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## Purification and changes in activities of $\beta$ -amylase from wheat seeds

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Changes in activities of activated and latent  $\beta$ -amylase were investigated in developing and germinating wheat seeds. Isozyme pattern in developing seeds was identical to that in dry seeds. No difference was found in the isozyme patterns of activated and total  $\beta$ -amylase. Isozymes from 6-h germinated seeds had the acidic isoelectric points, whereas those from 5-day germinated seeds had the neutral isoelectric points.

$\beta$ -Amylase was purified from dry wheat seeds. SDS-polyacrylamide gel electrophoresis of the purified  $\beta$ -amylase preparation showed two bands at molecular weight 61,300 and 64,500. From Oucetlony double immunodiffusion and immunoelectrophoresis experiments, two components of the purified  $\beta$ -amylase had common antigenic determinants.

Germinating cereals are known to contain starch degrading-enzymes,  $\alpha$ - and  $\beta$ -amylases.  $\alpha$ -Amylase appears little in developing seeds and is synthesized *de novo* in germinating seeds (VARNER and CANDRA, 1964; FILNER and VARNER, 1967; DAUSSANT and CORVAZIER, 1970). In contrast to  $\alpha$ -amylase,  $\beta$ -amylase are synthesized during seed development and stored in dry seeds. In germinating seeds, the enzyme occurs in two forms: activated and latent. The activated enzyme can be extracted with saline solution, whereas the latent one is extracted in the presence of reducing agent (ROWSELL and GOAD, 1962; DAUSSANT and CORVAZIER, 1970; BILDERBACK, 1971). Furthermore,  $\beta$ -amylase known to occur in isozymic forms such as  $\alpha$ -amylase (VISURI and NUMMI 1972; DAUSSANT and MACGREGOR, 1979).

In this study, changes in the activities of activated and latent  $\beta$ -amylase were investigated in developing and germinating wheat seeds. The enzyme was purified, and the properties were demonstrated.

### Materials and Methods

#### *Plant materials*

Winter wheat seeds (*Triticum aestivum* L. cv. Mukakomugi) were used in

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this experiment. Developing seeds were isolated from ears collected at one week interval after flowering from experimental plots. Seeds were germinated in the dark at 24°C, and separated into embryos and endosperms manually.

#### *Extraction of $\beta$ -amylase*

Samples of 0.5 g were homogenized in 50 mM acetate buffer (pH 4.8) with 0.2 M  $\beta$ -mercaptoethanol or without  $\beta$ -mercaptoethanol. The fraction extracting with  $\beta$ -mercaptoethanol was designated total  $\beta$ -amylase and that extracting without  $\beta$ -mercaptoethanol was designated activated  $\beta$ -amylase. Extraction of total  $\beta$ -amylase was carried out at 30°C and that of activated  $\beta$ -amylase was at 1°C for 2 h. The homogenate was centrifuged at  $10,000 \times g$  for 20 min. The extraction process was repeated twice, the extracts were combined and used for assay of  $\beta$ -amylase activity. In the base of sample containing  $\alpha$ -amylase, the extract was heated at 70°C for 15 min and centrifuged at  $10,000 \times g$  for 20 min. The supernatant solution was used for assay of  $\alpha$ -amylase activity, and  $\beta$ -amylase activity was taken away  $\alpha$ -amylase activity from total amylase activity.

#### *Assay*

Amylase activity was measured by the modified blue value method of FUWA (1954). Protein was determined according to the method of LOWRY *et al.* (1951).

#### *Purification of $\beta$ -amylase*

$\beta$ -Amylase was purified according to the procedure of KATO *et al.* (1974) with some modifications. Dry seeds were grinding in an Analytical Mill and sieving. Aleurone layers on the sieve were used.

