REVISTA ESPAÑOLA DE FISIOLOGIA, 34, 87-92. 1978

Starch Degradation During Germination of *Cicer arietinum* L. Seeds

J. Fernández-Tárrago, M. C. Rodríguez-Buján and G. Nicolás*

Departamento de Fisiología Vegetal
Facultad de Farmacia
Universidad de Santiago de Compostela

(Received on June 6, 1977)

J. FERNANDEZ-TARRAGO, M. C. RODRIGUEZ-BUJAN and G. NICOLAS. *Starch Degradation During Germination of* Cicer arietinum *L. Seeds.* Rev. esp. Fisiol., 34, 87-92. 1978.

The variations in starch and soluble sugar content, in phosphorylase and amylase activities in cotyledons of germinating seeds of *Cicer arietinum* L. are determined. Results from various experiments prove that the α-amylases are chiefly responsible for amylase activity. Phosphorylase plays an important rôle during the first two days of germination, but it is relegated to a secondary position as the amylase activity increases.

Disc electrophoresis on polyacrylamide gel shows the existence of a phosphorylase throughout germination, and detects two α-amylases after 48 and 96 h germination respectively. The increase in α-amylase activity during germination is due to *de novo* synthesis of the two isoenzymes, since both are inhibited by cycloheximide and actinomyces D. This *de novo* synthesis depends on some embryo produced factor, unreplaceable either by giberellic acid or by kinetin.

In a previous paper (23) has been found that during the germination of seeds of *Cicer arietinum* L. the carbohydrates were extensively depleted. These results suggest a very high amylolytic activity during germination. Since much of the knowledge of starch degradation comes from studies with cereals (2, 3, 10, 16, 19, 24, 29, 31) and studies with legumes are very scarce (17, 26, 28), has been considered interesting to determine the mechanism of breakdown of reserve starch in the cotyledons of germinating chick pea seeds. The influence of axis removal on starch degradation was also studied.

Materials and Methods

Chick pea sedds (*Cicer arietinum* L.) were soaked for 1 h in a disinfectant solution (0.2 % orthocide-50 wettable, a commercial fungicide) at room temperature, and rinsed several times with sterile distilled water. The seeds were then di-

* Dirección actual: Departamento de Fisiología Vegetal. Facultad de Ciencias. Universidad de Salamanca.

vided into two groups: in one group, the seeds were left intact (attached cotyledons) whereas in the other group the embryonic axis were removed from the seeds to produce the detached cotyledons. Sets of 10 intact seeds or 20 detached cotyledons were placed in petri dishes with filter paper moistened with 10 ml of sterile distillet water containing 7,000 units of penicillin per 100 ml to avoid bacterial contamination. Two sets of 10 intact seeds were incubated in the presence of cycloheximide (50 μg/ml) or actinomycin D (10 μg/ml) respectively. Two sets of 20 detached cotyledons were incubated in sterilized 100 ml-Erlenmeyer flasks containing in 15 ml of water, GA_3 (10^{-4} M; 10^{-5} M; 10^{-6} M) or Kinetin (10^{-4} M; 10^{-5} M; 10^{-6} M) and 7,000 units of penicillin per 100 ml, in a reciprocal shaker in the dark at 25° C for varying periods of time up to 10 days. In order to avoid bacterial or fungal contamination all the manipulations were performed in sterile hood.

Enzyme extraction and assay. Twenty cotyledons were homogenized with five times their weight of cold acetone (25) for 1 min at 0-4° C in a Sorvall Omnimixer. The slurry was filtered through a buchner funnel and the residue was again blended with half the amount of cold acetone and filtered again. This process was repeated once more. The powder was dried at 0-4° C and then suspended in 40 ml of unbuffered water and centrifuged in a Sorvall RC2-B refrigerated centrifuge at 31,000 *g* for 30 min at 2° C. The supernatant was used as the crude enzyme preparation. In order to differentiate between alpha and beta amylase the supernatant was selectively treated with 20 mM EDTA; 0.1 mM $HgCl_2$; and heated at 70° C for 5 min in the presence of 3 mM $CaCl_2$.

