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Effects of the germination time on enzyme stabilities in extracts from wheat endosperms

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Abstract

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Seeds of wheat (*Triticum aestivum* L.) were germinated and endosperm samples were harvested at daily intervals. Various enzymes were investigated with respect to their stability "in vitro" and their susceptibility to proteolytic inactivation by endopeptidase activity, which increases during germination. The stability of some enzymes in endosperm extracts was not obviously influenced by germination time (e.g. acid phosphatase, phytase, peroxidase, malate dehydrogenase). A decreasing stability during germination was observed for leucine aminopeptidase, pyrophosphatase and glucose-6-phosphate dehydrogenase. It appears likely that the accelerated inactivation of these enzymes in endosperm extracts from germinated wheat seeds was caused by the increased endopeptidase activity. Especially glucose-6-phosphate dehydrogenase can be considered as an interesting model enzyme to study proteolytic inactivation and its regulation.

Key words: endopeptidase – enzyme inactivation – germination – proteolysis – wheat

Introduction

A remarkable increase in endopeptidase activity during germination was observed in the seeds of various plant species (Sundblom and Mikola 1972, Harvey and Oaks 1974, Basha and Beevers 1975, Chrispeels and Boulter 1975, Ashton 1976). In the caryopsis of cereals this enzyme was found to be synthesized by the scutellum and by the aleurone layer (Jacobsen and Varner 1967, Okamoto et al. 1980). Endopeptidase is then released from the cells of synthesis into the whole endosperm (Okamoto et al. 1980). The main endopeptidase present in germinating seeds is most active around pH 5 (Sundblom and Mikola 1972, Chrispeels and Boulter 1975, Feller 1979). A minor activity in the neutral pH-range with other properties was detected in extracts from germinating seeds (Sundblom and Mikola 1972, Feller 1979). The activities of several other catabolic enzymes (e.g. α-amylase, ribonuclease, phytase, carboxypeptidase) in-

crease also in the storage tissues of germinating seeds, while some enzyme activities decrease during germination (Okamoto et al. 1980, Feller 1981, Murray 1984).

A rapid inactivation by endogenous endopeptidases was shown for several plant enzymes (Wallace 1978, Batt and Wallace 1983, Alpi and Beevers 1981, Feller 1981, Wray and Kirk 1981, Streit and Feller 1982). Some enzymes are very susceptible to proteolytic inactivation, while others remain active under the same conditions (Streit and Feller 1983). Leucine aminopeptidase was found to be relatively stable at pH 5.4 in extracts from ungerminated bean seeds, while the same enzyme was rapidly inactivated in extracts from cotyledons of beans germinated for several days (Feller 1981). From further experiments it was concluded that the accelerated inactivation was most likely due to the endopeptidase formed during germination. The actual pH, the presence of other proteins and the concentrations of solutes may affect the inactivation of a particular enzyme by a given endopeptidase activity (Holzer and Heinrich 1980, Streit and Feller 1982, Feller and Keist 1986). The susceptibility to proteolytic inactivation must be considered as an important property of enzyme proteins.

The objectives of this work were to compare the time courses during germination for the activities and the "in vitro"-stabilities of various enzymes extracted from wheat endosperms. It is of special interest to know for which enzymes the stability decreases when endopeptidase activity increases.

Materials and methods

Plant material

Seeds of winter wheat (*Triticum aestivum* L., cv. "MV 4") were imbibed for 4 h in tap water at 37 °C. The water was replaced in intervalls of one hour. The soaked seeds were germinated at 24 °C in the dark in containers with wet tissue paper. Samples were taken daily and stored frozen (–20 °C) prior to extraction.

Extraction

The seedlings and the scutella were removed and discarded. The endosperms from 30 grains were extracted in 10 ml acetate buffer (100 mM, pH 5.4) containing 1% (w/v) polyvinylpolypyrrolidone and 0.1% (v/v) mercaptoethanol. For extraction at pH 7.5 the acetate buffer was replaced by Tris/HCl buffer at the same concentration. The endosperms were homogenized with a Polytron mixer (Kinematica, Luzern) for 20 s at medium speed and for 5 s at full speed. The homogenates were passed through Miracloth (Calbiochem, San Diego) and centrifuged for 10 min at 2500 × g. The supernatants were desalted through Sephadex G-25 columns according to Feller et al. 1977. The columns were equilibrated with extraction buffer, but polyvinylpolypyrrolidone and mercaptoethanol were omitted. Extraction and desalting were performed at 0 to 4 °C. The desalted extracts were used for the investigation of enzyme stabilities.