About 600 g sample was frozen in liquid nitrogen and ground to a powder in a Universal Mill. The powdered sample was suspended in the extraction buffer {50 mM acetate buffer (pH 4.8), 2.5 ml/l  $\beta$ -mercaptoethanol} and stirred at room temperature for 3 h. The suspension was centrifuged at  $10,000 \times g$  for 20 min. The supernatant was added with 1/100 volume of saturated lead acetate, and the precipitate formed was filtered off with a Hyflo-super-cel. The filtrate was designated crude extract. All the procedures after this step were performed at 1-4°C. The fraction precipitating between 20 and 55% saturated with ammonium sulfate was dissolved in 10 mM Tris-HCl buffer (pH 8.0), and the solution was dialyzed against the same buffer overnight. After centrifugation at  $10,000 \times g$  for 20 min, the dialyzate was applied to a column (1.9  $\times$  26 cm) of DEAE-cellulose equilibrated with 100 mM Tris-HCl buffer (pH 8.0). The column was eluted with increasing NaCl concentration linealy from zero to 0.5 M in the same buffer. The enzyme

fractions were pooled, and the enzyme was precipitated by adding ammonium sulfate to 66% saturation. The precipitate was collected by centrifugation and dissolved in 50 mM acetate buffer (pH 4.8), then the solution was dialyzed against 10 mM sodium phosphate buffer (pH 6.8). The dialyzate was applied to a column (1.0×30 cm) of Phenyl-Sepharose CL-4B equilibrated with 10 mM sodium phosphate buffer (pH 6.8) containing 25% saturated ammonium sulfate. After washing with the same buffer containing ammonium sulfate, the column was eluted with decreasing ammonium sulfate concentration from 25% saturation to zero and increasing ethylene glycol concentration from zero to 50% in the same buffer, linearly. The enzyme fractions were pooled and dialyzed against the same buffer. The dialyzate was concentrated to 0.5–1.0 mg/ml by the ultrafiltration and applied to a column (1.0×30 cm) of Sephadex G-100 equilibrated with 10 mM sodium phosphate buffer (pH 7.8). Elution was performed by the same buffer and the enzyme fractions were pooled. The enzyme solution was concentrated to 1 mg/ml by the ultrafiltration and stored at  $-70^{\circ}\text{C}$ .

#### *Isoelectrofocusing*

Isoelectric focusing was performed in a slab gel containing 5% acrylamide and 2.4% Ampholine (LKB) in the pH range 4.5–7.0 as described previously (UCHIDA *et al.* 1987).

#### *Gel electrophoresis*

The enzyme preparations were analyzed on 12.5% (w/v) polyacrylamide slab gel containing SDS according to the method of LAEMMLI (1970).

#### *Preparation of antibody*

Antibody against  $\beta$ -amylase was obtained from rabbits immunized with the purified wheat  $\beta$ -amylase according to FUJIOKA *et al.* (1984).

#### *Immunological methods*

Immuno-electrophoresis was carried out as described by FUJIOKA *et al.* (1984).

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

## **Results**

#### *Changes in $\beta$ -amylase activities in developing and germinating wheat seeds*

The activities of total, activated and latent  $\beta$ -amylase during developmental stages of wheat seeds are shown in Fig. 1.  $\beta$ -Amylase activity appeared in the seeds at the first week after flowering, and there was an increase in total activity during development. Activated  $\beta$ -amylase activity

