Journ. Fac. Sci., Hokkaido Univ. Ser. V (Botany), **13** (3, 4): 281-292 1984.

Immunochemical studies of α-amylase from germinating wheat seeds

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α-Amylase was purified from 7-day germinated seedlings of wheat. Rabbit antiserum against the purified α-amylase was prepared. The purified α-amylase was separated into two components by immunoelectrophoresis.

Total amylolytic and α-amylase activities of wheat embryos and endosperms were assayed and α-amylase contents were estimated by the immunological method. Differences were found in the ratios of activity to the content of the enzyme from embryos and endosperms.

Changes in antigenic determinants of α-amylase were analyzed by the double immunodiffusion method. It was suggested that α-amylase of 7-day germinated seedlings had four antigenic determinants at least. The number of determinants was changed during the processes of germination at 24°C and cold treatment at 4°C. Although differences were found in the number of determinants of embryos and endosperms, changes in that were similar in both processes.

The activities of starch-degrading enzymes in cereals increase rapidly at the early stage of germination. Overall increase of the amylase during germination is attributed largely to α-amylase. In germinating barley and wheat seeds, α-amylase is synthesized *de novo*, whereas β-amylase is activated by limited proteolysis without new synthesis (VARNER *et al.* 1964, DAUSSANT *et al.* 1970). Many investigations concerning with α-amylase formation in cereals have been reported that the enzyme is synthesized in aleurone layer in response to gibberellic acid. However, it was recently demonstrated that the scutellum was considered to be a initial site of α-amylase synthesis (OKAMOTO *et al.* 1980). Further, it has been shown that the α-amylase exists in isozymic form in cereals (SCANDALIOS 1974). The composition of α-amylase isozymes varies in the species of cereals and the stages of development or germination (FRYDENBERG *et al.* 1965, TANAKA *et al.* 1970, DAUSSANT *et al.* 1972). Although the role of the each isozyme is uncertain at present, multiple α-amylase loci have been reported in wheat (NISHIKAWA *et al.* 1971), and the differential expression and regulatory mechanism of α-

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amylase genes during germination process are interesting.

In this paper, the properties of α-amylase and the changes in its contents were investigated in germinated and cold-treated winter wheat using the immunological methods for the specific detection of the enzyme. The changes in the specific activities and antigenic determinants of α-amylase in the embryos and endosperms of wheat during the periods of germination and cold treatment were demonstrated.

Materials and Methods

Plant materials

Winter wheat seeds (*Triticum aestivum* L. cv. Mukakomugi) were used throughout this experiment. Seeds were sterilized in 0.05% $HgCl_2$ solution for 15 min, rinsed with runnung water and germinated in the dark at 24°C. In cold treatment, seeds were kept at 4°C after 6 hr germination at 24°C.

Enzyme extraction

The seeds or seedlings in each stage of germination and cold treatment were separated into embryos and endosperms, then forzen at −70°C immediately. An adequate amount of the embryos or the endosperms was homogenized in a chilled mortar and pestle with two volumes of the extraction medium containing 0.1 M sodium acetate buffer (pH 4.8), 20 mM $CaCl_2$ and 5 mM 2-mercaptoethanol. The homogenate was centrifuged at 3,000×g for 15 min, and the supernatant was centrifuged again at 23,000×g for 15 min. The supernatant solution was used for assay of total amylase activity or soluble protein content. The supernatant solution was heated at 70°C for 15 min and centrifuged at 10,000×g for 10 min. The resultant supernatant was used for assay of α-amylase activity or immunological analysis.

Purification of α-amylase

α-Amylase was purified according to the procedure of CHEN *et al.* (1974) with some modifications. About 1 kg sample of 7-day germinated seedlings was homogenized with the extraction medium in a Waring Blender. The homogenate was centrifuged as above and the supernatant was heated at 70°C for 15 min to denature the β-amylase. The resulting precipitate was removed by centrifugation and the supernatant was fractionated with ammonium sulfate. The fraction precipitating between 20 and 60% saturated ammonium sulfate was dissolved in the extraction medium, then the solution was dialyzed. The solution was made to 40% with cold ethanol, the resulting precipitate was removed by centrifugation. The supernatant was treated with oyster glycogen at a concentration of 20 mg/m*l*. The glycogen-α-amylase

