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Amylase activity in barley grains and green gram seeds

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Abstract: *Cereal grains and pulses store abundant starch. During hydration and subsequent germination amylases are produced, and the amylases function in the mobilization of the stored starch. The sugars thus formed are utilized by the developing seedling. In the present investigation amylase activity as determined by the time taken to degrade the substrate starch using the iodine reagent test, and the amount of reducing sugars formed as a consequence of amylase action on starch using Benedicts's test have been studied. The study aims at comparing a monocot with a dicot; the cereal barley (Hordeum vulgare) and the pulse green gram (Vigna radiata) have been used as the experimental materials. Soaked barley grains and green gram seeds, and 2- and 4-day-old barley and green gram seedlings have been used in the study. The experimental design also allowed understanding the role of calcium as an activator of α-amylase. In 4-day-old barley seedlings the grain extract was still rich in starch compared to the axis extract whereas in green gram the axis extract had more starch than the cotyledon. The axis extract of barley seedlings had more reducing sugars than the grain in 2-day as well as 4-day-old seedlings. In green gram the cotyledon extract had more reducing sugars than the axis extract in 2-day-old seedlings whereas in 4-day-old green gram seedlings the axis extract had more reducing sugars than the cotyledon. The study supports the fact that stored starch is converted to glucose and made available to the developing seedling during seed germination.*

Keywords: *Barley, green gram, amylase, iodine reagent, Benedict's reagent*

1. INTRODUCTION:

Barley is a major cereal crop of the temperate regions, and hulled barley contains 73.5 g carbohydrates per 100 g [1]. Green gram (mung bean) is an important legume/ pulse crop and is cultivated in Asia on a large scale. Besides being a rich source of proteins (23.9 g per 100 g), the mature seed of green gram contains 62.6 g carbohydrates per 100 g [2]. Cereals and pulses constitute an important part of our diet. When cereal grains germinate the gibberellin present in the embryo diffuses into the aleurone layer of the endosperm inducing de novo synthesis of hydrolases in the aleurone cells. The hydrolases synthesized include α-amylase and protease which diffuse into the starchy endosperm. The protease converts the inactive form of β-amylase to the active form. Both α- and β-amylase, and other starch-degrading enzymes act on the starch stored in the endosperm, and ultimately sugars are released for use by the developing seedling [3,4]. Germinating green gram seeds are also rich in amylase [5,6], and α-amylase is mainly found in the cotyledons [7].

Amylases are hydrolases found in bacteria, fungi, plants and animals. The fungal and bacterial amylases are put to various industrial applications [8,9]. Being thermostable, α-amylase has a very significant place in commercial uses [10]. Three families of α-amylase genes have been recognized in higher plants; and each family is predicted to be directed to a cellular compartment. The compartments are the extracellular region, the cytosol and the plastids [11]. Both α-amylase and β-amylase act on α-1,4 glycosidic bonds in starch; however, α-amylase is an endoamylase whereas β-amylase is an exoamylase. Alpha-amylase acts at random on the glycosidic bonds to release limit dextrins, and some maltose and glucose. On the other hand, β-amylase acts on the glycosidic bonds from the non-reducing end of starch removing one maltose at a time and yielding maltose and dextrins [12]. Alpha-amylase is a calcium metalloenzyme [13]. Salivary amylase which initiates the breakdown of starch in the mouth is an α-amylase [14]. Sprouted wheat grains, chickpea and green gram seeds, radish root and ripe banana fruits are rich in amylase, and consumption of these plant materials assists in the digestion of starch in food [15,16].

2. MATERIALS AND METHODS:

A monocot and a dicot, namely barley grains and green gram seeds, which store abundant starch and are common food items, were used in the study. The following are the details of the materials studied.

Table 1: Plant materials studied.

S. No.	Common name	Scientific name	Family	Part used
1.	Barley	*Hordeum vulgare* L.	Poaceae	Soaked grains; grain and axis of 2- and 4-day-old seedlings
2.	Green gram (mung bean)	*Vigna radiata* (L.) R. Wilczek	Fabaceae	Soaked seeds; cotyledon and axis of 2- and 4-day-old seedlings

2.1 Germination of barley grains and green gram seeds:

About 50 g each of barley grains and green gram seeds were taken and rinsed thoroughly with tap water. Then the grains and seeds were soaked in water in separate beakers for 2 hours. Some of the soaked grains and seeds were used in conducting experiments. The remaining soaked grains of barley were tied in a cotton cloth and the cloth was then tied to a tap with water trickling down very slowly to ensure that the seedlings remained wet and received sufficient water for their growth. The soaked seeds of green gram were spread uniformly on garden soil taken in a tray. A thin layer of soil was laid on the seeds and then tap water was carefully sprinkled on the soil. The tray was placed near a window which received diffuse light.