Enzyme assays

All assays were adapted to microtitration plates by appropriate reductions of the volumes. The absorbances were read in a Multiskan multichannel photometer (Flow Laboratories, Baar).

The substrate solution for endopeptidase measurements contained 0.5% (w/v) azocasein and 0.1% (v/v) mercaptoethanol in 100 mM acetate buffer pH 5.4. For the assays 50 µl desalted extract was mixed with 100 µl substrate solution and kept at 25 °C for 3 h. In assays of the azocaseinase activity at pH 7.5 a 100 mM Tris/HCl buffer pH 7.5 replaced the acetate buffer and mercaptoethanol was omitted (Feller 1981). The reaction was terminated by the addition of 75 µl trichloroacetic acid 15% (w/v). For blanks the substrate solution was incubated separately and the enzyme

extract was added immediately before stopping. The plates were kept for 10 min at 2 °C before removing the denatured proteins by centrifugation for 25 min (750 × g, 6 °C) in a refrigerated bench centrifuge with special holders for microtitration plates. With a multichannel pipette 100 µl samples from the supernatants were transferred into a flat bottom microtitration plate and mixed with 100 µl 1 N NaOH. The enzyme activities were calculated from the absorbances at 450 nm.

The aminopeptidase substrate was prepared as suggested by Chrispeels and Boulter (1975) and contained 2 mM L-leucine-p-nitroanilide in 50 mM phosphate buffer pH 7.0 with 1% (v/v) dimethylsulfoxide. L-leucine-p-nitroanilide was first dissolved in dimethylsulfoxide and afterwards the phosphate buffer was added. For the enzyme assays 200 µl substrate solution was brought into each well of a microtitration plate and preincubated for 5 min at 37 °C. The reaction was initiated by the addition of 20 µl desalted extract and the plate was kept at 37 °C. The formation of the yellow product (p-nitroaniline) was detected by reading the absorbances at 405 nm with the multichannel photometer at various incubation times up to 2 h. The enzyme activities were calculated using a calibration curve with up to 50 nMol p-nitroaniline per well.

The substrate solution for the pyrophosphatase assays contained 50 mM Tris/HCl buffer pH 8.0, 50 mM $MgCl_2$ and 1.5 mM sodium pyrophosphate. Mixtures of 20 µl enzyme extract and 100 µl substrate solution were incubated for 30 min at 37 °C in a flat bottom microtitration plate. The reaction was terminated by the addition of 50 µl vanadate reagent according to Kali-Briefe (1971). Blanks were stopped immediately after mixing enzyme and substrate solutions. The liberation of inorganic phosphate was calculated from the absorbances at 405 nm.

Phytase activity was detected by the liberation of inorganic phosphate from phytic acid. The substrate solution (100 µl) with 100 mM acetate buffer pH 5.4, 1 mM sodium phytate and 2 mM magnesium sulfate was mixed with 30 µl enzyme extract and incubated for 60 min at 37 °C. The formation of inorganic phosphate was analyzed as described above for pyrophosphatase.

Acid phosphatase was assayed using a substrate solution with 20 mM p-nitrophenyl phosphate in 100 mM acetate buffer pH 5.4. From the 1:2 diluted enzyme extract 5 µl were mixed with 100 µl substrate solution and incubated for 30 min at 21 °C. After addition of 100 µl 1 N NaOH the absorbances were read at 405 nm.

The peroxidase substrate solution contained 42.5 mM phosphate buffer pH 7.5, 15 mM guajacol and 5 mM hydrogen peroxide (Fuhrer 1982). A mixture of 10 µl enzyme extract and 200 µl substrate solution was kept at room temperature (21 °C) and measured repeatedly up to 150 s. The enzyme activity was calculated from the measurements at 30 and at 150 s after mixing. These enzyme activities are shown in relative units.

