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Starch Content and Activities of Three Enzymes of Carbohydrate Metabolism in *Cassia senna* Seedlings

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Abstract. A study was made of physiological and biochemical changes taking place during germination of *Cassia senna*. Its seeds contain only a trace amount of reserve starch which increases in the cotyledons during the early stages of seed germination. This is followed by a rapid hydrolysis. The level of reducing sugars increases in the cotyledons throughout the germination period.

The activities of amylase, phosphorylase and invertase also increase in the cotyledons during seed germination. The electrophoretic patterns of amylase and phosphorylase isoenzymes undergo changes during the germination period.

Introduction

The process of seed germination is accompanied by the hydrolysis of reserve substances in the storage tissues to provide the essential soluble products for seedling growth. Storage starch has been shown to decline in the cotyledons of pea [1,2] and wheat grains [2]. Protein has been shown to decline in the cotyledons of *Cassia senna* [3], *Acacia laeta* [4], soyabean [5], pea [6], and in endosperm of *Euphorbia lathyris* [7]. In addition, lipids decline in the ensoperm of *E. lathyris* [7].

At the onset of reserve mobilization, the activities of certain hydrolases rise from an initially low or even undetectable level to a high level. Protease activity incrased manifold in pea [6, 8]. Such increases in activity also occur with anzymes involved in carbohydrate metabolism: amylase in pea [1,9], barley [10] and *Vigna mungo* [11] and phosphorylase in pea [12].

Unfortunately, despite the considerable amount of literature available on food mobilization of seeds, the hydrolysis of substances in seeds of plants native to the desert environments is less well understood. The work described here is a part of a program initiated to provide more information on seed metabolism of plants adapted to the arid environment of Saudi Arabia.

Materials and Methods

Seed collection, germination and harvesting were performed as described in [3].

Extraction and assay of starch and reducing sugars

Samples of 20 mg of finely ground seeds or freeze-dried etiolated germinated cotyledons were extracted with 10 ml 80% aqueous ethanol at 4°C overnight. After centrifugation at 1300 x g for 5 min, the supernatant was used for reducing sugar determination as described below. The residues were suspended in 10 ml distilled water in a glass tube and incubated in a boiling water bath for 2 h. After cooling to room temperature, the gelatinized starch produced was hydrolyzed by adding 2 mg α-amylase powder (BDH Chemicals), at room temperature for 1.5 h. After centrifugation, the liberated reducing sugars were determined according to the method of Bernfeld [13] using maltose as a standard. Since the amylase powder contained reducing sugars, a control tube was prepared by dissolving 2 mg α-amylase powder in 10 ml water and the reducing sugar content was measured and the experimental readings were corrected. In addition, before hydrolysis by α-amylase, the starch extract was analyzed for the possible presence of reducing sugars. No reducing sugars were detected in any extract.

Extraction of enzymes

Enzymes were extracted by homogenizing 20 mg of seeds or freeze-dried cotyledons in 1 ml 0.1 M TRIS/HCl buffer, pH 6.8 overnight or 1 h to minimize the possible effect of protease on isoenzyme patterns at 4°C. After centrifugation at 1300 xg for 7 min, the supernatant was used for enzyme assays. 20% (w/v) sucrose was included in the extraction buffer for samples used for electrophoresis analysis and the ratio between tissue and extraction buffer was 1 to 40.

Assay of enzymes

Amylase activity was measured at pH 5.0 by the method of Bernfeld [13; pp. 149-158], using maltose as a standard. The assay mixture contained 50 μl enzyme

extract, 0.5% starch (Fluka) and 50 mM sodium acetate buffer. The mixture was incubated at room temperature for 15 min.

The assay mixture for invertase contained 100 μl enzyme extract, 100 mM sodium acetate, pH 5.0, and 3.5% (w/v) sucrose. The mixture was incubated for 15 min at room temperature. The liberated reducing sugars were measured as above.

Effect of temperature on amylase activity

5 mM $CaCl_2$ was added to the extract to protect α-amylase from damage by heat. The extract was then incubated in a water bath at 70°C for 15 min. After cooling, enzyme activity was assayed as above.

Electrophoresis

Electrophoresis was performed using 7.5% polyacrylamide gel under non-denaturing conditions as reported in [14].

Gels were stained for amylase activity as in [14].