complex was collected by centrifugation at 10,000×g for 10 min and washed with 40% ethanol twice. The complex was dissolved in 15 mM Tris-HCl buffer (pH 7.8) containing 10 mM $CaCl_2$, and the solution was incubated at 25°C for 2 hr then dialyzed against 50 mM sodium acetate buffer (pH 5.5) containing 10 mM $CaCl_2$ overnight. Insoluble materials were removed by centrifugation, the supernatant solution was applied to a column (1.9×10 cm) of β-cyclohepta-amylose-epoxy Sepharose 6 B (SILVANOVICH *et al.* 1976). The column was eluted with 50 mM sodium acetate buffer (pH 5.5) containing 10 mM $CaCl_2$ and 8 mg/m*l* β-cyclophepta-amylose. The enzyme fractions were pooled and concentrated by the ultrafiltration. All procerures were carried out at 0–4°C.

Gel electrophoresis

The enzyme preparations in purification process were analysed on 12.5% polyacrylamide slab gel containing SDS as described by LAEMMLI (1970). After electrophoresis, the gels were stained with 0.05% Coomassie brilliant blue.

The purified enzyme preparations were fractionated on agarose gel. A melted 1.5% agarose solution containing 50 mM Tris-HCl buffer (pH 8.6) was poured as 5 mm thick layer on a glass plate (12×14 cm) and samples in 1% agarose prewarmed at 45°C were plated on the gel. After electrophoresis at 2 mA/cm for 5 hr, the gels were frozen, cut into 3 mm slices and extracted with acetate buffer (pH 4.8) by shaking.

Preparation of antibody

Antibody against α-amylase was obtained from rabbits immunized with the purified α-amylase. The enzyme solution (2 mg/m*l*) was mixed with an equal volume of Freund's incomlete adjuvant and the mixture was injected into the rear foot-pads of rabbits. Bleedings from the ear were begun at 3 weeks after immunization and continued at weekly intervals for further 8 weeks. The blood was allowed to clot for 1 hr at room temperature, chilled overnight and centrifuged at 3,000×g for 15 min. The antisera were incubated at 56°C for 30 min, then frozen at −70°C. Serum from non-immunized rabbit was similarly treated for use as a control.

Immunological methods

Immunoelectrophoresis was carried out on a glass plate (9×11 cm) coated with 1% agarose. Purified α-amylase was electrophoresed in 50 mM Tris-HCl buffer (pH 8.6) at 4°C for 90 min with a constant current of 2 mA/cm. Rabbit anti-α-amylase antiserum was placed in the trough, the plate was kept at 4°C overnight. After washing and drying, electrophoregrams were stained

with 0.05% Coomasie brilliant blue.

Single radial immunodiffusion tenchique for quantitation was carried out according to the procedure of MANCINI *et al.* (1965).

Agar gel double diffusion experiments were carried out by the method of OUCHTERLONY (1967).

Chemical assays

Protein was determined according to the method of LOWRY *et al.* (1951) using bovine serum albumin as the standard.

Amylase activity was measured by the modified blue value method of FUWA (1954). Soluble starch (0.6%) was used as the substrate and the reaction was performed at 40°C for 10 min.

Results

Purification of α-amylase

The purification process of α-amylase from 7-day germinated wheat seeds is summarized in Table 1. The specific activity of α-amylase preparation after affinity chromatography was 626.8×10^3 units/mg protein, representing overall purity of 140-fold from crude extract with 5% recovery. Each preparation of purification process was analyzed by electrophoresis in SDS-polyacrylamide gel. As shown in Fig. 1, the final preparation had a single band (mol wt, 43,000).

Rabbits were immunized by the purified α-amylase, and the antiserum was used for the specific detection of the enzyme. Purified α-amylase was separated into two fractions by immunoelectrophoresis in agarose gel (Fig. 2). The two fractions were antigenically different, since two distinct precipitin

Table 1. Purification procedure of wheat α-amylase

Step of purification	Volume (ml)	Total protein (mg)	Total activity (units × 10^{-5})	Enzyme recovery (%)	Specific activity (units × 10^{-3}/mg)	Purity
Crude extract	2,016	11,340	508.0	100	4.48	1.0
Heating at 70°C	1,975	9,875	422.7	83	4.28	1.0
$(NH_4)_2SO_4$ precipitation	182	1,775	342.5	67	19.30	4.3
Glycogen complex	17	23.4	38.1	8	162.92	36.4
Affinity chromatography	0.8	4.1	25.7	5	626.80	139.9

One enzyme unit of amylase activity is the enzyme activity causing 10% decrease in blue of 30 mg/ml starch solution for 10 min at 40°C.