Glucose-6-phosphate dehydrogenase and malate dehydrogenase were measured with the optical test at 366 nm (Bergmeyer 1970). The assays for glucose-6-phosphate dehydrogenase were prepared by mixing 150 µl phosphate buffer pH 7.5 (100 mM with 10 mM magnesium chloride), 20 µl NADP (10 mg/ml), 10 µl glucose-6-phosphate (10 mg/ml) and 20 µl desalted enzyme extract. For malate dehydrogenase 165 µl phosphate buffer pH 7.5 (100 mM), 20 µl oxaloacetate (2 mg/ml), 10 µl NADH (10 mg/ml) and 5 µl diluted (1 : 5) enzyme extract were combined. The dehydrogenase assays were incubated at 25 °C and measured repeatedly at 366 nm. The activities were calculated from the slopes.

Results

The endopeptidase acitivity at pH 5.4, which was low in ungerminated seeds and increased to a high level during the first 3 days of germination, was quite stable in extracts kept at 0 °C (Fig. 1). Most of this activity was lost when the same extracts were incubated at 37 °C. This endopeptidase may inactivate other enzymes. Therefore it should be noticed that this potentially inactivating enzyme is relatively unstable at elevated temperatures. About 50% of the initial activity were lost within 3 h at 37 °C.

Endopeptidase activity was also measured at pH 7.5 (data not shown). This activity was below the detection limit in endosperm extracts from ungerminated seeds, in-

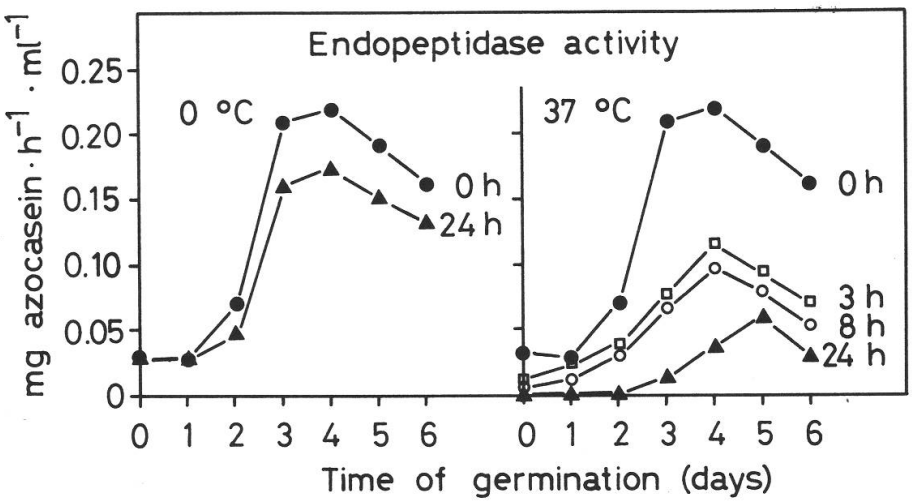

Fig. 1. Stability of endopeptidase in endosperm extracts from germinating wheat. Endosperms were harvested in intervals of one day during a germination period of 6 d. The samples were stored frozen (−20 °C) prior to extraction. The exracts (pH 5.4) were analyzed immediately after preparation (0 h) and after preincubation at 0 °C (left side) or 37 °C (right side). The preincubation times up to 24 h are indicated in the diagrams.