Gels stained for phosphorylase were incubated in 100 ml 0.2 M sodium citrate buffer, pH 6.0, containing 0.5 g glucose-1-phosphate and 50 mg starch, for 3 h at room temperature (25°C). Gels were stained with KI/I_2 solutions as for amylase.

Results

Starch and reducing sugar content

The changes in starch and reducing sugar content of the cotyledons during the eight days of seed germination are shown in Fig. 1. Ungerminated mature seeds contained very low amounts of starch: less than 3% of dry weight. The starch content of cotyledons increased rapidly in the first 4 days of germination, reaching a maximum on day 4. The starch content then began to decline rapidly.

The extracts from mature dry seeds and one-day old seedlings had only a trace amount of reducing sugars. As germination progressed, the amount of reducing sugars increased gradually in the cotyledons.

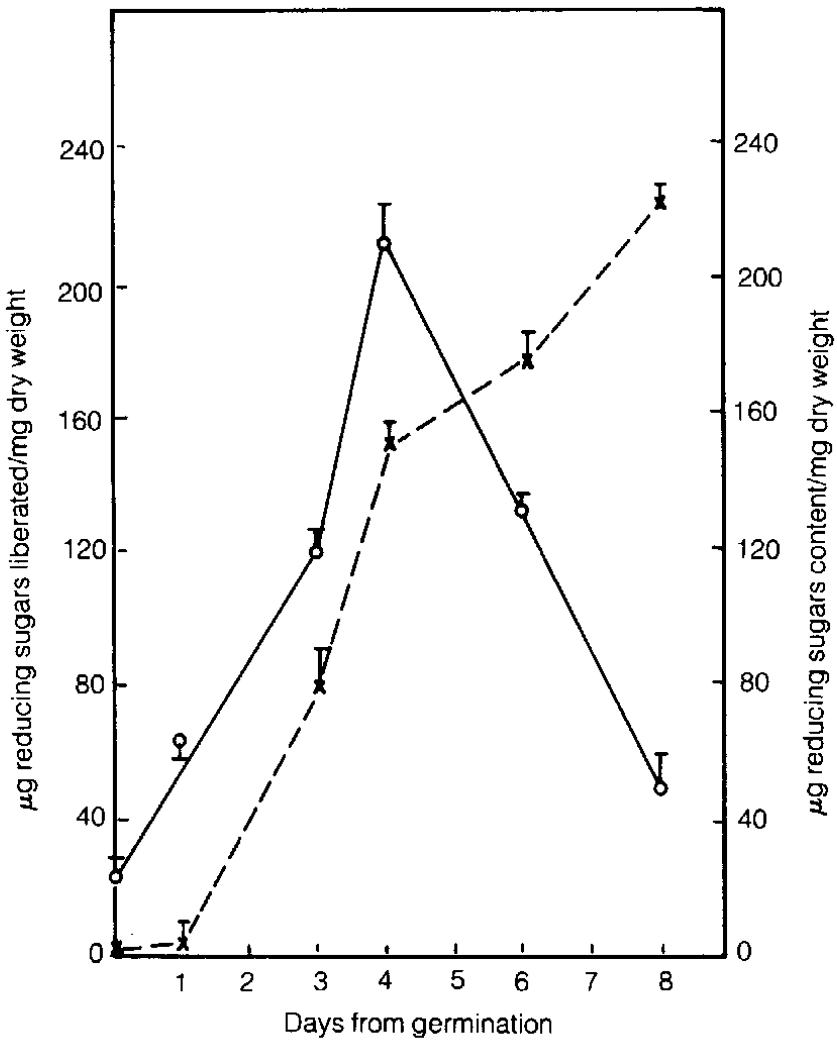

Fig. 1. Starch and reducing sugars content of the cotyledons after germination.
○——○ **starch content**
○.....○ **reducing sugars content**
Each point is the mean of three readings.

Activity of enzymes

Dry seeds contained a relatively low level of amylase activity [Fig. 2] and enzyme activity increased linearly between days 1 and 6, followed by a more rapid increased between days 6 and 8.

The ungerminated seeds had only a trace amount of invertase activity (Fig. 2). The enzyme activity in the cotyledons increased linearly during germination after the 1st day.

