DETERMINATION OF AMYLASE ACTIVITY IN COTYLEDONS OF *Phaseolus vulgaris* L. cv. carioca

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ABSTRACT

Determination of α- and β-amylase activity in the extracts of cotyledons of *Phaseolus vulgaris* L. cv. carioca was done using selective inactivation of α-amylase by lowering the pH of the incubation medium or by the use of EDTA as inhibitor or selective inactivation of β-amylase by the use of $HgCl_2$ or by heating to 70°C in the presence of $CaCl_2$; and still by using the reagent starch azure for specific determination of α-amylase. Results indicated that the methods used were inappropriate in this case, being indicated the determination of total amylase activity.

Key-words: α-amylase, β-amylase, determination, selective inactivation, *Phaseolus vulgaris*.

INTRODUCTION

Determination of the quantities of α- and β-amylase in the total amylase activity can be done by selective inactivation of β-amylase by heating the crude extract to 70°C or by the use of mercuric chloride, and inactivation of α-amylase by incubation of crude extract with EDTA, or by carrying out assay at low pH. These procedures were adopted by several authors, e.g. TÁRRAGO & NICOLÁS (1976), MONERRI & GUARDIOLA (1986) and CHRISPEELS & VARNER (1967).

DOEHLERT & DUKE (1983) and HIRASAWA (1989) used starch azure which allowed the quantification of enzymatic activity as a function of the color intensity, produced by the release of soluble fragments of starch linked to the Remazol Brilliant Blue dye as a result of the action of α-amylase.

In this work, we present results of tests conducted in accordance with the above procedures with the aim of showing the difficulties in the choice of a assured analysis of the contribution of α- and β-amylase for the total amylase activity in cotyledons of *Phaseolus vulgaris* L. cv. carioca.

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MATERIAL AND METHODS

Crude extracts (CE) were prepared according to the method of JULIANO & VARNER (1969). Cotyledons of *Phaseolus vulgaris* L. cv. carioca were ground in cold tris-maleate buffer (0.05M; pH 7.0), with the aid of a mortar and pestle. The homogenate was centrifuged for 20 min at 2.400 x g and the supernatant was considered as CE.

Amylase assays were done as follows:

(a) *With the use of inhibitors*. For the assay of total, α- and β-amylase the modified method of CHRISPEELS & VARNER (1967)was used. Reaction mixture contained 0.5 ml CE with 0.5 ml 40mM EDTA for α-amylase, 0.5 ml CE with 0.5 ml 0.4mM $HgCl_2$ for β-amylase or 1.0 ml CE for total amylase activity (CE was diluted with distilled water). To this, 1.0 ml starch solution (150 mg soluble starch, 600 mg KH_2PO_4 and 200 μmol $CaCl_2$ in 100 ml distilled water) was added and the reaction was carried for 5 min at 30°C. Reaction was stopped by adding 1.0 ml iodine reagent (3 mg de KI + 0.3 mg of I in 0.05N HCl). For the reading, the mixture was diluted with distilled water up to 13.0 ml and the absorbance read at 620nm in a spectrophotometer Spectronic 20D.

One unit of enzyme was considered as the quantity that causes an alteration of 0.1 in absorbance.

(b) *β-amylase assay with inactivation of α-amylase by lowering the pH of the incubation medium.* A modified method of MONERRI & GUARDIOLA (1986) was used which was similar as above, except that the starch solution was prepared in citrate-phosphate buffer, pH 3.0. The reaction was carried out and stopped as above.

(c) *α-amylase assay with inactivation of β-amylase by heating to 70°C.* This was done according to the method of TÁRRAGO & NICOLÁS, 1976. Aliquot of 5.0 ml of CE with 3, 6, or 9mM $CaCl_2$ were heated in water-bath at 70°C for 5, 10 or 15 min. After this treatment, the assay followed the procedure as described for the total amylase activity.

(d) *α-amylase assay with starch azure reagent.*

Two methods were tested. According to the first method of Hirasawa (1989) 2ml of 2% starch azure (w/v) were slowly heated before the assay until boiling. To this solution, 1 ml of sodium acetate buffer 0.2M (pH 5.4), 0.12 ml of $CaCl_2$ 0.1M 0.83 ml of deionized water and 50

µl of CE were added. The reaction was carried out for 5 min at 37°C under agitation and was stopped with addition of 1 ml of 50% trichloroacetic acid (TCA) (w/v). The resultant mixture was centrifuged at 1000 x g for 20 min, filtered and absorbance was read at 590nm.