Two aliquots of the crude enzyme preparation were removed, and one of them was heated at 70° C in the presence of added calcium ions to inactivate beta amylase. After 60 min incubation at 35° 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 (1) using 2% (w/v) potato starch solution in 0.05 M acetate buffer pH 5.0 as substrate.

Protein was determined by the method of LOWRY (21), and enzymatic activity was expressed as mg of maltose released per h per mg protein. Phosphorylase activity values were taken from the paper by DE LA FUENTE-BURGUILLO and NICOLÁS (8).

Starch and soluble sugars determination. The starch content of the cotyledons was determined by the method of DEKKER and RICHARDS (9). Soluble sugars were extracted and determined as previously described (23).

Polyacrylamide gel electrophoresis. Polyacrylamide gel disc electrophoresis was performed by the DAVIS method (7), during 1 h and 15 min at 4° C under a constant current of 3 mA per gel. The enzymatic extracts were prepared by homogenisation of 20 cotyledons in 40 ml of unbuffered distilled water. Before layering into the gel tubes, the samples were pre-incubated during 3 h at room temperature with water, 20 mM EDTA, 0.1 mM $HgCl_2$, or 3 mM $CaCl_2$. The latter sample was heated at the end of the pre-incubation time at 70° C for 5 min.

After electrophoresis, the zones of phosphorylase and amylase activity, were revealed by incubation of the gels vith a 1% (w/v) solution of soluble starch in 0.1 M acetate or phosphate buffer pH 5.0 for 2 h and 30 min at 30° C. After incubation, the gels were rinsed in distilled water, and then immersed for a few seconds in an I_2-IK solution and rinsed again in distilled water. The iodine stains the gel heavily, except in the zones of amylase or phosphorylase activity.

Results and Discussion

Figure 1 shows that the amylase level, which is very low in resting seeds, increased slowly during the first two days of germination. Beginning on the second day there was a drastic increase in amylase activity reaching a maximum on the fifth day. Similar behaviour has been found in lentil cotyledons (11) and in peas (17). DE LA FUENTE and NICOLÁS (8), whose results we reproduce in fig. 1. found in chick pea cotyledons a phosphorylase the activity of which is high during the first days of germination. These data, together with the low amylase activity during this time, and the rapid degradation of starch from the beginning of the germination period (fig. 2) suggest that phosphorylase plays an important role in the depletion of starch during the first two days of germination. From two days onwards, the alpha amylase becomes the chief starch degrading enzyme. As expected, the decrease in starch content coincided with an increase in the amount of soluble sugars liberated (fig. 2).

The results obtained after the treatment of the enzyme extracts with dif-

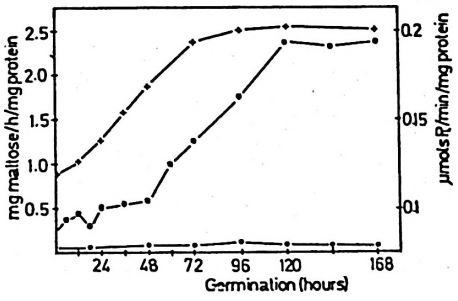

Fig. 1. *Phosphorylase and total amylase changes during germination of seeds of* Cicer arietinum *L.*
Amylase activity: ●—● attached cotyledons, O—O dettached cotyledons. The results obtained by incubation of attached cotyledons with cycloheximide and actinomycin D, were similars to those obtained with dettached cotyledons (O—O). Phosphorylase activity +—+ was expressed as µmoles of Pi/mg protein/min.