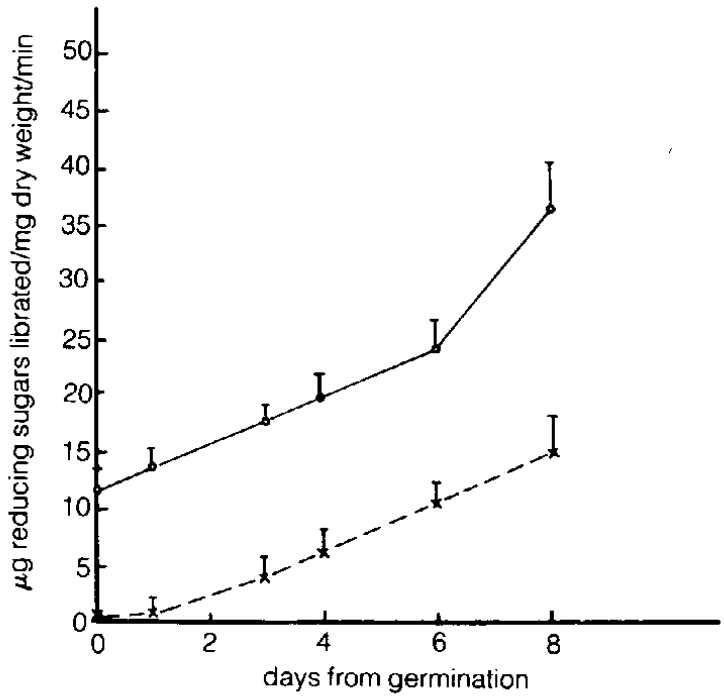

Fig. 2. Amylase and invertase activities of the cotyledons after germination.
○——○ **amylase activity**
○.....○ **invertase activity**
Each point is the mean of three readings.

Effect of temperature on amylase activity

Amylase activity in the extract, from dry seeds and from cotyledons of one-day-old seedlings was completely inhibited by incubation at 70°C in the presence of added calcium (Table 1). The extract from cotyledons of 3-day-old seedlings lost 85% of its activity, while extracts from cotyledons of 4-to 8 day-old seedlings lost 60% of their activity.

Table 1. Effect of incubation of extract at 70°C for 15 min in the presence of 5 mM $CaCl_2$ on amylase activity

Days from germination	% activity	%inhibition
0	0	100
1	0	100
3	15 + 4	85
4	40 + 2	60
6	40 + 3	60
8	39 + 2	61

Amylase patterns

No differences in amylase and phosphorylase patterns were detected in extracts incubated overnight or for 1 h.

The electrophoretic pattern of changes of amylase activity throughout the germination period is shown in Fig. 3. The extracts from cotyledons contained a non-specific stained band (Rf 0.09) and its staining intensity increased during germination.

The extracts from ungerminated seeds, soaked seeds and one-day-old seedlings, each resolved into 3 distinct electrophoretic bands; the slowest moving band (Rf 0.31) was colorless and the other two hands were pink in color. The intermediate moving component (Rf 0.35) was faint and had very low enzyme activity and could hardly be seen in the photograph. The fastest moving component (Rf 0.36) appeared to have the highest enzyme activity.

