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# Starch Degradation in the Cotyledons of Germinating Lentils

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## ABSTRACT

**Starch, total amylolytic and phosphorylase activities were determined in lentil cotyledons during the first days of germination. Several independent criteria show that the amylolytic activity is due mainly to an amylase of the α type. Starch is degraded slowly in the first days; during this time, α- and β-amylase activity are very low, while phosphorylase increases and reach a peak on the 3rd day. On the 4th day, there is a more rapid depletion of starch which coincides with an increase in α-amylase activity. By polyacrylamide gel electrophoresis of the crude starch-degrading enzyme, five bands were obtained: one phosphorylase, three α-amylases, and one β-amylase. Based on their heat lability or heat stability, two sets of α-amylase seem to exist in lentil cotyledons.**

The enzymic degradation of starch in higher plants is due mainly to the phosphorylases and the amylases. They have been studied particularly in seeds, where they are responsible for the breakdown of polysaccharide reserves. Much of our knowledge of starch degradation during seed germination comes from studies with cereals (6, 7, 15, 16, 18, 19, 23) where a number of amylase isozymes have been described. In contrast, available knowledge of starch catabolism in legumes is very limited and almost reduced to the studies with germinating peas (13, 20-22).

The major aim of the work presented here was to determine the mechanism of breakdown of reserve starch in the cotyledons of germinating lentil seeds, in order to improve our knowledge of the carbohydrate catabolism in this group of higher plants. Analyses of starch and activities of starch-degrading enzymes were carried out during the course of germination. Phosphorylases and amylases were separated by polyacrylamide gel electrophoresis to obtain information about the various starch-degrading enzymes of germinating lentil seeds.

## MATERIALS AND METHODS

Lentil seeds (*Lens culinaris*, Medik = *Lens esculenta*, Moench) were soaked for 4 hr in a disinfectant solution (0.12% Orthocide-50 wettable, a commercial fungicide) at room temperature. The seeds were then germinated in the dark at 25 C on moist filter paper in Petri dishes for different periods of time, and then separated into cotyledons and embryos. Only the cotyledons were used for all subsequent assays. All operations were performed at 0 to 4 C unless indicated otherwise.

**Preparation of Crude Enzyme.** The cotyledons (1 to 8/ml depending on the germination time) were homogenized with 20 ml of unbuffered distilled $H_2O$ in a Sorvall Omni-Mixer. The whole homogenate was strained through 2 layers of cheesecloth, and then centrifuged in a Sorvall RC2-B refrigerated centrifuge at 20,000*g* for 30 min at 2 C.

In order to differentiate between α- and β-amylase, the supernatant was treated selectively with 10 mM EDTA, 0.1 mM $HgCl_2$, 3 mM $CaCl_2$, and heated at 70 C in the presence of calcium ions.

**Amylase Assay.** Two aliquots of the supernatant were removed, and one was heated for 5 min at 70 C in the presence of 3 mM $CaCl_2$ to inactivate β-amylase. After 10-min incubation at 30 C in a water bath, total amylolytic activity in both fractions, and the separate activities of α- and β-amylase were assayed by the method of Bernfeld (2) using 1% (w/v) potato starch solution in 0.05 M acetate buffer (pH 5) as substrate.

β-Amylase was extracted and prepared from the cotyledons by the method of Swain and Dekker (21) by the addition to the homogenization media of 1 mM EDTA to inactivate α-amylase and 5 mM 2-mercaptoethanol. The activity was assayed as previously described.

Enzymic activity was expressed as mg of maltose released/10 min and /mg protein. Protein was determined by the method of Lowry *et al.* (17).

**Phosphorylase Assay.** The cotyledons of 40 seeds were homogenized in 20 ml of unbuffered distilled $H_2O$ containing 1 mM EDTA and 5 mM 2-mercaptoethanol. The enzymic activity was determined according to the method of Wheland (24). The reaction mixture contained: citrate buffer, 0.05 M (pH 6), 1 ml; glucose-1-P, 0.1 M, 0.25 ml; 0.25 ml of 5% soluble starch; 1 ml of crude enzyme extract (diluted if necessary). After 10-min incubation at 35 C in a water bath, the activity was expressed as mg of Pi liberated, determined by the method of Allen (1) per 10 min and per 2 seeds.

**Starch and Dry Weight Determinations.** The starch content of the cotyledons of lentil seeds was determined by the method of Dekker and Richards (4). Ten cotyledons (approximately 540 mg) were extracted three times with 10 ml of 0.5 N NaOH during 30 min; after neutralization with acetic acid and centrifugation, the three supernatants were pooled, and two aliquot portions were removed for subsequent starch and free glucose analysis.