According to the second method (Doehlert & Duke,1983) a solution of 2% starch azure (w/v) was prepared in 100mM potassium succinate (pH 6.0) with 3mM $CaCl_2$. This solution was slowly heated. After boiling, the solution was stirred continuously until use. The reaction mixture composed of 5 ml of the starch azure solution (placed in a test tube in a water-bath at 30°C) plus 1.0 ml of CE. In time designated as "zero", an aliquot of 1.0 ml was removed and placed in a test tube with 1.0 ml of 20% TCA (w/v). Additional aliquots were taken in times settled for the assay. After centrifugation, the absorbance of supernatant was read at 590 nm.

Protein determination: Protein concentrations were estimated according to LOWRY *et al.* (1951), using bovine serum-albumine as a standard.

Electrophoresis: Electrophoretic runs, for visualizing the enzymes present in the CE's of the different treatments, were done according to the method of LAEMMLI (1970), with modifications described in Table 1 and the exclusion of dodecyl sodium sulfate (SDS). The system comprises a 3% stacking gel (1.0 cm length) and 7.5% separation gel (6.0 cm length).

Running conditions: Electrophoretic runs were done in 80V constant voltage in a vertical slab, for about 2 hr.

Running buffer (pH 8.25): 3g of Tris + 14.4 g of glycine.

Visualization: After running, gels were incubated at 30°C in a 0.5% starch solution in 0.1M acetate buffer (pH 5.0) for 30 min and, thereafter, washed and transferred to iodine solution for about one min. After this time, a new washing of the gels was conducted. Bands with amylolytic activity appeared brightly while the rest of the gel was colored as a dark blue (JACOBSEN, SCANDALIOS & VARNER, 1970and TÁRRAGO & NICOLÁS, 1976).

TABLE 1 - Preparation of the polyacrylamide gels for electrophorectic runs, modified from the procedure proposed by Laemmli (8)

Stock Solution	Stacking gel	Separation gel
Acrylamide (30%) + bis-Acrymalide (0.8%) (ml)	1.0	2.13
Tris buffer 1M pH 8.8 (ml)	-----	3.18
Tris buffer 1M pH 6.8 (ml)	1.25	----
Glycerol 87% (ml)	----	0.5
Bi-distilled water (ml)	7.1	2.625
Ammonium persulfate 10% (µl)*	50.0	37.5
TEMED (µl)**	10.0	15.0

* prepared just before use
** N,N,N',N' - tetramethylethylenediamine

The procedure for the preparation of the samples is described in Table 2. An aliquot of 30µl was used for each gel well. Samples submitted to inhibitors of amylase activity were kept incubating for 1 hr before the addition of the buffer in the sample.

TABLE 2 - Preparation of the samples for electrophoresis in polyacrylamide gel. Sample buffer = 50 ml of tris-maleate buffer + 50 ml of glycerol 100% + 1 ml of bromophenol blue.

Substance	Sample A	Sample B	Sample C	Sample D	Sample E	Sample F
CE (µl)	100	100	100	100	100	100
10mM EDTA (µl)	----	----	----	----	----	100
0.1mM $HgCl_2$ (µl)	----	----	----	----	100	----
Tris-Maleate buffer (µl)	100	100	100	100	100	100
Sample buffer (µl)	200	200	200	200	200	200
$CaCl_2$	3mM	9mM	9mM	3mM	3mM	3mM
Identification	CE 8 days (dark)	CE 8 days (dark)	CE 9 days (dark)	CE 9 days (dark)	CE 9 days (dark)	CE 9 days (dark)

RESULTS AND DISCUSSION

According to DOEHLERT & DUKE (1983), inactivation of β-amylase through heating was described in barley, where the α-amylase was a stable enzyme in this condition. Our results indicated a reduction of α-amylase activity, mainly in extracts containing only 3mM $CaCl_2$ and after 15 minutes heating complete inactivation of the enzyme was observed (Figures 1 and 2). Heating the extract for 15 minutes with 9mM $CaCl_2$ maintained the activity at the same level of 5 min but lower than the control activity determined after selective inhibition by $HgCl_2$.