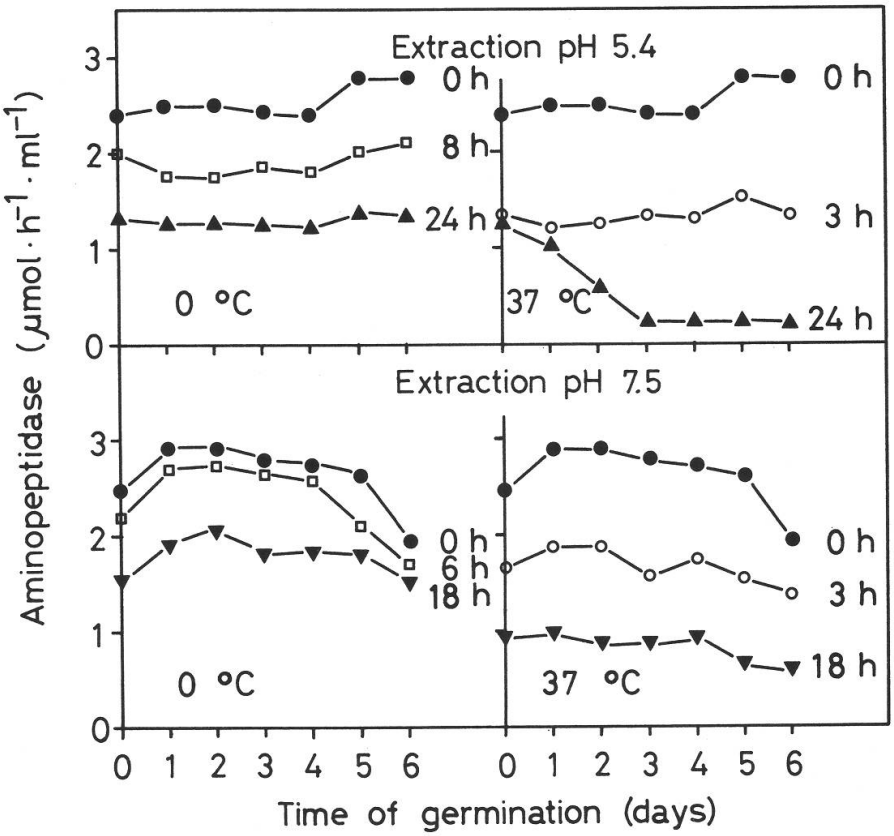

Fig. 2. Stability of aminopeptidase in endosperm extracts from germinating wheat. Endosperms were harvested in intervals of one day and the samples were stored frozen (−20 °C) prior to extraction. The extracts (pH 5.4 and pH 7.5) were analyzed immediately after preparation and after preincubation at 0 °C (left side) or 37 °C (right side). The preincubation times are indicated in the diagrams.

