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ACID PHOSPHATASE FROM MAIZE SCUTELLUM: PROPERTIES AS A FUNCTION OF SEED GERMINATION

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Key Word Index—*Zea mays*; Gramineae; maize; scutellum; germination; acid phosphatase; negative cooperativity.

Abstract—Acid phosphatase (optimum pH at 5.2) purified from maize scutellum both in a state of dormancy and during the first 24 hours of seed germination has a M_r of *ca* 65000, contains 6% neutral sugar, maintains its catalytic activity after succinylation of 52 free amino groups per molecule and does not show the apparent movement of optimum pH from 5.4 to 6.7 in the presence of 4 mM fluoride. Kinetic data showed Michaelian behaviour for the enzymatic hydrolysis of PNP-P and an apparent number of Pi bound per molecule equal to one. Our results also suggest that the increased acid phosphatase activity in maize scutellum as a function of seed germination could be the result of modifications in the enzyme molecule.

INTRODUCTION

We have shown that the acid phosphatase present in scutella of maize seeds after 96 hours germination is monomeric (M_r *ca* 65000), contains 16% neutral sugars, probably has only a single catalytic site for Pi and maintains its catalytic activity after succinylation of 52 free amino groups per molecule [1–3]. The enzyme shows a kinetic transition from negative cooperativity (at pH 5.4) to Michaelian behaviour (at pH 6.7). The negative cooperativity is also abolished by increasing Pi concentration, by succinylation, by lowering the pH of the assay and by high glucose concentrations [3, 4]. In the present paper we describe some kinetic and structural properties of the acid phosphatase present in maize scutellum both in the dormant state and during the first 96 hours of seed germination. Our results provide no evidence for synthesis *de novo* of this enzyme [5] and suggest that molecular modifications could be responsible for the increase in acid phosphatase activity occurring during seed germination.

RESULTS AND DISCUSSION

As expected [6], electrophoretic analysis showed a fast-migrating band with an electrophoretic mobility of *ca* 0.59 as the sole acid phosphatase activity present in the crude extract and ammonium sulphate fractions of scutellum tissue, both in the dormant state and during the first 48 hours of seed germination. In addition to this fast-migrating weakly-staining acid phosphatase activity, a slow-migrating enzyme with an electrophoretic mobility of *ca* 0.30 was also observed after 72 hours. This band was the only one present in scutellum after 96 hours or longer periods of seed germination, if the seeds were still viable and producing strong seedlings. Only the fast-migrating acid phosphatase was observed in scutellum of non viable seeds showing poor seedling growth (less than 1 cm after 120 hours of seed germination), suggesting that the presence of the slow-migrating enzyme is important for the full development of the maize seedling.

In an attempt to distinguish these two acid phosphatases, we modified slightly the purification procedure described earlier [1] and determined some of the kinetic and structural properties of the enzyme as a function of seed germination. The enzyme, purified from 10-hour-old maize scutellum tissue, eluted from DEAE-cellulose at pH 7.4 as Fraction I and purified 54-fold over the crude extract with an overall yield of 5%, showed heterogeneity on 7.5% PAGE at pH 8.3 with an electrophoretic mobility of *ca* 0.30 and a low specific activity with PNP-P (5.4 units · mg $^{-1}$) as compared to that described earlier [1]. The change in electrophoretic mobility was also observed after purification of the enzyme present in scutella both in a state of dormancy and during the first 72 hours of seed germination (Fraction I). This result suggests that possible molecular associations were eliminated and that the electrophoretic pattern of the crude extract was an insufficient criterion to establish the presence of distinct acid phosphatases in scutella of maize seeds. The neutral sugar content of the enzyme was 6% and the M_r was *ca* 65000 as determined by exclusion chromatography or by SDS-PAGE. Although electrophoretically nonhomogeneous, the enzyme was also found by titration with TNBS to contain 52 free amino groups per molecule [2]. After succinylation of all of these groups, no important variation was detected in the catalytic activity of the enzyme. The pH activity profile showed optimum pH at 5.2 when determined in the presence or absence of 4 mM fluoride, with an inhibitory effect of 60%. Thus, the apparent dislocation of the optimum pH from 5.4 to 6.7 in the presence of fluoride observed after 48 hours of seed germination could not be attributed to the ionization of substrate and/or fluoride [1]. In any case, if a modification did occur in the enzyme

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molecule, it had not yet affected the electrophoretic pattern when the gel was stained for activity. The acid phosphatase showed no significant activity toward bis-PNP-P or phytic acid at any time of seed germination. Furthermore, the rate of β-glycerylphosphate and glucose-6-phosphate hydrolysis was enhanced about 4- and 5-fold after 48 hours or longer of seed germination, respectively, in relation to that obtained with PNP-P. Thus, we may also assume that molecular modifications could be responsible for this kinetic transition.