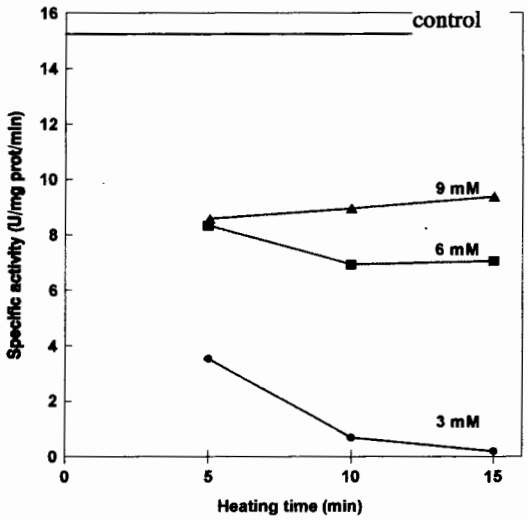

FIGURE 1 - Specific activities of α-amylase in CE prepared from cotyledons of intact seeds of *Phaseolus vulgaris* L. cv. carioca germinated in dark during 8 days at 25°C, submitted to 5, 10 or 15 min of heating at 70°C, with different concentrations of $CaCl_2$. Control refers to assay done with $HgCl_2$ as inhibitor of β-amylase, in the presence of 3mM $CaCl_2$.

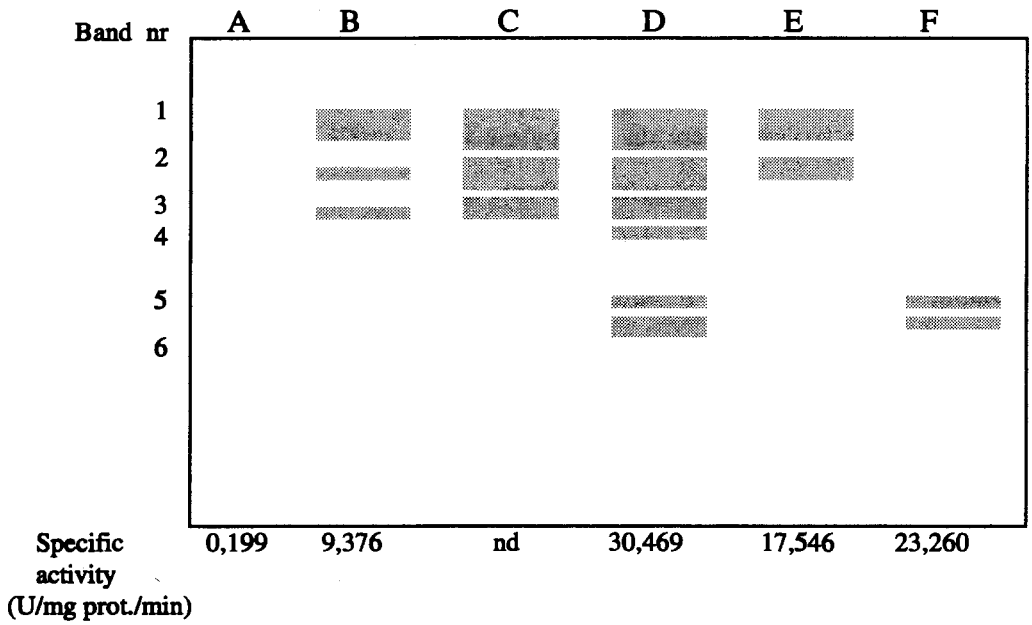

FIGURE 2 - Schematic representation of an electrophoretic run in polyacrylamide-plate gel, with samples prepared with CE of *Phaseolus vulgaris* L. cv. carioca with 8 (A and B) and 9 (C - F) days of germination in the dark, submitted to the following treatments: heating to 70°C for 15 min, with 3 (A) and 9 mM (B and C) $CaCl_2$; addition of 0.1mM $HgCl_2$ (E) and 10mM EDTA (F). Control refers to CE of 9 days with 3mM of $CaCl_2$ (D). Specific activities of each CE are related below each corresponding column. nd = not determined.

There are many other reports on the use of the heating method, e.g.by ADAMS *et al.* (1980) on seeds of *Glycine max*, PRISCO *et al.* (1981) on cotyledons of *Vigna unguiculata*, CHIBA *et al.* (1990) on *Oryza sativa*, and AKAEVA & FURSOV (1990) on *Triticum aestivum*. In all cases, concentration of $CaCl_2$ was fixed allowed the stability of α-amylase during the heating time used. MAC GREGOR et al. (1971), using ionic exchange chromatography, corroborated that treatments of heating to 70°C for 15 minutes completely inactivated the β-amylase in barley extracts, without altering the α-amylase properties.

BATTERSHELL & HENRY (1990), verified that the heating of wheat seeds extracts at 70°C for 15 min decreased α-amylase activity by more than 80%. These authors explained on the basis of a possible difference in the enzyme structure or in the absence of a α-amylase inhibitor as the one that existed in barley, which stabilized the enzyme during heating. In *Zea mays*, WARNER *et al.* (1991) related the complete loss of α-amylase activity by heating to 70°C for 15 minutes in the presence of 10mM $CaCl_2$.