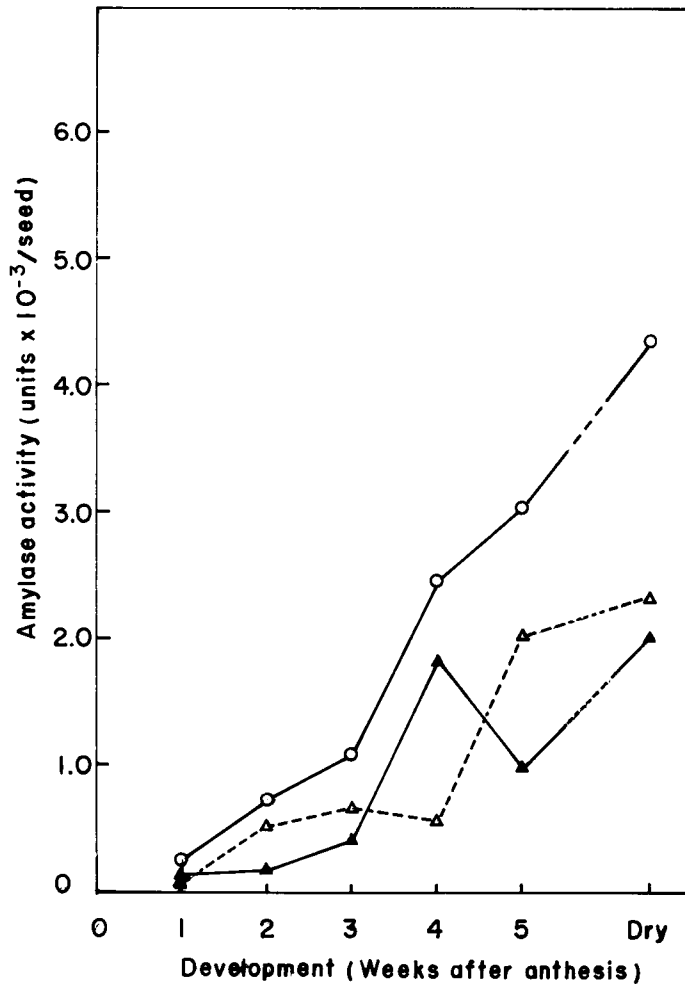
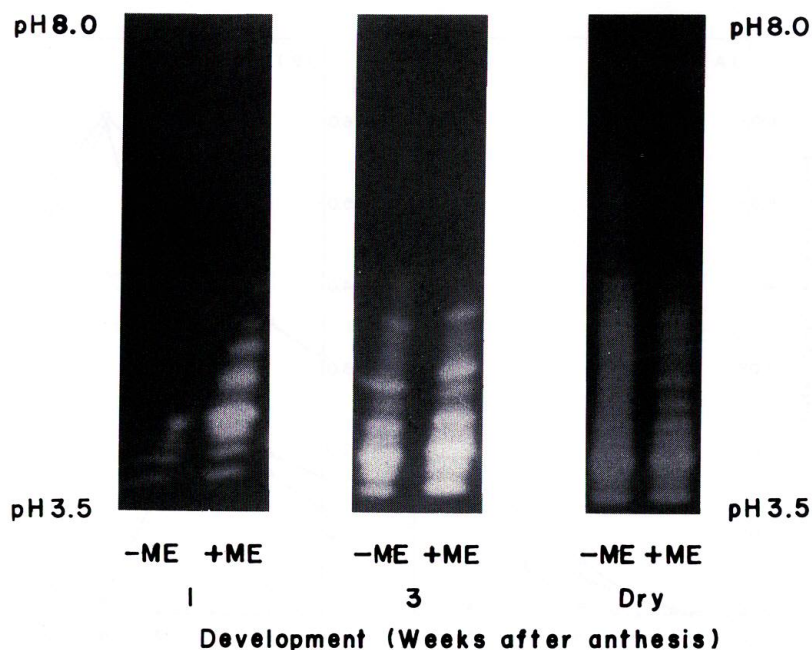


Fig. 1. Changes in  $\beta$ -amylase activities of developing wheat seeds.

Activities of total (—○—○—), activated (—▲—▲—) and latent (---△---△---)  $\beta$ -amylase. The extraction and assay conditions are described in **Materials and Methods**.

increased rapidly at the third to the fourth weeks after flowering and then decreased. Although an increase in latent  $\beta$ -amylase activity was a little until the fourth week, the activity increased rapidly at the fifth week of development.  $\beta$ -Amylases from developing and dry seeds were separated by