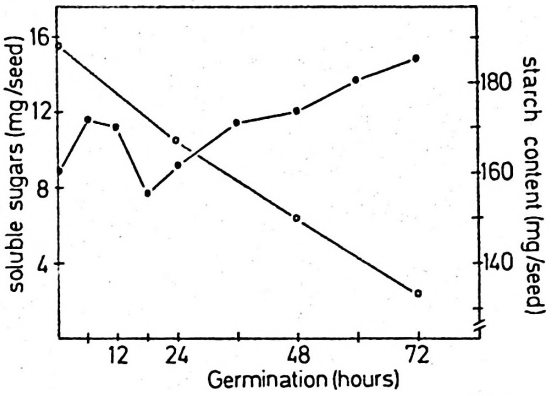

Fig. 2. *Changes in starch and soluble sugars during germination of seeds of* Cicer arietinum.
O—O stard content. ●—● soluble sugars.

ferent agents seem to indicate that this activity is due to an amylase of the alpha type. In the presence of 1.5 mM $CaCl_2$, 7, 55 and 70% of the total amylase activity remained after heating at 70° C for 5 min in extracts of 0, 48 and 144 h of germination respectively (data not shown). All activity was conserved after treatment with 5 mM $HgCl_2$ and finally, all enzymic activity was lost through the treatment with 10 mM EDTA (data not whown). Collectively, these results establish, according to FRYDENBERG and NIETSEN (12) and SWAIN and DEKKER (26) that the amylase(s) present in the extracts from chick pea seeds belong to the alpha type. According to these authors, beta amylase activity is irreversibly lost after heating at 70° C, is inhibited by heavy metal ions and is not affected by EDTA treatment. The total absence or low activity of beta amylases seems to be a general phenomenon in legume seeds, since similar results have been found in peas (17) and in lentils (11).

The zymogram pattern of the starch-degrading enzymes showed a maximum of three bands (fig. 3). The slowest band (band *a*) was the only one present in resting seeds, appeared only in the presence of Pi, and its activity was conserved after

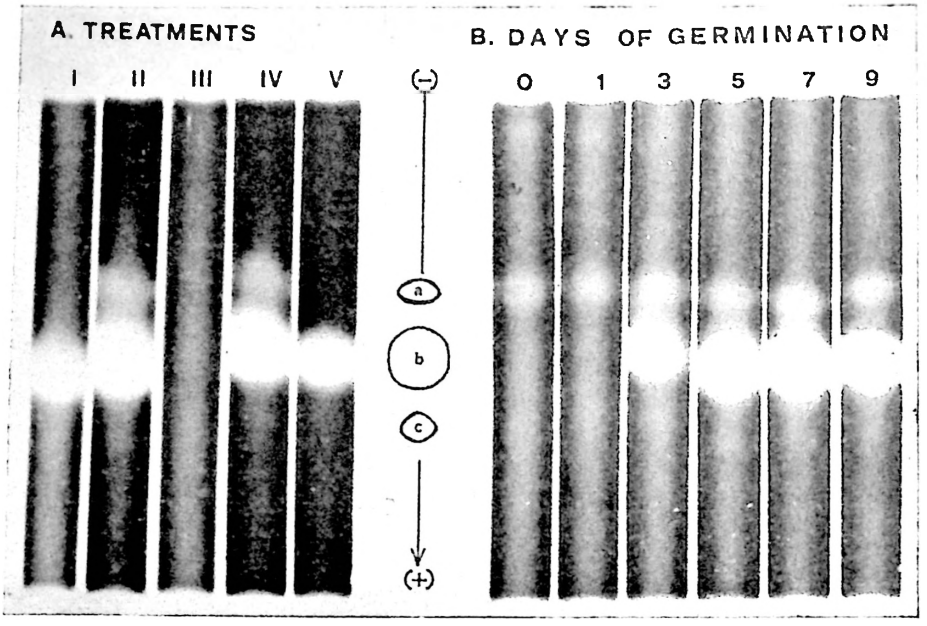

Fig. 3. *Zymogram pattern of starch-degrading enzymes in* Cicer arietinum *cotyledons. A*) Different treatments. I: extract in acetate buffer. II: extract in phosphate buffer. III: extract in phosphate buffer + EDTA. IV: extract in phosphate buffer + $HgCl_2$. V: extract in phosphate buffer + $CaCl_2$ heated a 70° C for 5 min. *B*) Days of germination.

