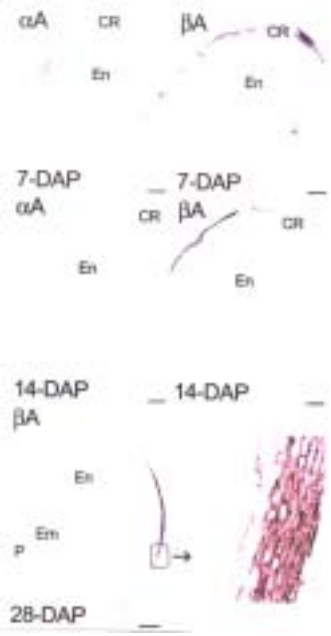

Fig. 5. Immunolocalization of α- and β-amylases in developing maize kernels. Kernels were collected at various developmental stages. Sample sections were incubated with specific antibodies against α-amylase or β-amylase. After the cross-reaction with the secondary antibody, a color development was followed to visualize the antigen-antibody interaction. Bluish brown color represented the positive signal. A sector in 28-DAP was amplified 10 fold to show the cytosolic location of β–amylase signal. αA: detected with α-amylase antibody; βA: detected with β-amylase antibody; BR: basal region; CR: crown region; Em: embryo; En endosperm; P: pericarp. Bar = 0.3 mm in 3-DAP; Bar = 0.5 mm in 14-DAP and 28-DAP.

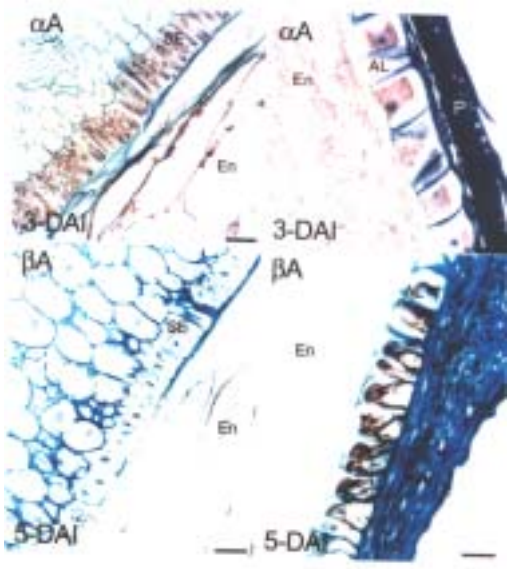

Fig. 6. Immunolocalization of α- and β-amylases in germinating maize kernels. Kernels were harvested on 3-DAI and 5-DAI. After fixation and sectioning, sample sections were incubated with specific antibodies against α-amylase or β-amylase, and then followed by the interaction with the secondary antibody and color development. Bluish brown color represented the positive signal, and greenish blue color was the result of counter staining with Fast Green to visualize tissue structure. αA: detected with α-amylase antibody; βA: detected with β-amylase antibody; AL: aleurone layer; En: starchy endosperm; SE: scutellar epithelium. Bar = 0.1 mm in 3-DAI; Bar = 0.5 mm in 5-DAI.

During seed development, the endosperm type β-amylase was actively synthesized in a latent form and deposited with starch inside endosperm. After seed imbibition, it was processed to an active carbohydrase in conjunction with α-amylases for mobilization of starch molecules. In maize, no endosperm type β-amylase was detected in either the germinating or developing endosperms, but the ubiquitous one was actively expressed during germination in the aleurone layer where no starch is present. If β-amylase synthesized in the aleurone layers played a role in amylolysis, it must be secreted into the starch endosperm in where the substrate resided. It was shown that a minor fraction (about 6%) of β-amylase activity was released to medium from maize aleurone layer *in vitro* (Laurière *et al*., 1992). Subbarao *et al.* (1998) reported that β-amylase was present in the isolated scutella of 4-day-old germinating kernels but not secreted into medium. However, we demonstrated that neither the enzymatic activity nor β-amylase mRNA was present in germinating maize scutellum (Wang *et al*., 1997). In this study, we further demonstrated by immuolocalization that the epithelial cells of maize produced α-amylases but not β-amylase after seed imbibition. The aleurone cells of germinating maize kernels were the only sites that could synthesize β-amylase *de novo*. Unlike α-amylase, the newly synthesized β-amylase protein molecules were retained in the

cytosol of the aleurone cell and not secreted into the starchy endosperm (Fig. 6). Our observations make the physiological functions of β-amylase in germinating maize seeds remained to be studied.

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玉米 β-澱粉酶不參與種子萌芽後之胚乳澱粉的降解作用

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摘　　要

大麥等小麥族的植物，於種子發育階段會在胚乳中合成並累積大量的 β-澱粉酶蛋白質分子，藉由種子萌芽後的活化作用才表現 β-澱粉酶的酵素活性；因此，β-澱粉酶被視為萌發種子中分解澱粉的重要酵素之一。玉米穀粒之 β-澱粉酶和大麥有不同的表現模式。利用免疫組織定位法，我們發現在發育中的玉米穀粒，只有果皮組織含有 β-澱粉酶分子，胚乳則無；而在萌芽的玉米穀粒中，盾片的上皮細胞是不會合成 β-澱粉酶的，只有糊粉層細胞能夠合成 β-澱粉酶。不同於 α-澱粉酶酵素分子在合成後會從糊粉層細胞分泌到胚乳，新合成的玉米 β-澱粉酶分子則被保留在糊粉層細胞內。由 PAS 和碘染的分析結果顯示，只有粉質胚乳組織含有澱粉粒，而糊粉層細胞則沒有澱粉的存在。由澱粉薄層和電泳活性分析結果推論，α-澱粉酶是分解玉米胚乳澱粉的重要酵素；而 β-澱粉酶雖在萌芽的糊粉層中合成，但自始它就留在糊粉層細胞內，不可能參與澱粉的分解作用。

關鍵詞：玉米、β-澱粉酶蛋白、免疫定位。

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