Dry weight was estimated on samples of 20 seeds from the difference in weight immediately after sampling, and after drying in an oven at 70 C until constant weight.

**Polyacrylamide Gel Electrophoresis.** Polyacrylamide gel disc electrophoresis was performed by the method of Davis (3) using a 7% small-pore gel. The electrophoresis was carried out for 1 hr and 15 min at 4 C under a constant current of 3 mamp/gel.

The enzymic extracts were prepared as previously described for the amylase assay. Before layering into the gel tubes, the samples were preincubated with 10 mM EDTA, 0.1 mM $HgCl_2$, or 3 mM $CaCl_2$ for 1 hr at room temperature. The sample treated with calcium ions was heated for 5 min at 70 C.

After electrophoresis, the zones of phosphorylase and amylase activity were revealed by incubation of the gels with a 1% (w/v) solution of soluble starch in acetate buffer, 0.1 M (pH 5), for 2 hr and 30 min at 30 C. After incubation, the gels were rinsed in distilled $H_2O$, immersed for a few seconds in an $I_2$-KI solution, and rinsed again in distilled $H_2O$. The iodine stains the gel heavily except in the zones of phosphorylase or amylase activity.

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## RESULTS

**Phosphorylase and Amylase Activity in Cotyledons of Germinating Lentils.** Amylase level in resting seeds was very low and remained constant during the first 2 days of germination and increased to a maximum level by the 6th day (Fig. 1A). By contrast, phosphorylase activity was higher in resting seeds, and its level increased to a maximum by the 3rd day, and then decreased slowly. The period of higher amylase activity coincided with the decrease in phosphorylase activity. Similar patterns of changes in both enzymes during germination have been found in pea (13) and rice (18).

Although the amylase assay used in this work is valid for both $\alpha$- and $\beta$-amylase, the results obtained after the treatment of the enzymic extracts with different agents seems to indicate that this activity is due to an amylase of the $\alpha$ type. In the presence of 1.5 mM $CaCl_2$, 0, 85, and 96% of the total amylase activity remained after heating at 70 C for 5 min in extracts of 0, 48, and 72 hr of germination, respectively (data not shown). All activity was conserved after treatment with 5 mM $HgCl_2$, and finally, all enzymic activity was lost by the treatment with 5 mM EDTA (data not shown). Collectively, these results establish according to Frydenberg and Nielsen (7) and Swain and Dekker (20) that the amylase(s) present in the extracts from lentil seeds belong to the $\alpha$ type. The total disappearance of activity in extracts from resting seeds by heating at 70 C for 5 min seemed to indicate a heat lability, although it also could be due to the concomitant low activity present in these extracts (0.03 mg maltose/mg protein·10 min), and the slight inactivation always obtained after heating. A further proof that calcium ions protect the $\alpha$-amylase against heat inactivation is shown in Figure 1D, 60% of the activity remained after 60 min of incubation at 70 C, whereas in the absence of calcium ions all enzymic activity was completely lost in 15 min. On the other hand the calcium ions had no effect on the amylase activity when added to the enzymic extracts (not shown). The effect of pH on the enzymic activity is shown in Figure 1C. A peak of maximal activity is obtained in the range of pH 4.6 to 5.4.

We were not able to detect $\beta$-amylase activity, probably due to the low amount present in our extracts.