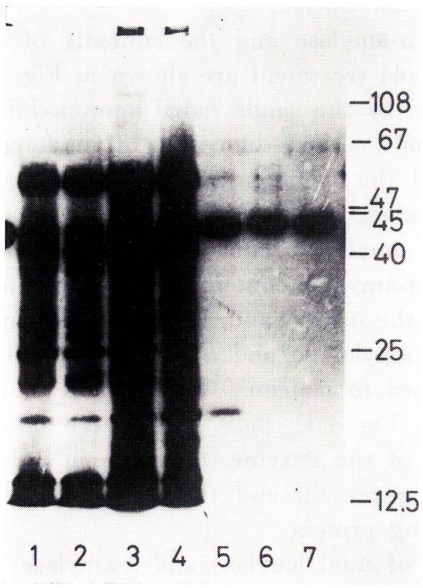

Fig. 1. SDS-polyacrylamide gel electrophoresis of fractions in α-amylase purification process and components separated by immunoelectrophoresis.

Track 1, crude extract; track 2, heating at 70°C; track 3, 20–60% $(NH_4)_2SO_4$ fraction; track 4, glycogen complex; track 5, affinity chromatography; track 6, component 1; track 7, component 2.

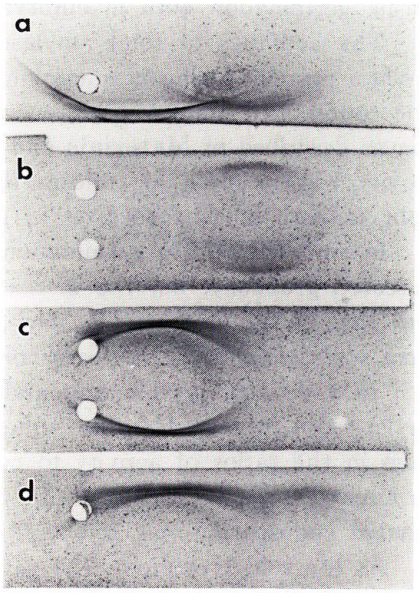

Fig. 2. Immunoelectrophoretic separation of purified α-amylase.

a and d, Purified α-amylase; b and c, immunoelectrophoretically separated α-amylase.

lines intersected. The fraction migrated more slowly was designated component 1 and that migrated more rapidly was designated component 2. Amylolytic activities of the two components were different, the activity of component 1 was higher than that of component 2 (data not shown). The mobility of two components in SDS-polyacrylamide gel electrophoresis was identical (Fig. 1). In further experiments the whole enzymes were used.

Contents and activities of α-amylase

Changes in fresh weight and soluble protein content of embryos and endosperms were studied during the periods of germination and cold treatment. During germination, as shown in Fig. 3, the fresh weight and the soluble protein content of embryos increased gradually, whereas those of endosperms increased early and then decreased. Changes in the fresh weight and the soluble protein content during cold treatment were similar to those

during germination both in embryos and endosperms.

The activities of total amylase and α-amylase and the contents of α-amylase protein during germination and cold treatment are shown in Fig. 4. The contents of α-amylase were estimated by the single radial immunodiffusion as described in **Materials and Methods**. More than 95% of amylolytic activity was localized in endosperms, and the remaining 5% was found in embryos both in the processes of germination and cold treatment.

In embryos during germination, total amylolytic and α-amylase activities increased gradually, but the increase of α-amylase content was not parallel with that of the activity (Fig. 4 (a)). On the other hand, in the endosperms there were remarkable increases of total amylolytic and α-amylase activities at 3 to 4 days of germination and reached to plateau. The content of α-amylase also increased at the same stage (Fig. 4 (a) (b)). There were differences in the ratios of activity to content of the enzyme, the ratio in 7-day germinated embryos was 2.3×10^4 units/mg protein and that in 7-day germinated endosperms was 16.6×10^6 units/mg protein.