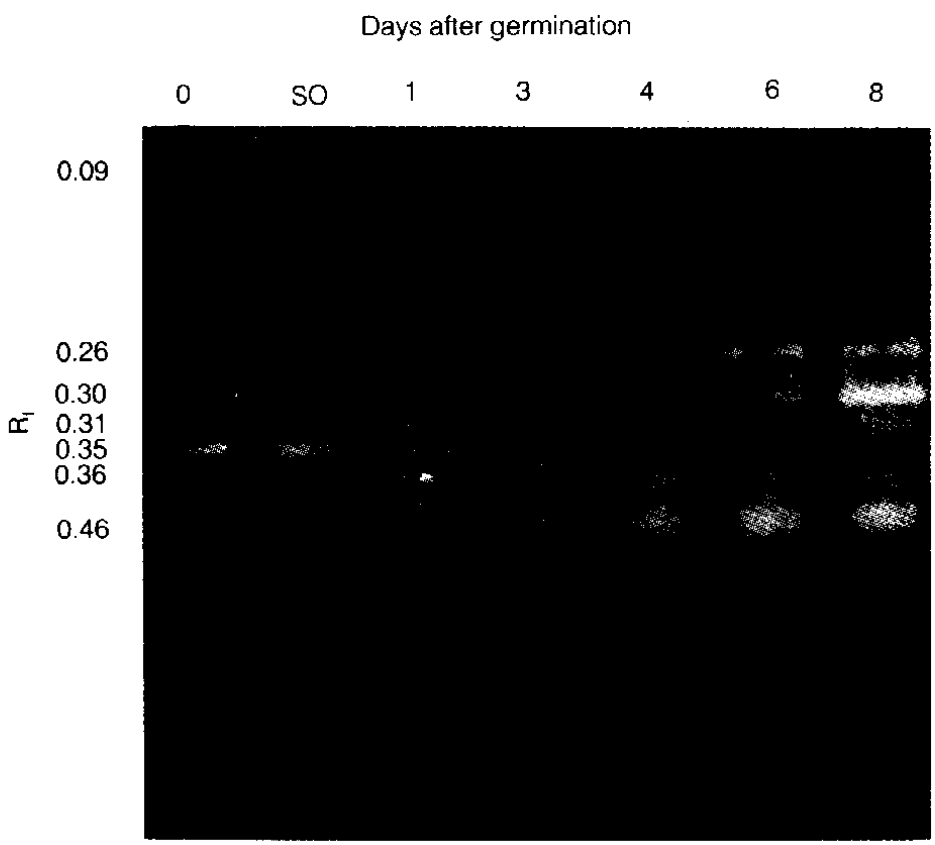

Fig. 3. Polyacrylamide gel electrophoresis (PAGE) of amylase of cotyledons at various stages of germination.
***Note* : Sample loaded was 80 μl**
SO : Soaked seeds

The amylase zymogram patterns of cotyledonary extracts of 3, 4, 6 and 8-day-old seedlings were almost identical. Each extract contained two slow moving bands (Rf 0.26 and 0.30 colorless), one fast moving, band (Rf 0.46, pink), in addition there were at least two other pink bands (Rf 0.38 and 0.39). It is clear from Fig. 3 that the activity of the two slower-moving components increased as seed germination progresses.

Phosphorylase patterns

As demonstrated in Fig. 4, each extract had a non-specific stained band (Rf 0.09) as for amylase.

