REVISTA ESPAÑOLA DE FISIOLOGIA, 37, 197-204, 1981

# Purification and Characterization of an α-Amylase from the Cotyledons of Germinating Lentils

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(Received on June 16, 1980)

J. FERNANDEZ-TARRAGO and G. NICOLAS. *Purification and Characterization of an α-Amylase from the Cotyledons of Germinating Lentils.* Rev. esp. Fisiol., 37, 197-204. 1981.

α-Amylase from the cotyledons of lentils germinated for 6 days (*Lens culinaris*, Medik) was purified by heat treatment, complexing with glycogen, Sephadex G-75 filtration and electrofocusing. Although three bands with α-amylase activity were separated in the purified extract from Sephadex G-75 filtration by polyacrylamide gel electrophoresis, only one α-amylase fraction was obtained by electrofucusing, which appeared free of contaminating proteins in the electrophoretic pattern. The purified enzyme had maximum activity at pH 5.4, an activation energy of 5.8 kcal/mol, a km for soluble starch of $3.4 \times 10^{-4}$ g/ml, an isoelectric point of 4.8 and a molecular weight of 43,000. The pH and temperature stability of the enzyme and the effect of calcium and mercuric ions on the enzyme activity and stability were also studied.

Starch metabolism during seed germination has been extensively studied in cereals, so that the pathways of enzymatic degradation and their physiological control are quite well understood. In contrast knowledge of the starch catabolism in leguminous seeds is very incomplete and confused, principally with respect to the physiological control of the evolution of α-amylases during germination.

The study of regulatory mechanisms of α-amylase activity in leguminous seeds has yielded different results from those obtained with cereal seeds, and different authors have reported contradictory results for the same species (13, 16). A better knowledge of the structural characteristics of the enzyme, as well as its properties, can contribute to the clarification of these physiological aspects. In this respect also, the study of cereal seeds is much more advanced and there are only few studies of purification of leguminous α-amylases (6, 14).

Lentil cotyledons have an α-amylase activity which starts at very low levels in ungerminated seeds and reaches maxi-

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mum specific activity after six days of germination (4). During the entire period of germination three bands of enzymatic activity are obtained with polyacrylamide gel disc electrophoresis. One characteristic of this seed is that the α-amylase appears to be the only amylase responsible for starch breakdown, since the β-amylase is practically inoperative and no amylopectin-1, 6-glycosidasic activity is detected. For these reasons, lentil seeds are an ideal material for the study of α-amylase activity. This paper will describe the process of purification of this enzyme and some characteristics which can be compared with those of other seeds.

## Materials and Methods

*Extractions and purification of the α-amylase.* Lentil (*Lens culinaris*, Medik) seeds were germinated by the method previously described (4). After germinating for six days, the cotyledons were separated (200 g fresh weight) and were homogenized in 500 ml of 0.025 M TrisHCl pH 7.4. The homogenate was filtered through a double layer of cheesecloth and centrifuged at 20,000 *g* for 30 minutes at 2° C. The precipitate was discarded, and 0.5 M $CaCl_2$ was added to the supernatant until a final concentration of 0.01 M was reached.

The extract was heated at 70° C for ten minutes by the method described for purification of wheat α-amylase (8) without changing the pH of the extract. The resulting precipitate was eliminated by centrifugation at 500 g.

Next a glycogen complex was formed according to the method of SCHRAMM and LOYTER (12). The enzyme solution obtained by heat treatment was raised to pH 7; cold absolute ethanol was added slowly, while stirring, to a final concentration of 40% v/v. The precipitate formed was removed by filtration and 25 mg glycogen dissolved in 5 ml of the original buffer was added dropwise to the supernatant. It was stirred slightly for ten minutes, to allow precipitation of the enzyme-glycogen complex, and then centrifuged at 2,000 *g* for ten minutes. The precipitation was repeated until no activity was detected in the supernatant and the precipitates were dissolved in a total of 100 ml of 0.05 M Tris-HCl buffer, pH 6.7, containing $5 \times 10^{-3}$ M$CaCl_2$. This enzyme solution was left at room temperature overnight to allow the digestion of the glycogen.

The treatment with heat and the formation of the glycogen complex were performed immediately after the extraction.

Two different methods were used for the final purification process: Sephadex G-75 chromatography: a 60 × 3 cm column was prepared and 45 ml of glycogen complex solution were eluted with 0.05 M.Tris HCl, pH 7.4, containing 0.01 M $CaCl_2$, and 5 ml fractions were collected. Electrofocusing: by the method described by VESTERBERG and SVENSSON (17), introducing 20 ml of glycogen complex solution into a 110 ml column with a gradient of pH 4-6 at 300 V for 36 h at 4° C. The content of the column was collected in 2.6 ml fractions.