As for cold treatment, the activities of total amylase and α-amylase in embryos increased with duration of cold treatment though they decreased temporarilly (Fig. 4 (c)). These activities in endosperms increased rapidly

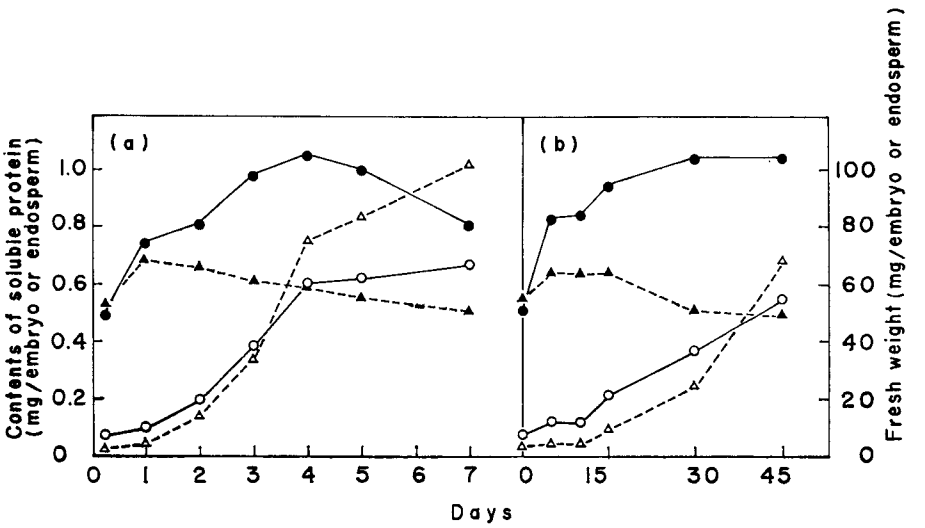

Fig. 3. Fresh weights and soluble protein contents of germinated and cold-treated wheat embryos and endosperms.

(a) Germinated at 24°C, (b) cold-treated at 4°C.

Fresh weight (-△--△-) and soluble protein content (-○—○-) of embryos, fresh weight (-▲--▲-) and soluble protein content (-●—●-) of endosperms.

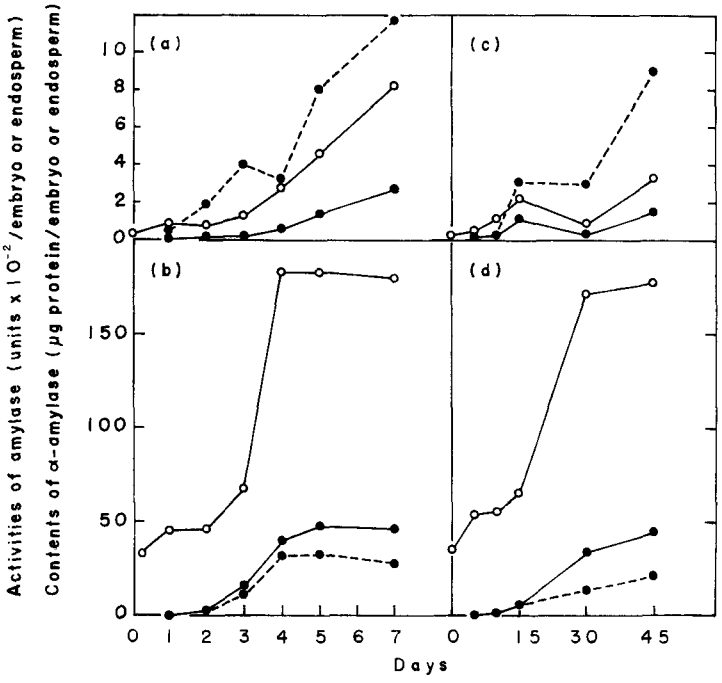

Fig. 4. Total and α-amylase activities and α-amylase contents of germinated and cold-treated wheat embryos and endosperms.

(a) Germinated embryos, (b) germinated endosperms, (c) cold-treated embryos and (d) cold-treated endosperms.

Activities of total (-○—○-) and α-amylase (-●—●-), contents of α-amylase (-●- -●-).

Total and α-amylase activities were measured in soluble protein fractions and those heated at 70°C, respectively. α-Amylase contents were measured according to the method of MANCINI *et al.* as described in **Materials and Methods**.

from 15 to 30 days (Fig. 3 (c) (d)). The α-amylase contents in both embryos and endosperms increased gradually. These changes during cold treatment were similar to those of the early stage of germination.

The α-amylase contents in soluble protein were 2% in embryos and 4% in endosperms at maximum.

Immunochemical properties of α-amylase

Changes in immunological properties of germinating wheat seeds were analyzed utilizing rabbit antiserum against α-amylase purified from 7-day germinated seedlings. Fig. 5 shows the reactions of crude extracts from embryos and endosperms at the different stages of germination with the antiserum in the double immunodiffusion method.