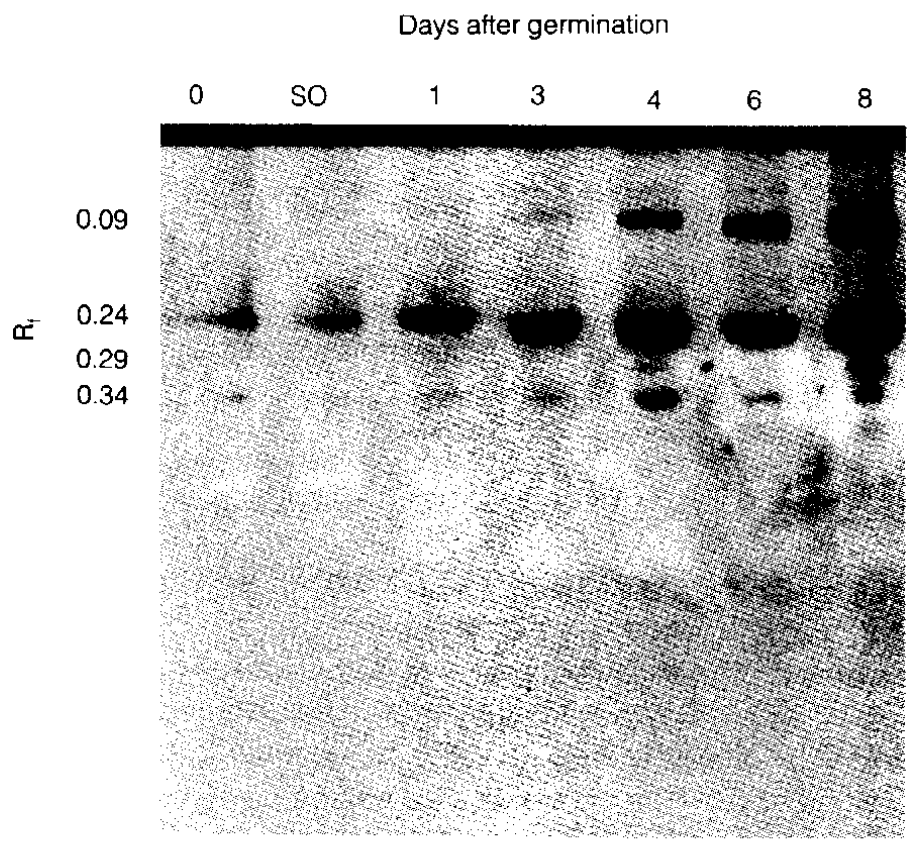

Fig. 4. Polyacrylamide gel electrophoresis (PAGE) of phosphorylase of cotyledons at various stages of germination.
***Note* : Sample loaded was 80 μl.**
So : Soaked seeds

The extracts from dry seeds, soaked seeds, one-day-old seedling and cotyledon of 3-day-old seedlings each displayed two phosphorylase bands (Rf 0.24 and 0.34). The cotyledon extracts of 4, 6 and 8-day-old seedlings had an intermediate moving band (Rf 0.29). It is clear from the photograph that the activities of all the components, especially the slowest moving one, increased during germination.

Discussion

The results of the present investigation demonstrated that dry seeds of *C. senna* contain only a trace amount of starch. This observation supports the previous one [3] that protein is probably the major reserve food in *C. senna* seeds. The reason for this is unknown at the present time but it might have an ecological adaptation value to the arid environment and it might also be due in part to the small size of the seeds which might be for their benefit to store large quantity of protein as a nitrogen source for seedlings growth and as an energy source.

The early accumulation of starch during the first days of germination in *C. senna* seed and its rapid hydrolysis thereafter is in good agreement with the results reported for soyabean seeds [15], but not with results reported for other species [1, 10], where starch was hydrolyzed from the early stages of seed germination.

The physiological importance of the early increase of amylase activity during the period of starch synthesis cannot be assessed at present, since the major role of amylase is starch hydrolysis. However, these findings may indicate that amylase activity in the early stages of germination of *C. senna* seeds is unrelated to starch metabolism. It has been suggested that some forms of amylase such as, β-amylase, probably have no essential role in starch metabolism [15]. In addition, the results suggest that all amylase extracted from dry seeds and soaked seeds and most of its activity extracted from cotyledons of 3-day-old seedling was probably β-amylase, since it was not heat stable in the presence of added calcium. The role of β-amylase in plant metabolism is under question [15].

The more rapid increase in the activity of cotyledon amylase between days 6 and 8 of germination coincided with rapid starch hydrolysis. In addition electrophoretic analysis of amylase isoenzymes showed that this period was characterized by the increase in the amylolytic activity of the two slowest moving components. This might suggest the important role of these two components in starch hydrolysis.

It is surprizing to find an increase in the activity of the slowest moving component of phosphorylase during the period of rapid starch synthesis, since it is known that the major role of phosphorylase is starch degradation [13, 149-158 pp]. This observation required further investigation.

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المحتوى النشوي ونشاط ثلاثة أنزيمات محللة للنشا في بادرات العشرق

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(سُلِّمَ في ٦محرم ١٤١٣هـ، وقُبل للنشر في ١٠ ربيع الآخر ١٤١٤هـ).

ملخص البحث. لقد تمت دراسة كل من المحتوى النشوي والسكريات المختزلة ونشاط كل من الأنفرتيز والفوسفورليز والأمليز في فلقات بادرات العشرق الشاحبة في مراحل مختلفة من الإنبات.

أوضحت نتائج الدراسة أن المحتوى النشوي في البذور الجافة منخفض جدًّا وتزداد كميته في الأيام الأولى من الإنبات ثم يأخذ في التناقص بشكل سريع. محتوى الفلقات من السكريات المختزلة يزداد بزيادة عدد أيام الإنبات. نشاط كل من الأمليز والأنفرتيز يزدادان بزيادة عدد أيام الإنبات، كذلك تم فصل أيزوأنزيمات الفوسفورليز والأمليز بوساطة الفصل الكهربائي PAGE وأوضحت النتائج أن الحزم الأيزوأنزيمية لهذين الأنزيمين يحدث لها تغيرات في المراحل المختلفة من الإنبات.