When the enzymes present in 72-hour-old maize scutellum tissue were purified, one of them was eluted as Fraction I and the other two as Fraction II. Fraction I, purified 141-fold over the crude extract with an overall yield of 8.4%, showed heterogeneity on 7.5% PAGE at pH 8.3, an electrophoretic mobility of *ca* 0.30 and a high specific activity with PNP-P (71 units·mg^{-1}) as compared to that reported earlier [1]. The neutral sugar content was about 7–12%, corresponding to intermediate values between those shown by the enzymes purified from 10- and 96-hour-old scutellum tissue [1]. No variation was observed in M_r, in the number of free amino groups per molecule, in the catalytic activity after extensive succinylation of the enzyme, in activity towards various phosphomonoesters, or in the pH dependence for fluoride inhibition when these properties were compared to those shown by the enzyme purified from 96-hour-old scutellum tissue [1, 2]. The presence of two enzyme forms, eluted as Fraction II and electrophoretically indistinguishable, also seems to be related to time of seed storage since they were still observed in 96-hour-old scutellum tissue from recently harvested seeds (maximum storage time of 30 days). Furthermore, only one enzyme form (Fraction II) was observed in 72-hour-old scutellum tissue of seeds stored for long periods of time (minimum of 6 months) but still viable and showing strong seedling growth.

The enzyme purified from 96-hour-old maize scutellum tissue of seeds stored for at least 6 months, eluted as Fraction II and purified 118-fold over the crude extract with an overall yield of 7.8%, appeared to be homogeneous on 7.5% PAGE at pH 8.3, with the protein band being superimposable on acid phosphatase activity. These results indicate that, in this procedure, purification of the enzyme to electrophoretic homogeneity depends on storage time and on seed germination. This dependence was also observed when the electrophoretic patterns of the ammonium sulphate fraction stained for proteins and obtained from 10- and 96-hour-old scutellum tissue of seeds stored for 6 months were compared. All properties found for the enzyme purified by the procedure described in this paper were similar to those previously found for the enzyme described earlier [1], except for the higher specific activity with PNP-P at pH 5.4 (71 units·mg^{-1}). A similar specific activity value was found for the enzyme after extensive succinylation, rechromatography on Sephadex G-75 [1, 2] and concentration by ultrafiltration. The acylated enzyme showed a neutral sugar content of 9% and was detected in the dialysable fraction along with possible low-M_r glycoproteins (absorption at 200 nm, a characteristic reaction with the Folin–Ciocalteau reagent [7] and a neutral sugar content of 40%). Thus, the elevated concentration of sodium chloride in the elution buffer of the Sephadex G-200 column (see Experimental) seems to replace the effect of succinylation in terms of increased specific activity of the enzyme. It seems clear that this molecular association does not affect the electrophoretic pattern or M_r of the enzyme.

Heat inactivation at 65° and at pH 5.4 showed half-lives of 2.5 min (k = 0.27 min^{-1}), 4 min (k = 0.17 min^{-1}) and 5 min (k = 0.14 min^{-1}), respectively, for the acid phosphatase purified from 10-, 72- (Fraction I) and 96-hour-old scutellum tissue. This increase in thermal stability could be attributed to the increased percentage of neutral sugars attached to the enzyme molecule during germination. The half-life of 5 min shown by the enzyme purified from 96-hour-old scutellum tissue is similar to that shown by the succinylated enzyme [2]. Thus, the higher half-life value (6.8 min) obtained for the native enzyme purified as described earlier [1] could be attributed to the molecular association described above. The time course for inactivation of the acid phosphatase present in scutellum tissue of dormant seeds and during the first 24 hours of seed germination follows simple first-order kinetics (half-lives of 2.5 min), independent of the stage of enzyme purification. After 48 hours or longer periods of seed germination, the time course for the enzyme inactivation follows simple first-order kinetics only if it is purified until the final step of the procedure described here. This result also suggests the existence of molecular associations with the enzyme.