**Changes in Dry Weight and Starch Content in Germinating Lentil Seeds.** The changes in dry weight consisted of a slow

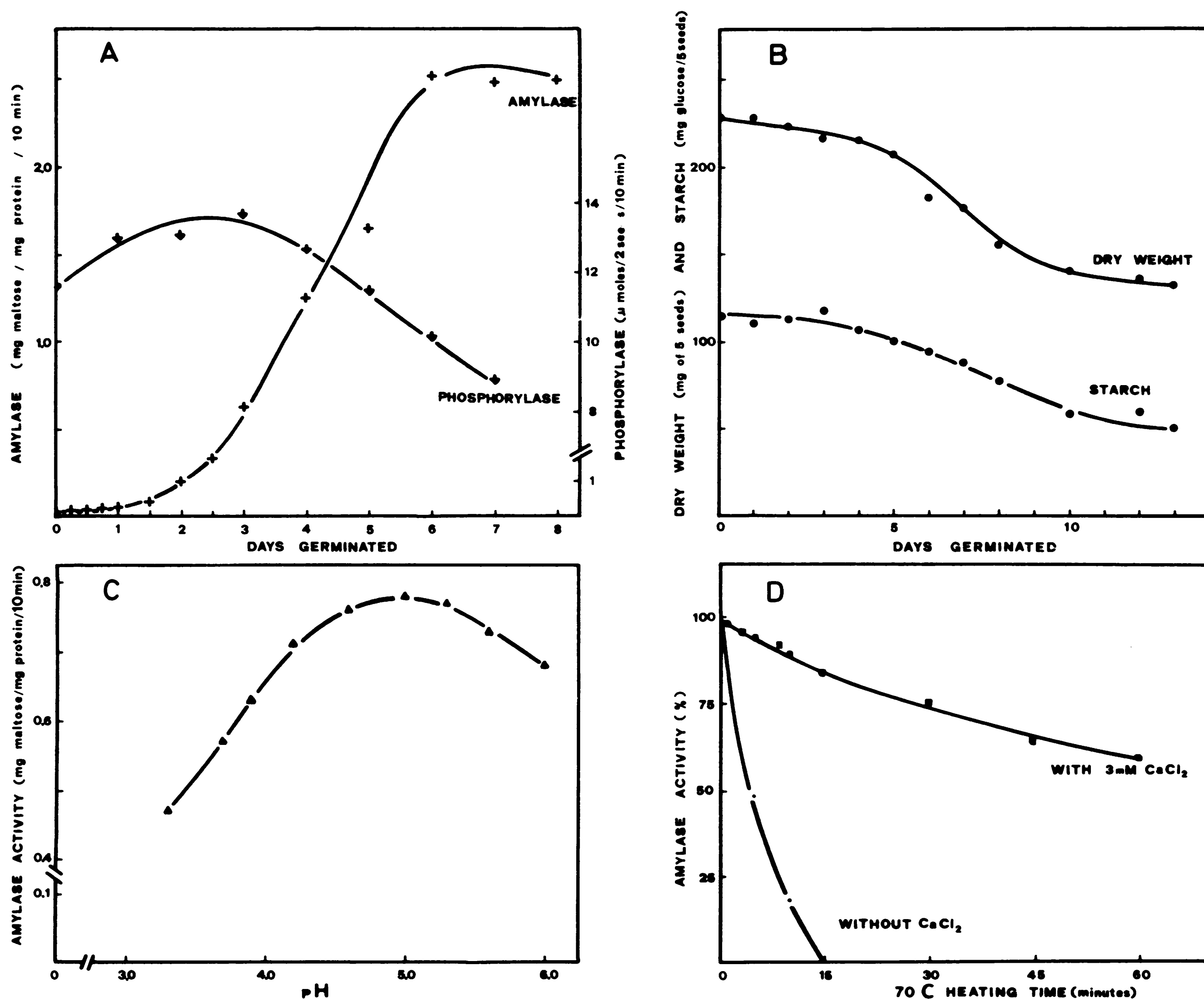


FIG. 1. A: changes in total amylase and phosphorylase in the germinating lentil cotyledons. B: changes in total dry weight and starch in the germinating lentil cotyledons. C: activity of lentil cotyledon amylase as a function of pH. D: effect of calcium ions on the stability of $\alpha$-amylase against heating at 70 C.

phase during the first 4 days of germination, a fast phase between 4 and 10 days, and finally another slow phase from 10 days onward (Fig. 1B). The degradation of starch in germinating lentil cotyledons followed a similar pattern of decrease. In contrast with the results obtained in rice (18), barley (14), and pea (13) in which the dry weight changes and starch degradation are very fast processes, in lentil cotyledons these processes are very slow. The residual starch after 13 days of germination was still 42% of the original starch content.

**Electrophoretic Study of Starch-degrading Enzymes in Lentil Cotyledons.** The zymogram pattern of the starch degrading-enzymes showed a maximum of five bands (Fig. 2). The slowest band (band a) appeared only in the presence of Pi, was insensitive to EDTA and mercuric chloride, and disappeared when the extracts are heated at 70 C for 5 min. These results are consistent with the identification of this band as a phosphorylase. The three middle bands (b, c, and d) were colorless and were present in starch-incubated gels regardless of the buffer used. They did not appear in gels treated with EDTA, and were insensitive to the heat and mercuric chloride treatments. These bands were identified as $\alpha$-amylases. The fastest migrating band (band e) was pink, EDTA-resistant, and disappeared when treated with mercuric chloride or heated at 70 for 5 min. This suggests that it represents a $\beta$-amylase isozyme. The slight and thin appearance of this band, which suggests a low activity, is in good agreement with the failure to detect it by the colorimetric method used in the enzymic assay.