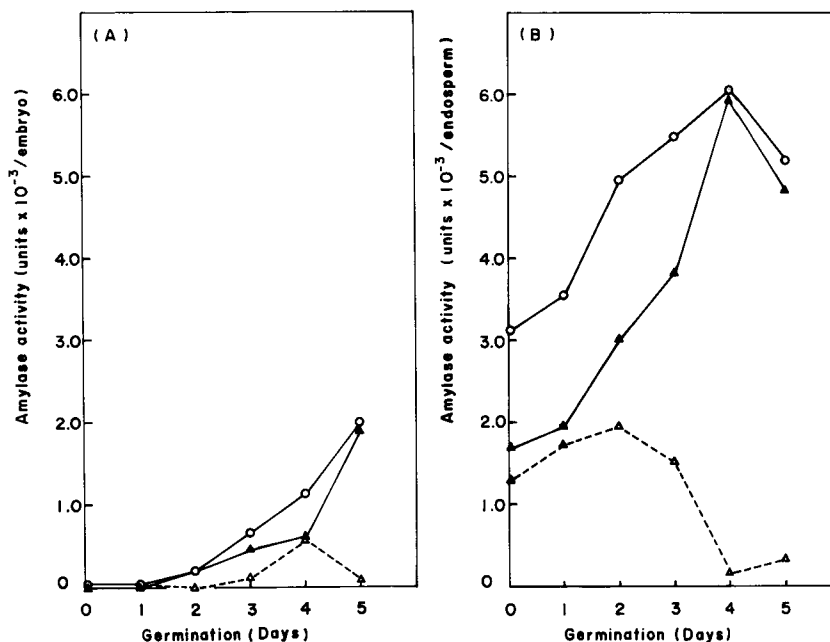


**Fig. 2.** Isoelectric focusing of  $\beta$ -amylase from developing and dry wheat seeds.

+ME: isozymes of total  $\beta$ -amylase, -ME: isozymes of activated  $\beta$ -amylase. The experimental conditions are described in **Materials and Methods**.

isoelectric focusing, and changes in  $\beta$ -amylase isozymes are shown in Fig. 2. Amylase activities were detected in the pH range 3.5 to 5.5, and the isozyme patterns in developing seeds were identical to those in dry seeds except for the first week after flowering. Furthermore, the difference was not detected in the isozyme patterns of activated and total  $\beta$ -amylase.

Changes in activities of  $\beta$ -amylase from wheat embryos and endosperms during germination are shown in Figs. 3(A) and (B). In the embryos, there was an increase in total  $\beta$ -amylase activity after the second day of germination, the activities could be attributed largely to activated  $\beta$ -amylase activity. Total  $\beta$ -amylase activity in endosperms increased gradually until the fourth day, whereas activated  $\beta$ -amylase activity increased rapidly and then decreased. In contrast to activated  $\beta$ -amylase activity, latent  $\beta$ -amylase activity decreased on and after the third day of germination. Changes in  $\beta$ -amylase isozymes in embryos and endosperms during germination are

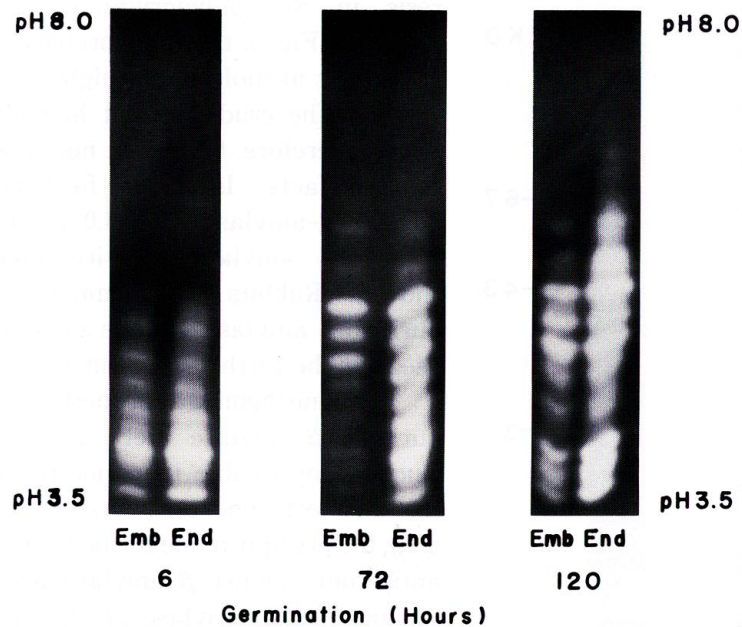


**Fig. 3.** Changes in  $\beta$ -amylase activities of germinating wheat embryos (A) and endosperms (B). Activities of total (○-○-), activated (▲-▲-) and latent (△-△-)  $\beta$ -amylase. The extraction and assay conditions are described in **Materials and Methods**.