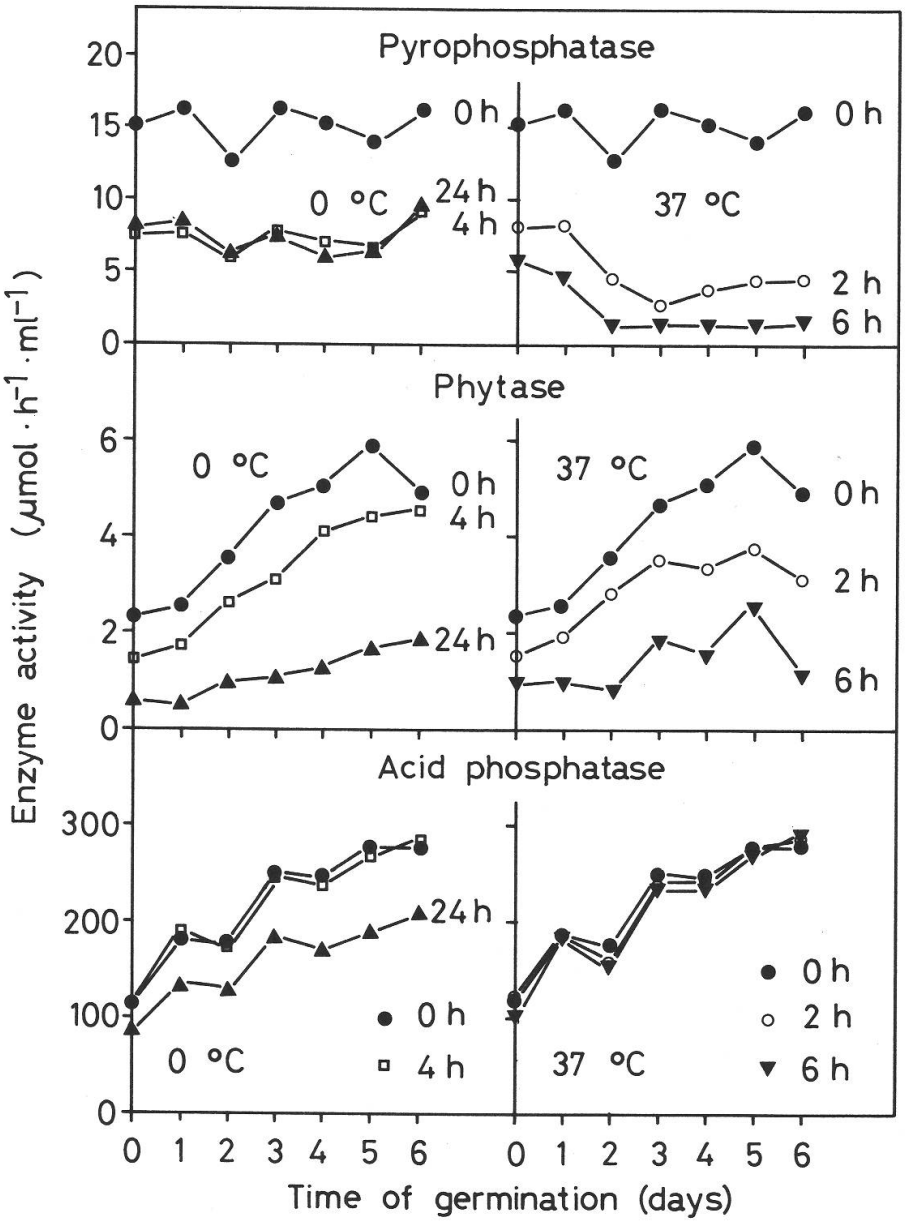

Fig. 3. Stability of phosphatases in endosperm extracts from germinating wheat. Endosperms were harvested in intervals of one day and the samples were stored frozen (–20 °C) prior to extraction. The extracts (pH 5.4) were analyzed immediately after preparation and after preincubation at 0 °C (left side) or 37 °C (right side). Alkaline pyrophosphatase, phytase and acid phosphatase activities were measured using standard procedures. The preincubation times are indicated in the diagrams.

creased during germination and reached a maximum (0.087 mg · ml^{-1} · h^{-1}) at day 5 and remained active during preincubation at 0 °C and at 37 °C for 24 h. Azocaseinase activity was always considerably lower at pH 7.5 than at pH 5.4 in freshly prepared extracts, but the stability "in vitro" was better for the activity measured at pH 7.5.

Leucine aminopeptidase is most active in the neutral pH range. This enzyme activity remained high in wheat endosperms during a germination time of 6 d (Fig. 2). The stability "in vitro" was better at 0 °C than at 37 °C. No major effects of the germination time on aminopeptidase inactivation were observed in extracts at pH 7.5. On the other

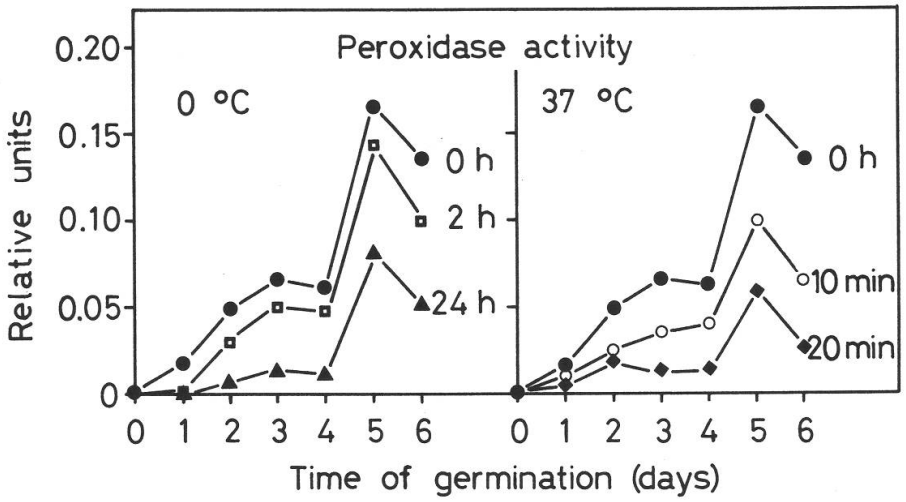

Fig. 4. Stability of peroxidase in endosperm extracts of germinating wheat. Endosperms were harvested daily during germination and kept at –20 °C prior to extraction. The extracts (pH 5.4) were analyzed immediately after preparation and after preincubation at 0 °C (left side) and at 37 °C (right side). The preincubation times are indicated in the diagrams.