**Changes in Enzyme Pattern during Germination.** The phosphorylase and all the amylase isozymes appear in resting seeds, although the three bands designated as $\alpha$-amylases with very low intensity, show a progressive increase during germination (Fig. 2). The phosphorylase band remained almost unchanged during germination time. The $\beta$-amylase band disappeared by the 6th day of germination. We did not detect the appearance of any new band during germination.

## DISCUSSION

A maximum of five bands with amylolytic activity appeared in cotyledons of lentil seeds during germination. According to their characteristics, the slowest band is a phosphorylase, the three middle bands are $\alpha$-amylases, and the fastest migrating band seemed to be $\beta$-amylase.

All were present in resting seeds, although with low activity. During germination the total amylolytic activity increases, but $\alpha$-amylase became by far the principal enzyme, whose activity reaches a maximum (80-fold) level by the 6th day. This increase coincided with the disappearance of starch, which began on the 5th day of germination. Similar results have been reported (6, 13).

Although the increase in $\alpha$-amylase activity during germination seems to be a general phenomenon in all the seeds studied, the presence of this enzyme in resting seeds is not so general. In sorghum (8), rice (23), maize (9), and pea (13) $\alpha$-amylase activity did not appear until the germination process had started. In agreement with our results, $\alpha$-amylase has been found also in resting seeds of rye (10), oats (10), barley, and wheat (11).

In oats, Smith and Bennett (19) suggest the presence of two sets of $\alpha$-amylase, one heat-labile set in resting seeds, formed during development of the grain, and a second heat-stable set of similar electrophoretic mobility formed during germination. As can be deduced from the results obtained (identical electrophoretic mobility in resting and germinated seeds, and disappearance of activity by heating in resting seeds) a similar pattern seems to exist in lentil cotyledons; but since the specific activity of amylase in resting seeds is so low, the possibility that both supposed sets are identical and heat-stable cannot be ruled out. In fact, as has been stated above, a slight inactivation was always obtained after heating in the presence of calcium ions.

The phosphorolytic pathway for the degradation of starch reserves in lentil cotyledons seems not to be very important, since during its maximal activity the starch content remains almost intact, and since its activity begins to decline when $\alpha$-amylase increases and starch depletion begins to be significant. The role of phosphorylase during the first steps of germination should be to provide the substrate for glycolysis and respiration without using ATP.

$\beta$-Amylase activity in lentil cotyledons was very low and has been detected only by electrophoresis, declining in activity during germination until complete disappearance. Since we have found in axis tissue an active $\beta$-amylase (unpublished data) we conclude, in agreement with Swain and Dekker (21) that any oligosaccharides formed by $\alpha$-amylolysis should be translocated to axial tissue and degraded there to glucose.

In lentil cotyledons the amount of starch by the 13th day of germination was still about 50% of the total, whereas in other seeds (12–14, 18) by this time, the starch content had been almost completely depleted. This could be due to the already stated low $\beta$-amylase activity and to the apparent lack of amylopectin-1,6-glucosidase (we could not detect it) whose function, according to Dunn (5) is to degrade dextrins released into solution by the $\alpha$-amylolytic activity.

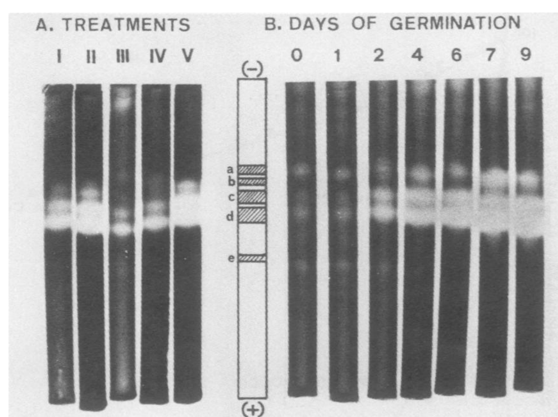


FIG. 2. Zymogram pattern of starch-degrading enzymes in lentil cotyledons. A: different treatments: I: extract in acetate buffer; II: extract in phosphate buffer; III: extract in phosphate buffer + EDTA; IV: extract in phosphate buffer + $HgCl_2$; V: extract in phosphate buffer + $CaCl_2$ heated at 70 C for 5 min.

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