Figure 1 shows the double reciprocal plots of the initial velocities for acid phosphatases purified from scutellum tissue as a function of PNP-P concentration. It can be seen that the enzyme purified from 10- and 72-hour-old (Fraction I) scutellum tissue showed no deviation from Michaelian behaviour [8], with the respective K_m values being 2.6×10^{-4} M (n = 1.02) and 1.4×10^{-4} M (n = 1.02) at pH 5.4. The interaction constants for Pi [9] determined at 1.4 mM PNP-P against Pi concentration were 1.0 and 1.1 for the enzyme purified from 10- and 72-hour-old (Fraction I) scutellum tissue, respectively, indicating that these enzymes also have only a single catalytic site [3]. The K_i values were quite similar for both enzymes (2.5×10^{-4} M and 1.5×10^{-4} M, respectively, for the enzyme purified from 10- and 72-hour-old scutellum tissue). As shown in Fig. 1, the double reciprocal plots of the initial velocities for the acid phosphatase purified from 96-hour-old scutellum tissue are compatible with the negative cooperativity phenomenon ($K_{0.5}$ = 4.8 $\times 10^{-4}$ M and n = 0.70), indicating that the deviation

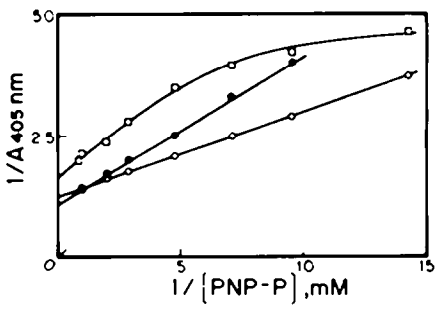

Fig. 1. Double-reciprocal plots of velocity of purified maize scutellum acid phosphatase as a function of PNP-P concentration at pH 5.4. ●, ○, □ acid phosphatase purified from 10-, 72- (Fraction I) and 96-hour-old maize scutellum tissue, respectively. Each tube contained 5 μg (●), 380 ng (○) and 290 ng (□) of purified enzyme. For details see Experimental.

from Michaelian behaviour [8] could not be attributed to the molecular associations described above. This kinetic phenomenon was also shown by all enzymes retained by DEAE-cellulose at pH 7.4 (Fraction II), further supporting our hypothesis that the cause of this kinetic phenomenon at pH 5.4 could be the ionization of the enzyme molecule [3].

The enzyme present in scutellum tissue at any time of seed germination (0- to 96-hour-old) and at any purification step (including crude extracts) did not catalyse the transfer of Pi from 6 mM PNP-P to glucose, glycerol or inositol, when the acceptor concentrations were 0.05, 0.20, 0.50 and 1.0 M and the assay system had been buffered at pH 5.4, 6.1 or 7.1. In all assays, the *p*-nitrophenoxide ion/free Pi ratio was near 1.00.

The results reported here do not support the hypothesis that the increased acid phosphatase activity detected in scutellum tissue of maize seeds during germination [1] is the result of synthesis *de novo* [5]. Thus, it is possible that the enzyme is modified during germination (including carbohydrate attachment) and that these modifications play a role in the development of maize seedlings. Considering that the cytoplasmic pH of scutellum cells is alkalinized during sucrose transport [10, 11], no nutritional role could be attributed to this cytoplasmic enzyme [1], which does not hydrolyse phytic acid at any pH or other possible natural substrates at pH 8.0, and does not catalyse the transphosphorylation reaction. Thus, it is possible that the enzyme, which might be synthesized during seed formation, is transported to another site to exert its catalytic function in the germination of the seeds. It is well known that scutellum tissue plays an important role in transport as a whole, and specifically in the secretion of α-amylase into the endosperm during the germination of maize seeds [12].