hand, in extracts prepared at pH 5.4 this enzyme activity was more rapidly lost at 37 °C, when the seeds were germinated for several days. This result is consistent with findings reported earlier for bean seeds (Feller 1981). The rather constant loss of activity at 0 °C and also during the first 3 h at 37 °C is surprising. Perhaps the low endopeptidase activity present in ungerminated seeds was sufficient or this inactivation was caused by factors other than proteolysis catalyzed by the main endopeptidase. In this context it must be considered that several aminopeptidase forms are present in legume seeds (Collier and Murray 1977, Kruger and Preston 1978, Murray and Waters 1985) and these forms may behave differently with respect to proteolytic inactivation.

Carboxypeptidase activity (measured with N-carbobenzoxy-L-phenylalanine-L-alanine as substrate) remained relatively constant in our samples during germination (data not shown). This exopeptidase was very stable "in vitro" at pH 5.4 as well as at pH 7.5. More than 80% of the initial activity was detected after preincubation for 24 hours at 0 °C or at 37 °C. Therefore this peptide hydrolase was not further investigated in our system.

The time courses and the stabilities "in vitro" at pH 5.4 were compared for 3 phosphate liberating enzymes (Fig. 3). Pyrophosphatase plays a major role in biosynthetic processes and is highly active in the alkaline pH-range (Walsh 1979). This enzyme behaved similarly to aminopeptidase. Its activity remained high during the first 6 days of germination. At 37 °C its stability was better in endosperm extracts of ungerminated seeds than in those prepared after germination. As already mentioned for aminopeptidase, pyrophosphatase stability at 0 °C was similar in extracts from germinated and from ungerminated seeds. Phytase and acid phosphatase are most active in the slightly acidic pH-range. The activities of these two enzymes increased remarkably during germination and reached their maximum later than endopeptidase. Their stabilities were not influenced by the germination time. Despite the similarities observed between phytase and acid phosphatase they differ clearly in their inactivation rates under given preincubation conditions. While acid phosphatase was very stable "in vitro", phytase was much more rapidly inactivated in the same extracts.