α-Amylase activity was determined by method described by BERNFELD (1), using 1% (w/v) potato starch in 0.05 M acetate buffer, pH 5, as substrate and incubating at 30° C for ten minutes.

Protein was determined by the method of LOWRY *et al.* (9).

*Characterization of the α-amylase.* The polyacrylamide gel disc electrophoresis was carried out by the DAVIS method (3), using 7% small-pore gel. To localize the bands with α-amylase activity a technique previously described was used (4).

To study the influence of pH on the activity, α-amylase activity was determined by dissolving the substrate in 0.1 M acetic-bisodium phosphate buffer, pH 3.1-

7.4, and 0.1 M Tris HCl buffer, pH 8.0-8.9. Its influence on stability was studied by incubating 0.1 ml of the enzymatic extract with 0.5 ml of the same buffers for two hours at 27° C; subsequently the activity was assayed at pH 5.

The effect of temperature on the rate of enzymatic hydrolysis of the starch was studied between 15°-17° C and calculating the apparent energy of activation by plotting the results on an Arrhenius graph. To determine the thermal stability of the enzyme the extract was incubated for one hour and thirty minutes at the same temperatures, and the activity assayed at 30° C.

The apparent Michaelis constant, $k_m$ was determined using soluble starch (Merck) as a substrate, and incubating at different concentrations for 3 min under the conditions already described. The results were plotted on a Lineaweaver-Burk graph.

To determine the molecular weight SDS gel electrophoresis were used according to the ZAHLER method (18), using trypsin, alcohol dehydrogenase, ovoalbumin, bobine serum albumin and catalase as standards. The gels were dyed overnight with Coomassie Blue.

## Results

*Purification of the α-amylase.* To purify the α-amylase of lentil cotyledons, seeds that have been germinated for six days were used; this is the point at which there is maximum specific activity during germination and there are no indications of β-amylase activity; therefore, the BERNFELD (1) method may be used as measure of α-amylase activity.

The results obtained in the purification process are shown in table I. In the precipitation by formation of an enzyme glycogen complex a substantial increase in specific activity is obtained, but disc electrophoresis a large amount of protein without α-amylase activity is observed (fig. 1). Final purification was assayed by Sephadex G-75 filtration and electrofocusing. In a first experiment the two methods were used in succession and a similar degree of purity of the enzyme was obtained, although with a very low yield.

Enzyme characteristics were studied using the enzyme solution obtained with Sephadex G-75 filtration, since the three α-amylase isoenzymes are present, the degree of purity is good according to what was observed in electrophoresis (fig. 1), and optimal volume with a sufficient degree of enzyme concentration was obtained. To determine the molecular weight an electrofocusing extract was used.

In the first attempts of purification ion-exchange chromatography was used as final step, but DEAE-cellulose chromatography, and more markedly DEAE-Sephadex A-50 chromatography, resulted in

Table I. *Purification of α-amylase from lentil cotyledons.*
* A unit of α-amylase activity is that amount of enzyme which produces reducing groups from starch hydrolysis equivalen to 1 mg of maltose in 10 min. **, ***. Carried out with 45 ml and 20 ml of glycogen-complex extract respectively, yield was calculated proportionally.

| Extract | Volume ml | Concentration units*/ml | Total units | Protein mg/ml | Specific activity units/mg prot. | Yield % | Purification |
|---|---|---|---|---|---|---|---|
| Initial | 500 | 32.2 | 16,625 | 9 30 | 3.5 | 100 | 1.0 |
| Heat treated | 450 | 30.4 | 13,702 | 3.82 | 7.9 | 83 | 2.2 |
| Glycogen complex | 92 | 93.5 | 8,602 | 0.55 | 170.0 | 52 | 47.6 |
| Sephadex G-75 ** | 92 | 26.4 | 2.430 | 0.10 | 254.0 | 30 | 71.2 |
| Electrofocused *** | 13 | 57.5 | 748 | 0.14 | 386.1 | 20 | 108.1 |