shown in Fig. 4. In the case of the enzyme extracts containing  $\alpha$ -amylase activity, the extracts were partially purified until Phenyl-Sepharose CL-4B column chromatography (see **Materials and Methods**). There was no difference in the isozyme patterns from embryos and endosperms during germination. The  $\beta$ -amylase isozymes from 6-h germinated embryos and endosperms had the acidic isoelectric points and were identical to those from dry seeds. However, the isozymes from embryos and endosperms after 3 days germination had the acidic and neutral isoelectric points.

#### *Purification and some properties of $\beta$ -amylase from wheat seeds*

The purification process of  $\beta$ -amylase from dry wheat seeds is summarized in Table 1. The specific activity of  $\beta$ -amylase preparation after Sephadex G-100 column chromatography was 16,845 units/mg protein, representing overall purity of 6.31-fold from crude extract with 2.6% recovery. The crude extract and the purified preparation were analyzed by electropho-

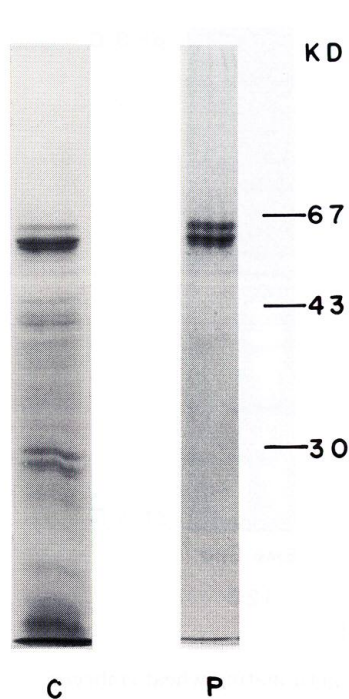


**Fig. 4.** Isoelectric focusing of  $\beta$ -amylase from germinating wheat embryos and endosperms.  
 Emb : isozymes of  $\beta$ -amylase from embryos, End : isozymes of  $\beta$ -amylase from endosperms. The experimental conditions are described in **Materials and Methods**.

**Table 1.** Purification procedure of wheat  $\beta$ -amylase

Step of purification	Total protein (mg)	Total activity (units $\times 10^{-5}$ )	Enzyme recovery (%)	Specific activity (units/mg)	Purity
Crude extract	$7.6 \times 10^3$	203	100	2670	1.00
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation	$4.9 \times 10^3$	172	84.9	3499	1.30
DEAE-Cellulose chromatography	768	44	21.7	5719	2.14
Dialyzate	297	16	7.9	5361	2.01
Hydrophobic chromatography	28.6	4.3	2.5	15047	5.64
Sephadex G-100	26.5	4.5	2.6	16845	6.31

One enzyme unit of amylase activity is the enzyme activity causing 10% decrease in blue of 20 mg/ml starch solution for 10 min at 40°C.



**Fig. 5.** SDS-polyacrylamide gel electrophoresis of the crude extract and the purified  $\beta$ -amylase. C: crude extract, P: purified  $\beta$ -amylase.