It was interesting to note that values obtained for α-, and β-amylase activities with inhibitors (EDTA and $HgCl_2$) together exceeded the total amylase activity (cf. figure 3).

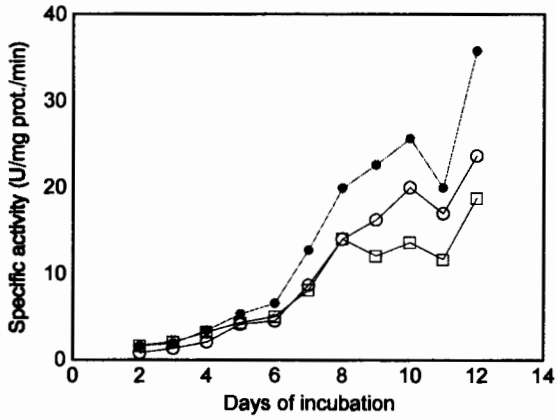

FIGURE 3 - Specific activities of total amylase and sum of α- and β-amylases assayed separately, using EDTA or pH 3,0 for inactivation of alpha-amylase and $HgCl_2$ as β-amylase inhibitor in cotyledons of *Phaseolus vulgaris* L. cv. carioca incubated in the dark at 25°C. • - α- + β-amylase (with EDTA and $HgCl_2$), - α- + β- amylase (pH 3 and $HgCl_2$), O - total amylase.

ROBBINS & EGAN (1992) reported that mercuric chloride was not effective in the selective inhibition of β-amylase in sorghum, although TÁRRAGO & NICOLÁS (1976) used successfully 5mM EDTA and 5mM $HgCl_2$ for the differentiation between α- and β-amylase in cotyledons of *Lens* sp.

KOSHIBA & MINAMIKAWA (1981) presented data showing the inactivation of α-amylase by EDTA in cotyledons of *Vigna mungo*, while NORBY & RINNE (1985) reported that treatment with EDTA did not inactivate completely in cotyledons of *Glycine max*.

The results obtained when the β-amylase activity was estimated by submitting the CE to a substrate prepared in pH 3.0 are presented in Figure 3. This procedure, according to MONERRI & GUARDIOLA (1986) would be preferable to the use of inhibitors, since the EDTA would lead to an incomplete inactivation, superestimating β-amylase activity. However, it was verified that the sum of α- and β-amylase does not correspond always to the total amylase activity.

The two methods tested here with the starch azure were also inadequate, since the quantification of the reaction was impossible due to a high viscosity of the reagent and the

coefficients of variation exceeding the 10% limit settled for assays with inhibitors. The pattern obtained in these assays did not follow the one obtained for the total amylase activity with methodologies used previously (Figure 4).

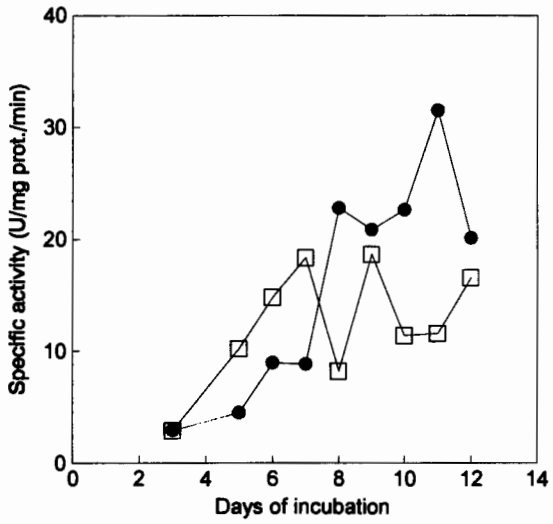

FIGURE 4 – Specific activity of α-amylase in cotyledons of *Phaseolus vulgaris* L. cv. carioca incubated in the dark and exposed to 1 hour of red light. • - $HgCl_2$ and □ - starch azure

Based on the use of inhibitors (EDTA and $HgCl_2$) and in the procedure of heating to 70°C, it was verified that two bands (1 and 2) showed characteristics of α-amylase (inhibition by EDTA, stability under heating to 70°C in the presence of 9mM $CaCl_2$) and two (5 and 6) of β-amylase (inhibition by $HgCl_2$, sensibility to heating). Bands 3 and 4 did not allow a well-defined characterization, since they were inhibited by the several procedures (figure 2).

As a conclusion, we suggest the determination of total amylase activity, without distinction between α- and β-amylase in the assays with cotyledons of *P. vulgaris* L. cv. carioca, with methodologies tested in the present work.

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