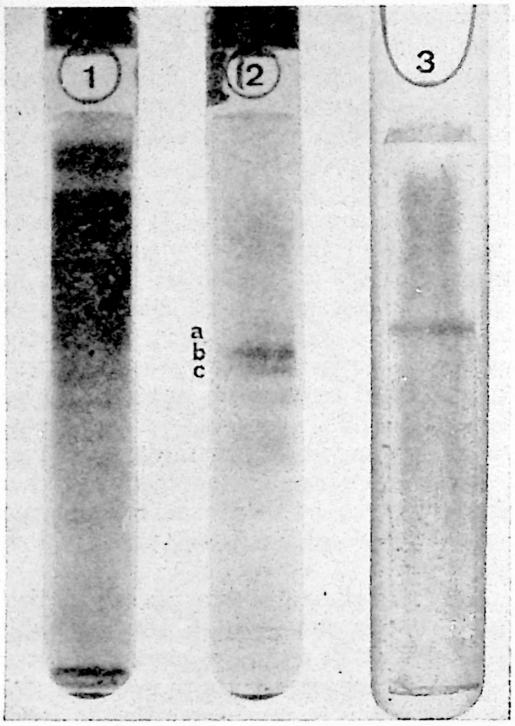


Fig. 1. *Polyacrylamide gel disc electrophoresis of purified α-amylase.*
(1) Glycogen-complex extract; (2) Sephadex G-75 extract; (3) electrofocused extract. *a, b* and *c* protein bands with α-amylase activity. (1) and (2) stained with amido Schwartz, (3) stained with Coomassie blue.

a considerable loss of enzyme activity and the degree of purification was lower than the degree obtained by electrofocusing.

When various bands with α-amylase activity are detected by electrophoresis, they generally correspond to isoenzymes which can be separated by ion-exchange chromatography or by electrofocusing (2, 11, 15). Whit lentil cotyledons, although three bands with α-amylase activity were detected in the crude extract (4) and in the enzyme solution from Sephadex G-75 filtration, only one activity peak was obtained, either with ion-exchange chromatography (data not shown) or electrofocusing (fig. 2).

Aliquots obtained from the various steps of purification, containing 0.01 M $CaCl_2$, completely retain their α-amylase activity after six month of storage at 2° C at —18° C.

*Characterization of the purified α-amylase.* The electrofocusing carried out on a gradient of pH 4-6 gives a pH of 4.8 for lentil α-amylase (fig. 2).

Figure 3 *a* illustrates the activity curve of lentil α-amylase at different pH values. This enzyme is inactive above pH 8.5 and below pH 3, with maximum activity at pH 5.4. The effect of pH on enzyme stability is shown in figure 3 *b*. The enzyme retains all activity after two hours of incubation at pH 5-8 and is completely inactivated below pH 3.5 and above pH 9.

Investigation of enzyme activity at different temperatures revealed maximum activity at 50°-55° C; represented on the Arrhenius plot, a lineal relation was observed between 25°-45° C (fig. 4). The energy of activation calculated from the slope is 5.8 Kcal/mol.

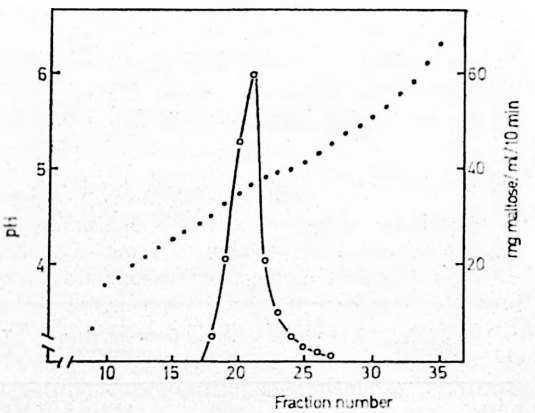


Fig. 2. *Electrofocusing of α-amylase purified to the glycogen-complex stage.*
(●—●) pH gradient; (○—○) α-amylase activity.

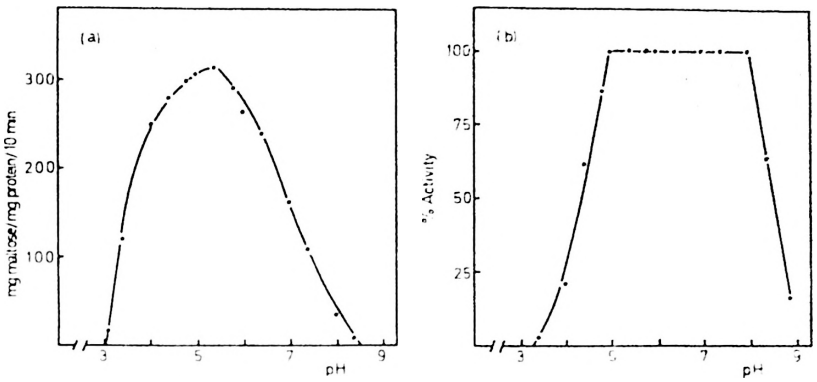


Fig. 3. *Effect of pH on α-amylase purified to the Sephadex G-75 stage.* (*a*) on the activity; (*b*) on the stability. Stability of the enzyme is expressed as percentage of original activity at pH 5 remaining after 2 h at each pH at 27° C.