treatment with $HgCl_2$ and disappeared after treatment with EDTA and by heating at 70° C for 5 min in the presence of calcium ions. These results are consistent with the identification of this band as a phosphorylase. A second band (band *b*) appeared after 48 h of germination. It did not appear in gels treated with EDTA and was insensitive to the heat and mercuric chloride treatments. Finally a third band (band *c*) appeared after 96 h of germination with the same characteristics as band *b*. Both bands were identified as alpha amylases. Band *c* presented a very slight activity and it was very difficult to make it visible in gels. Tests were performed in order to detect the presence of other enzymes, mainly beta amylases, but all of them were without success. The appearance of the two bands of alpha amylase, which coincided with the sharp increase in amylolytic activity was due to *de novo* synthesis, and not to the enzyme activation, since cycloheximide and actinomycin D (fig. 1) inhibited the increase in total amilolytic activity. *De novo* synthesis of alpha amylase has also been found in rice endosperm (22) and in pea cotyledons (17). SWAIN and DEKKER (28) and GOODWIN and CARR (14) found that puromycin and actinomycin D inhibited alpha amylase synthesis in pea cotyledons and barley aleurone layers respectively.

Fig. 3 B shows the changes in enzyme pattern during germination. The phosphorylase was the only enzyme which appeared in resting and 1 day germinated seeds. The slight amylase activity shown in fig. 1 during the first two days of germination could not be detected by electrophoresis. The other two bands, *b* and *c*, appeared as mentioned above at 48 and 96 h of germination respectively. The phosphorylase was also the only enzyme which appeared in electrophoresis of detached cotyledons (not shown). These re-

Table I. *Effect of gibberellic acid and kinetin on the amylolytic activity in detached cotyledons of* Cicer arietinum *L.*

Incubation h	Amylase activity			
	Attached cotyledons	Detached cotyledons	Detached cotyledons + GA_3 (10^{-4} M)	Detached cotyledons + Kinetin (10^{-4} M)
0	0.22 *	0.07	—	—
72	1.27	0.08	0.07	0.06
240	2.15	0.10	0.09	0.09

* Specific activity.

sults suggest in agreement with those of CHIN *et al.* (5) that an axis component may regulate the capacity for protein synthesis during germination.

It is a well known fact that some plant hormones act similarly to the embryo axis in inducing hydrolytic activity in embryoless seeds. Gibberellin produced in the embryos of germinating seeds of barley and other cereals, moves to the aleurone layers and promotes the formation of amylases, proteases and other hydrolases (6, 15, 18, 22, 32). Kinetin promotes starch degradation in wheat endosperm (4) and induces amylase activity in detached cotyledons of germinating peas (20).

Table I shows the results obtained when detached cotyledons were incubated with GA_3 or Kinetin. None of these substances had any promotive influence on amylolytic activity in chick peas cotyledons. Since about 40 different gibberellins have been characterized in higher plants it is possible that a different type of gibberellin might be active in this legume. Another possibility is that some unknown factor released by the embryo is the promoting agent of amylase activity in chick pea cotyledons. More work is needed in order to explain this problem.

Resumen

Se determinan las variaciones del contenido en almidón y azúcares solubles y de las actividades fosforilásica y amilásica en cotiledones de semillas de *Cicer arietinum* L. durante la germinación. Las α-amilasas son las principales responsables de la actividad amilásica. Durante los dos primeros días de germinación la fosforilasa es importante en la degradación del almidón, y pasa a un lugar secundario al aumentar la actividad amilásica. Por medio de electroforesis de disco en gel de poliacrilamida se evidencia la existencia de una fosforilasa durante toda la germinación y de dos α-amilasas que se detectan después de 48 y 96 h de germinación, respectivamente. Se comprueba que el aumento de actividad α-amilásica durante la germinación es debido a síntesis *de novo* de las dos isoenzimas, ya que ambas son inhibidas por cicloheximida y actinomicina D. Esta síntesis *de novo* depende de algún factor producido por el embrión que no puede ser sustituido ni por el ácido giberélico ni por kinetina.