2.2 Preparation of enzyme extracts:

Twenty grams of the experimental material was ground in 100 mL distilled water using a mixer-grinder. The homogenate was filtered using a plastic sieve. The filtrate was used as the crude enzyme extract. Two and four days from sowing (referred to as 2- and 4-day-old seedlings) the seedlings were used to prepare separate extracts of the grains/ cotyledons and the axes. The ratio of the plant material to distilled water was 1:5. The extracts were used immediately in experiments.

2.3 Detection of amylase activity by iodine reagent test:

The degradation of the substrate starch (1%) by the amylase in the extracts was studied by the iodine reagent test [17,18]. Starch and iodine reagent form a physical coloured complex which is blue-black. More the starch more intense will be the blue-black colour developed. The gradual action of amylase on starch will cause the fading and finally disappearance of the blue-black colour. The time taken for complete fading of the blue-black colour is the end point. Six test tubes were set up (Tables 2, 3). The ingredients were added in the order as given in the tables. The time taken to reach the end point in each test tube was recorded.

2.4 Detection of reducing sugars by Benedict's test:

Using a mortar and pestle 0.5 g of the experimental material was made into a paste in 1 mL distilled water. The paste was transferred into a test-tube, 2 mL of Benedict's reagent was added and shaken, and then the test tube was heated and cooled. The amount of brick red precipitate formed in the reaction mixture was noted [17]. A change in colour from the clear blue colour of Benedict's reagent, and the formation of a precipitate indicate the presence of reducing sugars. Arbitrary plus (+) marks were given for the amount of brick red precipitate formed.

3. RESULTS AND DISCUSSION:

3.1 Morphology of the seedlings:

The barley grains and green gram seeds germinated readily; and the 4-day-old seedlings were longer than the 2-day-old seedlings. Two days from sowing in most grains of barley both the roots and the coleoptile had emerged. In many of the 4-day-old seedlings of barley the coleoptile had ruptured and the leaf was visible (Figure 1). Green gram showed epigeal germination. In most of the 2-day-old green gram seedlings the plumular hook had not opened, in quite a few seedlings the first pair of leaves had begun to emerge, and the radicle showed the initiation of lateral roots. In 4-day-old green gram seedlings the leaves were expanded and the cotyledons had begun to shrivel (Figure 1).

3.2 Detection of amylase activity by iodine reagent test:

Amylase activity was present in both the experimental materials and at all the stages studied, i.e., in soaked grains of barley, soaked seeds of green gram, in the grains and axes of 2- and 4-day-old barley seedlings, and in the cotyledons and axes of 2- and 4-day-old green gram seedlings (Tables 2, 3). Presence of calcium chloride ($CaCl_2$) enhanced amylase activity in both the taxa in all the enzyme extracts studied substantiating the fact that calcium is an activator of α-amylase (test tubes 1, 6; Tables 2, 3). In the control without the enzyme the starch could not be degraded and the blue-black colour persisted (test tube 2; Tables 2, 3). The test tube without the substrate starch (test tube 3) took less time compared to the test tube with starch (test tube 1). This was because the amylase had to act only on the internal starch present in the grain/ seed. In the control without both the enzyme and starch the yellow colour of iodine reagent persisted (test tube 5; Tables 2, 3). In general, the extracts of soaked grains and seeds took less time in reaching the end point compared to that of the seedlings when the iodine reagent test was conducted. The boiled and cooled enzyme extract took too long a time to reach the end point (test tube 4). This was because boiling the enzyme extract denatured amylase. However, as it has been reported that α-amylase is thermostable [10], it is quite possible that very few molecules of amylase had withstood the boiling and were able to act on starch. The time taken to reach the end point was the least in soaked grains and seeds, intermediate in 2-day-old seedlings, and maximum in 4-day-old seedlings (test tube 1).

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Figure 1: Representative 2- and 4-day-old seedlings of barley (A, C) and green gram (B, D).

In 2-day-old barley seedlings the grain and the axis extracts took nearly the same time to reach the end point. But the grain extract took much more time than the axis extract in reaching the end point in 4-day-old barley seedlings meaning thereby that the starch content in the grain was still high and that the starch was getting mobilized to the axis. Soaking barley grains in gibberellic acid for 12 hours increased the germination and α-amylase activity by 156% and 90%, respectively [19]. Six isozymes of α-amylase have been identified in germinating barley, and the isoenzymes are formed gradually and all six are present six days after the beginning of germination [20]. Also, the addition of gibberellic acid in the culture medium enhanced the production of α-amylase isozymes to a great extent whereas the addition of kinetin did not [20]. In green gram the cotyledon extract took less time than the axis extract in reaching the end point in 2-day-old as well as 4-day-old seedlings. This was because the starch in the cotyledon was mobilized as sugar to the axis, and the seedlings had also begun photosynthesis and it is likely that starch formation was initiated in the axis.