Table II. *Effect of various substances on α-amylase activity.*

The effect is expressed as percentage of specific activity remaining after treatment of a Sephadex G-75 extracts. To study the influence of $CaCl_2$, the excess of $Ca^{2+}$ was first removed from the Sephadex G-75 extract by filtering it through a small Sephadex G-25 column. * $CaCl_2$ $10^{-1}$ M was added before activity assay.

| $CaCl_2$ | | EDTA | | $HgCl_2$ | |
|---|---|---|---|---|---|
| Molarity | % activity | Molarity | % activity | Molarity | % activity |
| 0.0 | 87.7 | $10^{-3}$ | 0 | $10^{-5}$ | 100 |
| $10^{-3}$ | 100 | $10^{-3}$(+)* | 0 | $10^{-5}$ | 67 |
| $10^{-2}$ | 100 | | | $10^{-4}$ | 0 |

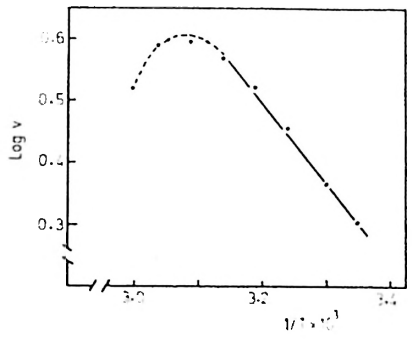


Fig. 4. *Arrhenius plot for α-amylase purified to the Sephadex G-75 stage.*

The thermal stability of the purified α-amylase is shown in figure 5. The extract obtained from filtration with Sephadex G-75 is stable for 90 min at temperatures up to 50° C, the stability decreasing at higher temperatures (figure 5 *a*). Since this extract contains 0.01 M $CaCl_2$ the influence of $Ca^{2+}$ on the thermal stability was studied (fig. 5 *b*). A marked decline was noted upon removing the ions from the extract by filtering it through a small Sephadex G-25 column: there was a 20 % decrease in activity after 60 minutes at 40° C, and activity disappeared altogether after 40 minutes of heating at 60° C.

Filtration by means of a Sephadex G-25 column produced a decrease in activity to 87.7 % from the origin, which was reversed with the addition of $10^{-3}$ M $CaCl_2$, however the activity of the crude extract was the same before and after the addition of $CaCl_2$. Incubation of the enzyme for 30 min with $10^{-3}$ M EDTA produced total inactivation which was not reversed despite the subsequent addition of $CaCl_2$. Incubation with $10^{-6}$ M $HgCl_2$ for 60 min did not affect activity; however, at greater concentrations there was some inactivation which was com-

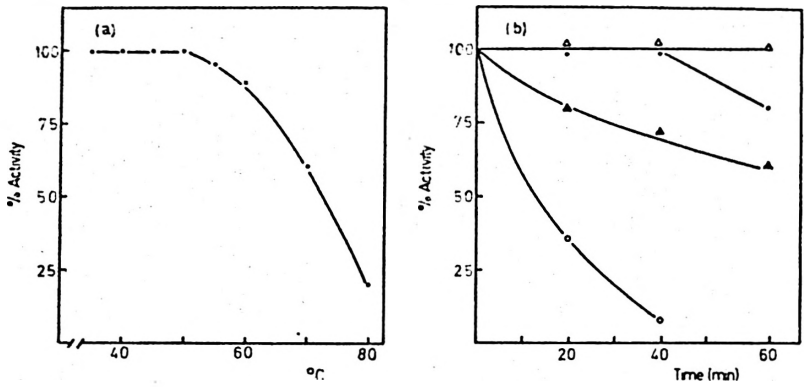


Fig. 5. *Effect of temperature on the stability of α-amylase purified to the Sephadex G-75 stage.*
(*a*) with $CaCl_2$; (*b*) without $CaCl_2$. Stability with $CaCl_2$ is expressed as percentage of original activity at 30° C remaining after 1 h 30 min at each temperature. Stability without $CaCl_2$ is expressed in the same way but varying the incubation time at each temperature: (△—△) 30° C; (●—●) 40° C; (▲—▲) 50° C; (○—○) 60° C.

plete at a concentration of $10^{-4}$ M (table II).