References

1. BERNFELD, P.: In «Methods in Enzymology». Vol. 1 (S. P. COLOWICK and N. O. KAPLAN, eds.). Academic Press. New York, 1955, pp. 149-158.
2. BERNSTEIN, L.: *Amer. J. Bot.*, **30**, 517-526, 1943.
3. BILDERBACK, D. E.: *Plant Physiol.*, **48**, 331-334, 1971.
4. BOOTHBY, D. and WRIGHT, T. C.: *Nature*, **196**, 389-390, 1962.
5. CHIN, T. Y., POULSON, R. and BEEVERS, L.: *Plant Physiol.*, **49**, 482-489, 1972.
6. CHRISPEELS, M. J. and VARNER, J. E.: *Plant Physiol.*, **42**, 1008-1016, 1967.
7. DAVIS, J. B.: *Ann. N.Y. Acad. Sci.*, **121**, 404-427, 1964.
8. DE LA FUENTE-BURGUILLO, P. and NICOLÁS, G.: *Pl. Sci. Lett.*, **3**, 143-148, 1974.
9. DEKKER, R. H. F. and RICHARDS, G. N.: *J. Sci. Fd. Agric.*, **22**, 441-444, 1971.
10. DURE, L. S.: *Plant Physiol.*, **35**, 925-934, 1960.
11. FERNÁNDEZ-TARRAGÓ, J. and NICOLÁS, G.: *Plant Physiol.*, **58**, 618-621, 1976.
12. FRYDENBERG, O. and NIELSEN, G.: *Hereditas*, **54**, 123-139, 1965.
13. GEPSTAIN, A. and ILAN, I.: *Plant Cell. Physiol.*, **11**, 819-822, 1970.
14. GOODWIN, P. B. and CARR, D. J.: *Planta*, **106**, 1-12, 1972.

15. GOODWIN, P. B. and CARR, D. J.: *J. Exp. Bot.*, **23**, 1-7, 1972.
16. ILIEV, S. V.: *Compt. rend. acad. Bulg. Sci.*, **26**, 117-120, 1973.
17. JULIANO, B. O. and VARNER, J. E.: *Plant Physiol.*, **44**, 886-892, 1969.
18. KHAN, A. A., VERBEEK, R., WATERS, E. C. and VAN ONCKELEN, H. R.: *Plant Physiol.*, **51**, 641-645, 1973.
19. KRUGER, J. E.: *Cereal Chem.*, **49**, 391-398, 1972.
20. LOCKER, A. and ILAN, I.: *Plant Cell Physiol.*, **16**, 449-454, 1975.
21. LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. and RANDALL, R. J.: *J. Biol. Chem.*, **193**, 265-290, 1951.
22. PALMIANO, E. P. and JULIANO, B. O.: *Plant Physiol.*, **49**, 751-756, 1972.
23. RODRÍGUEZ-BUJÁN, M. C., BATALLÁN, I., BERMÚDEZ, M. T., SÁNCHEZ-TAMES, R. and NICOLÁS, G.: *Rev. esp. Fisiol.*, **30**, 21-26, 1974.
24. SMITH, J. B. and BENNETT, M. D.: *J. Sci. Fd. Agric.*, **25**, 67-71, 1974.
25. SURENDRANATHAN, K. K. and MADHUSUDANAN NAIR, P.: *Phytochemistry*, **11**, 119-125, 1972.
26. SWAIN, R. R. and DEKKER, E. E.: *Biochem. Biophys. Acta*, **122**, 75-86, 1966.
27. SWAIN, R. R. and DEKKER, E. E.: *Biochem. Biophys. Acta*, **122**, 87-100, 1966.
28. SWAIN, R. R. and DEKKER, E. E.: *Plant Physiol.*, **44**, 319-325, 1969.
29. TANAKA, Y., ITO, T. and AKAZAWA, T.: *Plant Physiol.*, **46**, 650-654, 1970.
30. TKACHUK, R. and KRUGER, J. E.: *Cereal Chem.*, **51**, 508-528, 1974.
31. WAGENAAR, S. and LUGTENBORG, T. F.: *Phytochemistry*, **12**, 1243-1247, 1973.
32. YOMO, H. and VARNER, J. E.: *Plant. Physiol.*, **51**, 708-713, 1973.