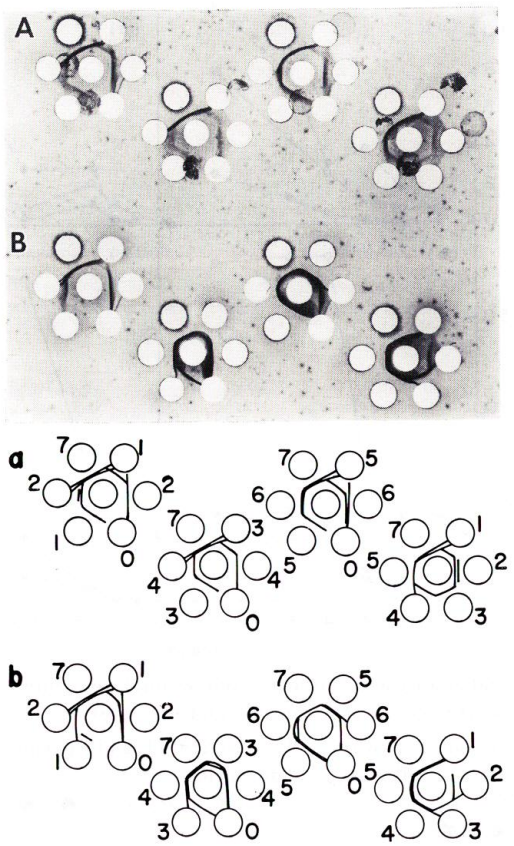

Fig. 5. Reactions in gel diffusion of α-amylase from embryos and endosperms at different germination stages with rabbit antiserum.

(Above) A, embryos; B, endosperms. (Below) a and b, schematic representation of A and B respectively.

0, none; 1, 2, 3, 4, 5 and 6, crude enzyme from 1-, 2-, 3-, 4-, 5- and 7-day germinated embryos or endosperms; 7, α-amylase purified from 7-day germinated seedlings.

The precipitin lines corresponding to the purified α-amylase from 7-day germinated seedlings fused completely with that of the crude extracts of 7-day germinated embroys, showing that the purified α-amylase was antigeni cally identical to the enzyme of crude extracts from the embryos. However, the precipitin line corresponding to the enzyme of 7-day-embryos fused partially with that of the enzyme extracted from 5-day germinated embryos and formed a spur, showing that the antigenic determinants of the enzyme from 5-day-embryos lack some of the determinants present in the enzyme of

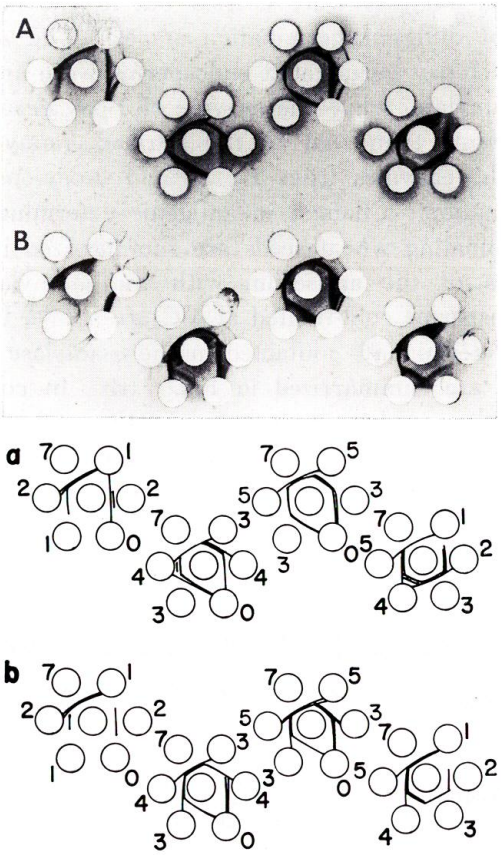

Fig. 6. Reactions in gel diffusion of α-amylase from embryos and endosperms cold-treated at 4°C with rabbit antiserum.

(Above) A, embryos; B, endosperms. (Below) a and b, schematic representation of A and B respectively.

0, none; 1, 2, 3, 4 and 5, crude enzyme of 5-, 10-, 15-, 30- and 45-day cold-treated embryos or endosperms; 7, α-amylase purified from 7-day germinated seedlings.

7-day-embryos.

The α-amylase of 5-day-embryos had more determinants than that of 4-day-embryos, which had equal determinants to that of 3-day-embryos. The determinants of α-amylase from 2-day-embryos are more than those of the enzymes from 3- and 1-day-embryos. These immunochemical analyses on the extracts from embryos of different germination stages suggested that the α-amylase of 7-day germinated seedlings had four antigenic determinants at least.