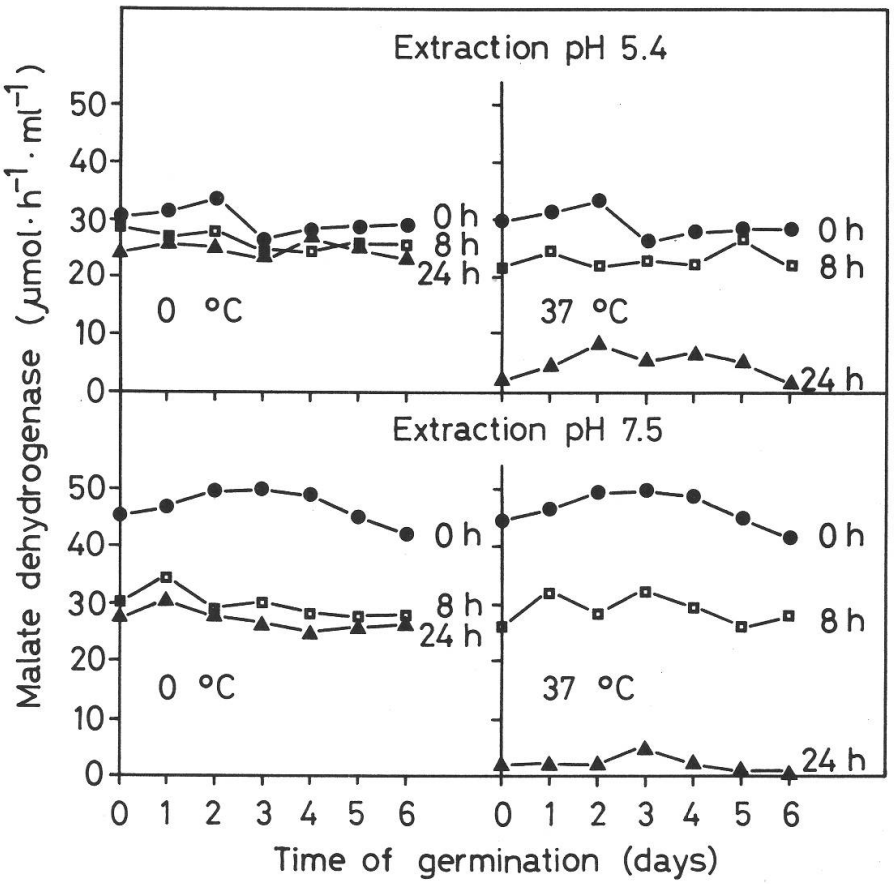

Fig. 5. Stability of malate dehydrogenase in endosperm extracts from germinating wheat. Endosperms were harvested in intervals of one day and the samples were stored frozen (–20 °C) prior to extraction. The extracts (pH 5.4 and pH 7.5) were analyzed immediately after preparation and after preincubation at 0 °C (left side) or 37 °C (right side). The preincubation times are indicated in the diagrams.

Peroxidase (measured at pH 7.5) was found to be interesting from different points of view (Fig. 4). The most pronounced increase during germination was observed for this enzyme activity. Peroxidase was very unstable in extracts (pH 5.4). More than 50% of the initial activity were lost within 24 h at 0 °C. When the same extracts were incubated at 37 °C, the activity dropped below 50% within 10 to 20 min. In extracts at pH 7.5 peroxidase stability was found to be even poorer: 70–90% were lost within 1 h at 0 °C and no activity was detectable after 10 min at 37 °C (data not shown). Under the conditions used peroxidase was the most unstable of the enzymes tested, but no major effect of the germination time on this very rapid inactivation was observed in our experiments.

Total malate dehydrogenase activity remained relatively constant in wheat endosperms during germination (Fig. 5). The activity was always about 50% higher in freshly prepared extracts at pH 7.5 than in those at pH 5.4. In extracts at pH 5.4 this activity was stable at 0 °C, but decreased below 20% within 24 h at 37 °C. At pH 7.5 the activity dropped within 8 h at 0 °C to the level observed in fresh extracts at pH 5.4 and remained quite stable afterwards. At 37 °C the initial inactivation up to 8 h was faster at pH 7.5 than at pH 5.4 and between 8 and 24 h the loss of activity was similar in both pH-ranges. It appears likely that the behaviour observed for total malate dehydroge-