Table 2: Amylase activity in barley grains studied using the iodine reagent on the day of sowing, and in 2- and 4-day-old seedlings.

S. No.	$CaCl_2$ (mL)	Enzyme extract (mL)*	Distilled water (mL)	Starch (mL)	Iodine reagent (drops)	Time taken to reach end point (seconds)**				
						Day of sowing	2-day-old seedlings		4-day-old seedlings	
						Soaked grain	Grain	Axis	Grain	Axis
1	5	5	-	5	6	15.67	46.67	43.33	332.33	92.33
2	5	-	5	5	6	Blue-black colour	Blue-black colour	Blue-black colour	Blue-black colour	Blue-black colour
3	5	5	5	-	6	7.33	31.00	9.33	32.33	8.67
4	5	5 (boiled & cooled)	-	5	6	868.33	437.33	235.00	907.33	683.67
5	5	-	10	-	6	Yellow colour	Yellow colour	Yellow colour	Yellow colour	Yellow colour
6	-	5	5	5	6	20	55.00	54.00	412.67	143.67

*After adding the enzyme to $CaCl_2$ the reaction mixture was incubated for 10 minutes.
** Average of three replicates.

Table 3: Amylase activity in green gram seeds studied using the iodine reagent on the day of sowing, and in 2- and 4-day-old seedlings.

S. No.	$CaCl_2$ (mL)	Enzyme extract (mL)*	Distilled water (mL)	Starch (mL)	Iodine reagent (drops)	Time taken to reach end point (seconds)**				
						Day of sowing	2-day-old seedlings		4-day-old seedlings	
						Soaked seed	Coty ledon	Axis	Coty ledon	Axis
1	5	5	-	5	6	9.67	12.33	56.67	79.33	432
2	5	-	5	5	6	Blue-black colour	Blue-black colour	Blue-black colour	Blue-black colour	Blue-black colour
3	5	5	5	-	6	4.67	10.00	11.67	14.00	16.00

4	5	5 (boiled & cooled)	-	5	6	338.33	159.33	354.67	609.33	792.33
5	5	-	10	-	6	Yellow colour	Yellow colour	Yellow colour	Yellow colour	Yellow colour
6	-	5	5	5	6	11.00	15.67	63.67	89.67	508.67

*After adding the enzyme to $CaCl_2$ the reaction mixture was incubated for 10 minutes.
** Average of three replicates.

3.3 Detection of reducing sugars by Benedict's test:

Table 4: Amount of reducing sugars determined using the Benedict's reagent on the day of sowing, and in 2- and 4-day-old seedlings.

Plant	Plant part	Amount of brick red precipitate*		
		Day of sowing	2-day-old seedlings	4-day-old seedlings
Barley	Grain	+2.67		
	Grain		+1.67	+3.33
	Axis		+2.67	+4.33
Green gram	Seed	+1.67		
	Cotyledon		+3.33	+2.00
	Axis		+1.67	+4.67

*Average of three replicates.

On the day of sowing the barley grains have more reducing sugars than green gram seeds (Table 4). The axis had more reducing sugars than the grain in 2-day-old as well as 4-day-old barley seedlings, which showed that starch was getting enzymatically digested and mobilized from the grain (bulk of the grain is made up of starchy endosperm) to the growing seedling axis which includes the coleoptile and roots. In green gram the 2-day-old seedlings had more reducing sugars in the cotyledon compared to the axis. But in 4-day-old green gram seedlings the axis had more reducing sugars than the cotyledon. This can be explained based on the fact that the starch in the cotyledon was digested and then transported to the axis. Moreover, many 2-day-old and all 4-day-old seedlings had shown the emergence of the first pair of leaves; this meant that gradually the seedlings had begun photosynthesis. An increase in the concentration of reducing sugars and an approximate 50% hydrolysis of starch has been reported in chickpea and green gram seeds 96 hours after germination [21].

4. CONCLUSIONS:

Barley grains and green gram seeds store abundant starch as the reserve food material. During germination the starch is digested by amylases and other starch-degrading enzymes. The glucose that is formed is mobilized to the seedling axis thus helping in the rapid growth of the seedling. Therefore, the amount of reducing sugars in the seedling axis increases with time.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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