The analogous analyses were carried out in the crude extracts from the endosperms of different germination stages. The α-amylase in crude extracts of 4- and 5-day germinated endosperms were antigenically identical to the purified α-amylase, while the enzyme of 7-day germinated endosperms had less determinants than that of the purified α-amylase. Further, the determinants of the enzymes from 1-, 2- and 3-day-endosperms increased by days of germination. Changes in antigenic determinants present in the α-amylase of germinating wheat seeds are summarrized in Fig. 7 (A).

The reactions of the antiserum with the α-amylase extracted from embryos and endosperms cold-treated at 4°C are shown in Fig. 6. Changes in the antigenic determinants contained in the α-amylase during the process of cold treatment are summarrized in Fig. 7 (B). In comparison with the enzymes from wheat seeds germinated at 24°C and cold-treated at 4°C, differences were found in the number of antigenic determinants present in α-amylase, while patterns of the determinant formation were similar in both processes.

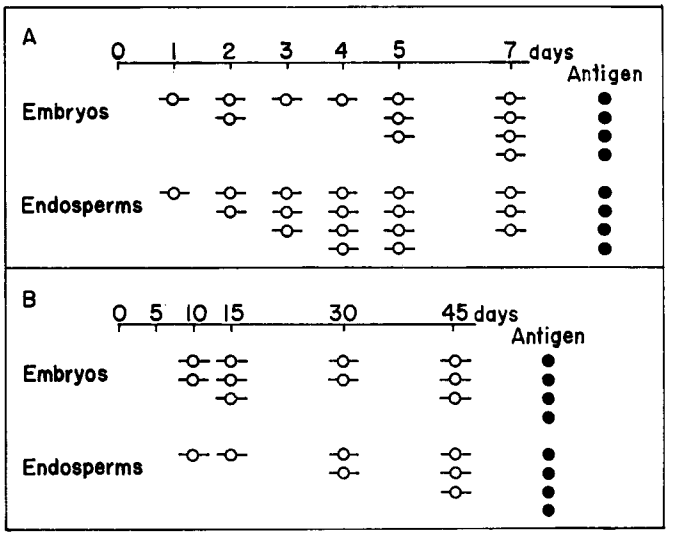

Fig. 7. Changes in the number of antigenic determinants of α-amylase from embryos and endosperms during the periods of germination and cold treatment.

A, germination at 24°C; B, cold treatment at 4°C.

Discussion

Activities of the α-amylase were not parallel with contents of the enzyme. Rarios of the activity to the content in endosperms were higher than those in embryos in both periods of germination and cold treatment. Therefore, the α-amylase might largely exist as an inactive form or a precursor in embryos.

α-Amylase have been shown to be isozymic form in cereals, and changes in the isozyme patterns were also shown in developing and germinating barley and wheat seeds (BILDERBACK 1971, STODDART *et al.* 1971, SARGEANT 1979). It was further, reported that the α-amylase in maturing wheat seeds was antigenically different from that synthesized during germination (DAUSSANT *et al.* 1972).

We showed that the α-amylase purified from 7-day germinated seedlings was separated into two components by the immunoelectrophoresis which were antigenically different. The results of double diffusion method using a rabbit antiserum against α-amylase showed the changes in antigenic determinants of the enzymes from wheat embryos and endosperms during the processes of germination and cold treatment. It was suggested that the α-amylase of 7-day germinated seedlings had four antigenic determinants at least. The experiments using a antiserum against the α-amylase purified from 3-day germinated seedlings suggested that the enzyme from 3-day-seedlings had no more different determinant (data not shown). Although differences were found in the number of determinants of embryos and endosperms, changes in that were similar during the both processes of germination at 24°C and cold treatment at 4°C.

There are three possibilities on changes in the number of antigenic determinants, 1) antigenic determinants of the enzyme protein increased, 2) the number of the isozymes increased, 3) both of the enzyme proteins and the antigenic determinants increased. Further experiments on the relation between isozymes and antigenic determinants are now under investigation.

Acknowledgements

A part of this work was performed in the Research Institute of Immunological Science, Hokkaido University. We wish to express our gratitude to Dr. M. KAKINUMA and Dr. T. KIMURA for their helpful advice. We also thank the Kitami Agricaltural Experimental Station for supplying the wheat seeds.

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