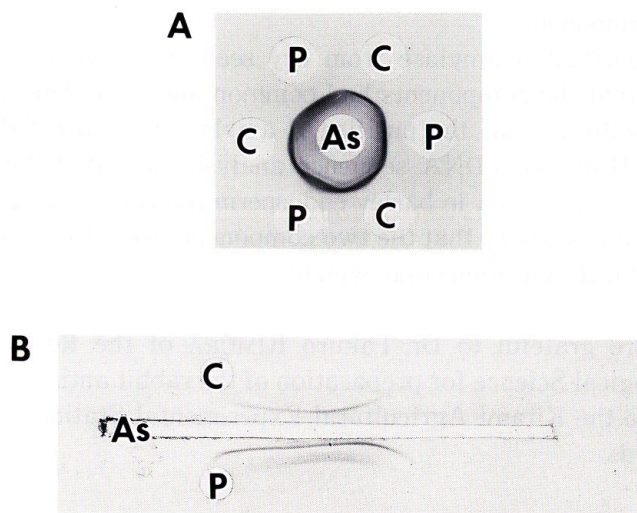
resis in SDS-polyacrylamide gel. As shown in Fig. 5, the final preparation has two bands at molecular weight 61,300 and 64,500. The crude extract had also two bands, therefore, they were not considered to be artifacts. Isoelectric focusing of the purified  $\beta$ -amylase at pH 4.0–7.5 showed 9 bands of amylase activity (data not shown). Rabbits were immunized by the purified  $\beta$ -amylase, and the antiserum was used for the further experiments.

Immunochemical properties of the purified  $\beta$ -amylase were examined by Ouchterlony double immunodiffusion and immunoelectrophoresis. As shown in Fig. 6(A), the precipitin line formed between the antiserum against  $\beta$ -amylase and either the purified  $\beta$ -amylase or the crude extract completely fused. Immunoelectrophoresis of the purified  $\beta$ -amylase and crude extract is shown in Fig. 6(B). A single arc with each the purified  $\beta$ -amylase and the crude extract formed. These data suggest that two components in the purified  $\beta$ -amylase preparation had the common antigenic determinants. In-

distinct line formed on the inside of precipitin line in double immunodiffusion and on the outside of arc in immunoelectrophoresis considered to be due to polymerization of  $\beta$ -amylase.

### Discussion

Activated  $\beta$ -amylase activity increased rapidly at the fourth week of development and decreased at the fifth week. Alternatively, there was an increase in latent activity at the fifth week. In germinating endosperms, activated  $\beta$ -amylase activity increased after the second day, whereas latent activity decreased after the third day. In zymograms of activated and total  $\beta$ -amylase, no difference was found. OKAMOTO and AKAZAWA (1979) demonstrated the association of  $\beta$ -amylase with the starch granules of rice. HARA-NISHIMURA *et al.* (1986) also showed that  $\beta$ -amylase is associated with



**Fig. 6.** Ouchterlony double immunodiffusion (A) and immunoelectrophoresis (B).  
C: crude extract, P: purified  $\beta$ -amylase and As: rabbit anti- $\beta$ -amylase antiserum.

starch granules of dry barley seeds, the association occurs during the desiccation phase of seed development. Therefore, it was suggested that the activated enzyme synthesized during development was stored in dry seed at latent form, and the latent enzyme was converted to the activated form during germination.

In isoelectric focusing of  $\beta$ -amylase isozymes from dry seeds and 6-h germinated embryos and endosperms, isoelectric points of main bands were acidic, whereas they were neutral in 5-day germinated seeds. DAUSSANT and MACGREGOR (1979) observed a change in electric mobility of  $\beta$ -amylase during germination. Zymogram of the purified  $\beta$ -amylase treated with papain was similar to that of 5-day germinated seeds (data not shown). BILDERBACK (1971) demonstrated that the treatment of strachy endosperm with patain yield four forms of  $\beta$ -amylase. Therefore, it was suggested that the  $\beta$ -amylase from 5-day germinated seeds would be affected by proteolytic activity. Recently, KREIS *et al.* (1987) isolated  $\beta$ -amylase cDNA clones from a barley endosperm cDNA library, and deduced four glycine-rich repeat

sequences of 11 residues at the C-terminus. It was suggested that the repeat sequences may be involved in the interaction with proteolytic enzyme and cellular component.