Knowing the variety of substrate on which α-amylase acts, and the variability that can exist within the same substrate depending on its origin, a significant Michaelis constant value cannot be assigned for the enzyme. To determine the $K_m$ of lentil cotyledon α-amylase, soluble starch, the most widely cited substrate in the literature, is used; with it a value of $3.2 \times 10^{-4}$ g/ml is obtained.

Molecular weight was 43,000 with the α-amylase mobility being identical to that of ovoalbumin.

## Discussion

The heat treatment at 70° C followed by precipitation of a glycogen complex and filtration with Sephadex G-75 is a fast simple method to purify α-amylase of germinated lentil cotyledons, while obtaining enough pure extract for use in subsequent experiments. For works requiring a high degree of purification, electrofocusing can be used, because as shown in the electrophoretic pattern the resulting fraction is free of contaminating proteins, although this method gives a lesser yield and can only be used with small quantities of enzyme.

The electrophoretic pattern of the Sephadex G-75 extract reveals the presence of three protein bands which correspond to three bands of α-amylase activity. After electrofocusing only one band of protein appears. If the three original bands represent true isoenzymes, two of them are lost in the electrofocusing and they are not separated by ion-exchange chromatography.

No important differences are noted when the α-amylase of lentil cotyledons is compared with those of ungerminated barley and malted barley (5); ungerminated oats, rye and wheat (7); sorghum malt (2); malted wheat (15); immature wheat (11); immature barley (10); broad beans (6); and peas (14), with respect to the optimal pH for enzymatic activity, pH stability, $k_m$ for soluble starch, the isoelectric point and molecular weight. One should only point out that the isoelectric points of the four fractions of α-amylase of malted

wheat are higher (between 6.05 and 6.2), while those of immature wheat and immature barley are very similar to that of lentil αamylase.

The energy of activation is the only characteristic which appears to establish a difference between the α-amylase of cereals and legumes, that of legumes being slightly lower: 5.8 kcal/mol for lentils, 5.0 kcal/mol for broad bean (6) and 7.6 kcal/mol for peas (14). Only two fractions of malted wheat α-amylase have a similar value (7.01 kcal/mol) (15), while for the other cereals the values range from 8.47 kcal/mol for another malted wheat fraction to 13 kcal/mol for ungerminated barley and malted barley (15). Although the data available in the literature are not extensive enough to make a valid comparison, this seems to indicate that the α-amylases of legumes are more efficient as catalysts than those of cereals.

All the α-amylase have a high thermal stability which is dependent on the presence of calcium ions in the extract. Filtration with Sephadex G-25 eliminates not only excess calcium ions from the purified extracts, but also some of those necessary for activity, since there is a 14 % loss in activity of lentil α-amylase. Malted wheat α-amylases (15) lose 91 % of their activity after filtering with Sephadex G-10, which is reversed with the addition of $Ca^{2+}$; this appears to indicate that either the lentil α-amylase requires less $Ca^{2+}$ for its activity or that these ions are more firmly bound to protein.

EDTA causes total, irreversible inactivation of lentil α-amylase, despite the subsequent addition of $Ca^{2+}$. The same occurs with α-amylase of pea cotyledons (14); however, with the α-amylase of other seeds (6, 7) there is partial recuperation of enzymatic activity upon addition of $Ca^{2+}$. It is possible that the $Ca^{2+}$ — enzyme structure relationship is distinctive for each species.

α-amylase activity is generally inhibited to a greater or lesser degree, depending on enzyme origin, by $Hg^{2+}$ but, according to MARCHYLO *et al.* (11), this seems to be unrelated to the need for free sulphydryl groups for the maintenance of enzyme activity.

## Resumen

Se purifica la α-amilasa de cotiledones de lenteja (*Lens culinaris*, Medik) germinadas durante 6 días, mediante tratamiento a 70° C, formación de un complejo con glucógeno, filtración por Sephadex 6-75 y electroenfoque. Mediante electroforesis en gel de poliacrilamida se separan tres bandas con actividad α-amilásica en el extracto purificado por filtración por Sephadex 6-75, obteniéndose sólo una fracción con actividad en el electroenfoque libre de proteínas contaminantes. La enzima purificada tiene máxima actividad a pH 5,4, una energía de activación de 5,8 kcal/mol, una $k_m$ para el almidón soluble de $3,4 \times 10^{-4}$ g/ml, un punto isoeléctrico de 4,8 y un peso molecular de 43.000. Se estudia también el efecto del pH y la temperatura sobre la estabilidad de la enzima y el efecto de los iones de calcio y mercurio sobre su actividad y estabilidad.

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