EXPERIMENTAL

Enzyme preparation. Acid phosphatase present in scutella was extracted, fractionated with $(NH_4)_2SO_4$ and chromatographed on a DEAE-cellulose column (2.4 × 43 cm) previously equilibrated with 10 mM NaOAc, pH 5, as described in ref. [1]. The enzyme was dialysed for 20 hr against 12 l. of 10 mM Tris–HCl buffer, pH 7.4, containing 40 mM NaCl (with two buffer changes), applied to a DEAE-cellulose column (2.4 × 70 cm) previously equilibrated with the buffer used for dialysis, and the unabsorbed proteins were eluted at a flow rate of 120 ml/hr (20 ml fractions). When the eluate showed acid phosphatase activity the enzyme peak (Fraction I) was dialysed against 10 l. of 10 mM Tris HCl buffer, pH 7.4, for 20 hr with two buffer changes and concd by chromatography on a DEAE-cellulose column (2 × 2 cm) previously equilibrated with the buffer used for dialysis. After washing with 50 ml of the same buffer, the enzyme was usually eluted with the first 6 ml of 0.1 M NaOAc buffer, pH 5, at a flow rate of 120 ml/hr. Solid NaCl at a final concn of 0.5 M was added to this enzyme fraction. The proteins absorbed on DEAE-cellulose at pH 7.4 (see above), were eluted with 10 mM NaOAc (pH 5) at a flow rate of 60 ml/hr (6 ml fractions). When the eluate showed acid phosphatase activity the enzyme peak (Fraction II) was concd by ultrafiltration, with NaCl and NaOAc added at final concns of 0.5 M and 0.1 M, respectively. Both fractions I and II were independently chromatographed on a Sephadex G-200 column (1.2 × 130 cm) previously equilibrated and eluted with 0.1 M NaOAc–(pH 5) containing 0.5 M NaCl, at a flow rate of 7 ml/hr (2.5 ml fractions). The enzyme peaks were dialysed for 20 hr against 8 l. of 10 mM NaOAc, pH 5 (with two buffer changes), concd by ultrafiltration and stored at 4°.

Assay procedures. *p*-Nitrophenylphosphate or bis-PNP-P hydrolysis was carried out at pH 5.4 as described in ref. [3]. G-6-P, β-glycerylphosphate and phytic acid hydrolysis was carried out as described in ref. [1]. The liberated Pi was measured by the method of ref. [13]. Incubations were carried out at 37°, and all enzyme activities were measured in duplicate for at least two time intervals. One unit of acid phosphatase is defined as 1 μmol substrate hydrolysed·min^{-1}. The buffers used to cover the pH range required for determination of the optimum, in the presence or absence of 4 mM KF, were 0.1 M NaOAc (pH 4–5.8), 0.1 M Na·maleate (pH 5.8–7.2) and 0.1 M Tris HCl (pH 7.2 to 8.5). The extent of transphosphorylation from PNP-P to glucose, glycerol and inositol was estimated by measuring the liberated *p*-nitrophenol and Pi from the same reaction tube as described in ref. [14]. After incubation for 5 min at 37°, the reaction was stopped by the addition of 1 ml cold 10% TCA. Samples were taken and *p*-nitrophenoxide ion and Pi were measured as described in refs [3] and [13], respectively. Protein was measured by the method of ref. [7] using BSA as a standard. Neutral sugars were measured by the method of ref. [15] using glucose as a standard. Disc electrophoresis was carried out by the method of ref. [16] as described in ref. [17]. The enzyme activity bands were developed by the method of ref. [18] as described in ref. [6]. The ratio of the distance covered by the enzymes to the distance covered by Bromophenol Blue (electrophoretic mobility) was measured. M_r was measured as described in ref. [19] (1.5 × 116 cm Sephadex G-200 column equilibrated and eluted with 50 mM NaOAc buffer, pH 5, 2.5 ml fractions, at a flow rate of 10 ml/hr) and by the method of ref. [20] as described in ref. [1], using appropriate M_r markers. The number of free amino groups in the protein was determined by using TNBS [21]. Succinylation of the molecule was performed as described in ref. [2]. Thermal inactivation at 65°, pH 5.4, was performed as described in ref. [3].

Kinetic studies. Initial velocities were plotted as described in ref. [22]. Values of apparent inhibition constants for Pi(Ki) were determined from Dixon plots as described in ref. [23] or from plots of log $v_0 - v_i/v_i$ vs log Pi concns as described in ref. [9]. Interaction constants for the substrate (n) and Pi (n') were determined by the methods of refs [24] and [9], respectively. The kinetic constants given in this paper were obtained from linear-square analysis. When biphasic kinetics was observed, $K_{0.5}$ was determined by the Hill procedure as described in ref. [25].

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