The purified  $\beta$ -amylase from dry seeds had two components, and it appeared that the components had common antigenic determinants. There was a possibility that the purified  $\beta$ -amylase was affected by proteolytic activity. However, cDNA sequence analysis indicated the presence of at least two types mRNA in barley endosperm (KREIS *et al.*, 1987). Therefore, there was a possibility that the two components were isozymes of  $\beta$ -amylase which had different molecular weight.

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#### References

- BILDERBACK, D. E. 1971. Amylases in developing barley seeds. *Plant Physiol.* **48**: 331-334.
- DAUSSANT, J. and CORVAZIER, P. 1970. Biosynthesis and modifications of  $\alpha$ - and  $\beta$ -amylases in germinating wheat seeds. *FEBS Letters* **7**: 191-194.
- and MACGREGOR, A. W. 1979. Combined immunoabsorption and isoelectric focusing of barley and malt amylases in polyacrylamide gel. *Anal. Biochem.* **93**: 261-266.
- FILNER, F. and VARNER, J. E. 1967. A test for *de novo* synthesis of enzymes: density labeling with  $\text{H}_2\text{O}^{18}$  of barley  $\alpha$ -amylase induced by gibberellic acid. *Proc. Nat. Acad. Sci. USA* **58**: 1520-1526.
- FUJIOKA, T., UCHIDA, K., ISHIKAWA, H. A. and ISHIKAWA, K. 1984. Immunochemical studies of  $\alpha$ -amylase from germinating wheat seeds. *J. Fac. Sci. Hokkaido Univ. Ser. V (Botany)* **13**: 281-292.
- FUWA, H. 1954. A new method for microdetermination of amylase activity by the use of amylose as the substrate. *J. Biochem.* **41**: 583-603.
- HARA-NISHIMURA, I., NISHIMURA, M. and DAUSSANT, J. 1986. Conversion of free  $\beta$ -amylase to bound  $\beta$ -amylase on starch granules in the barley endosperm during desiccation phase of seed development. *Protoplasma* **134**: 149-153.
- KATO, M., HIROMI, K. and MORITA, Y. 1974. Purification and kinetic studies of wheat bran  $\beta$ -amylase. Evaluation of subsite affinities. *J. Biochem.* **75**: 563-576.
- KREIS, M., WILLIAMSON, M., BUXTON, B., PYWELL, J., HEJGAARD, J. and SVENDSEN, I. 1987. Primary structure and differential expression of  $\beta$ -amylase in normal and mutant barleys. *Eur. J. Biochem.* **169**: 517-525.
- LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of

- bacteriophage T4. *Nature* **227** : 680-685.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193** : 265-275.
- OKAMOTO, K. and AKAZAWA, T. 1979. Enzymic mechanism of starch breakdown in germinating rice seeds. 8. Immunohistochemical localization of  $\beta$ -amylase. *Plant Physiol.* **64** : 337-340.
- OUCHTERLONY, O. and NILSON, L. A. 1967. Immunodiffusion and immunoelectrophoresis. *In* : Handbook of Experimental Immunology ed. WEIR, D. M., 655-706. Blackwell Science Publication, *Oxford*.
- ROWSELL, E. V. and GOAD, L. J. 1962. The constituent of wheat binding latent  $\beta$ -amylase. *Biochem. J.* **84** : 73.
- UCHIDA, K., ISHIKAWA, H. A. and ISHIKAWA, K. 1987. Immunological characterization of  $\alpha$ -amylase isozymes in developing and germinating wheat seeds. *J. Fac. Sci. Hokkaido Univ. Ser. V (Botany)* **140** : 89-94.
- VARNER, J. E. and CHANDRA, G. R. 1964. Hormonal control of enzyme synthesis in barley endosperm. *Proc. Nat. Acad. Sci. USA* **52** : 100-106.
- VISURI, K. and NUMMI, M. 1972. Purification and characterization of crystalline  $\beta$ -amylase from barley. *Eur. J. Biochem.* **28** : 555-565.