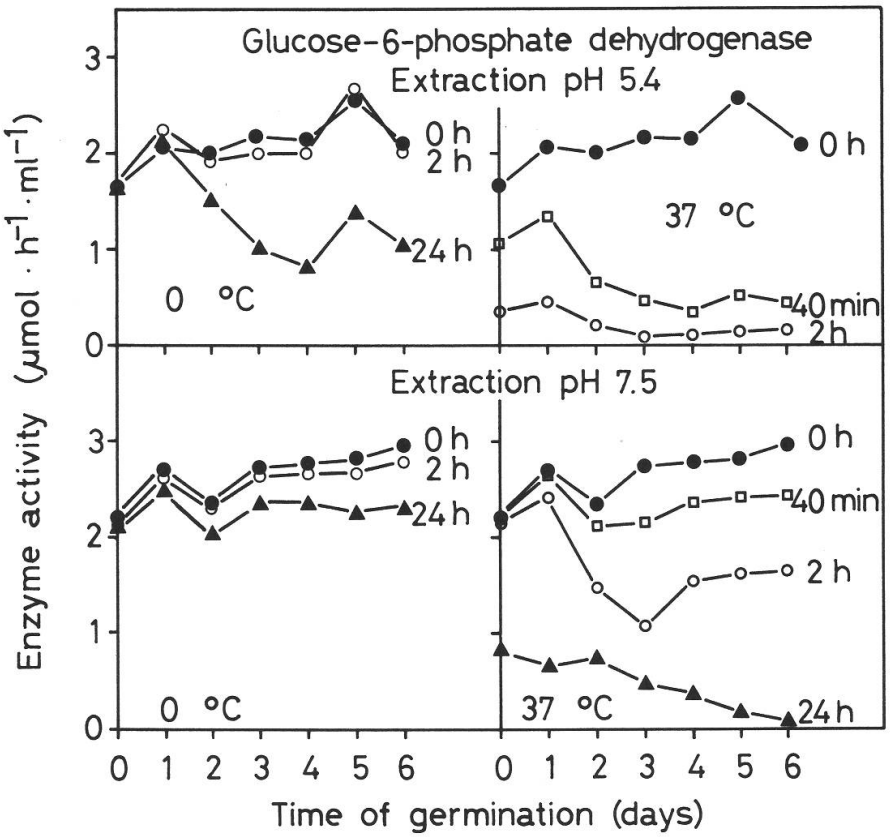

Fig. 6. Stability of glucose-6-phosphate dehydrogenase in endosperm extracts from germinating wheat. Endosperms were harvested in intervals of one day and the samples were stored frozen (−20 °C) prior to extraction. The extracts (pH 5.4 and pH 7.5) were analyzed immediately after preparation and after preincubation at 0 °C (left side) or 37 °C (right side). The preincubation times are indicated in the diagrams.

nase activity during preincubation at pH 7.5 was caused by different properties of various forms present in the extracts. In all cases, the inactivation of malate dehydrogenase was not accelerated by the increased endopeptidase activities present in endosperm extracts from seeds germinated for several days.

Glucose-6-phosphate dehydrogenase is a very interesting enzyme with respect to its inactivation behaviour (Fig. 6). The activities were slightly higher in extracts at pH 7.5 than in those at pH 5.4. No major activity changes occured during germination. The stability was always much better in endosperm extracts from ungerminated than in those from germinated wheat seeds. This enzyme was more rapidly inactivated at pH 5.4 than at pH 7.5. The stability was considerably higher at 0 °C than at 37 °C. The remarkable decrease in the stability of glucose-6-phosphate dehydrogenase after 2 d of germination concurs with an increase in endopeptidase activity. The accelerated inactivation is most likely due to proteolysis catalyzed by the endopeptidase formed.

Discussion

The stabilities of the different enzymes tested vary in a wide range and are differently affected by pH and temperature during preincubation. In the group of en-

zymes which show increasing activities in wheat endosperms during germination, acid phosphatase is very stable, while others (e.g. phytase, peroxidase) are much more labile under the same conditions. An accelerated inactivation by the endopeptidase formed in germinating seeds was not obvious for this group of enzymes, since the relative stabilities were similar throughout the germination period investigated. These enzyme stabilities may be affected "in vivo" by the actual environment (e.g. by redox conditions, pH, presence of other proteins, concentrations of substrates). Since there was no clear correlation between the stabilities of these enzymes and the harvest time, the activity of the major endopeptidase appears to be uncritical. Perhaps the inactivation was initiated in these cases by factors other than proteolytic attack.

The other enzymes investigated, which do not increase in activity during germination, behave interestingly "in vitro". The stability of malate dehydrogenase was not dependent on germination time. On the other hand, leucine aminopeptidase, pyrophospatase and glucose-6-phosphate dehydrogenase became more rapidly inactivated in endosperm extracts from germinated than in those from ungerminated seeds. This effect was most pronounced for glucose-6-phosphate dehydrogenase. The results mentioned suggest an inactivation by the endopeptidase activity increasing during germination as it was observed earlier for a series of enzymes in different systems (Alpi and Beevers 1981, Feller 1981). The inactivation of glucose-6-phosphate dehydrogenase by endogenous and exogenous endopeptidases remains to be further elucidated in future experiments. The inactivation by endopeptidases could play an important role in the regulation of enzyme patterns.

Nitrate reductase and glutamine synthetase were found to be protected from proteolytic inactivation by their cofactors (Streit and Feller 1982). Effects of low molecular weight compounds on the hydrolysis of a particular enzyme protein should generally be considered as important factors for the control of proteolysis "in vivo". From preliminary experiments it can be concluded that glucose-6-phosphate dehydrogenase extracted from wheat endosperms represents an interesting model system in this context.

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