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Purification and Properties of Acid Phosphatase from Plump and Shriveled Seeds of Triticale[1]

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ABSTRACT

A major triticale (X *Triticosecale* Wittmack) endosperm acid phosphatase (EC 3.1.2.2) (APase) from sib-lines producing plump and shriveled seed was purified 140- and 230-fold to a specific activity of 94 and 153 micromoles per minute per milligram protein respectively, by ammonium sulfate fractionation, ion-exchange chromatography, chromatofocusing, affinity column chromatography, and gel filtration. The purified enzyme from both materials is a monomeric glycoprotein with an apparent molecular weight of 45,700 ± 500 containing 12% carbohydrate and an apparent isoelectric point of pH 5.9. It hydrolyzes tri- and di-phosphate of nucleosides as well as phosphate esters and exhibits characteristics of ATP-hydrolase and phosphatase. About 2-fold more of the APase was isolated from shriveled seeds, and the purified enzyme exhibited 3- and 5-fold higher V_{max} for *p*-nitrophenyl phosphate and ATP, respectively, than that of plump seed. The I_{50} for Pi concentration was 5.5-fold higher in APase of shriveled seed than the plump one. These varied quantitative and kinetic properties substantiate the role of APase in lines with shriveled seeds being reduction of starch accumulation by depleting substrates and energy supply in the cytosol.

The shrunken endosperm mutants of corn are known to be related to low ADP-Glc pyrophosphorylase activity (bt2) (10), to defective sucrose synthase (sh2) (9), or to high ribonuclease activity at the later stages of seed development (bt2:02) (12). One of the shrunken endosperm barley mutants is due to a premature chalazal necrosis causing physical blockage of assimilate transport to endosperm tissue, and others are caused by abnormal endosperm development (17). The shriveling of triticale kernels has been attributed to aberrant nuclei formed by mitotic errors in the endosperm during the early coenocytic period, and not caused by a poor supply of photosynthate to the kernel (24). Recently, the triticale endosperm ADP-Glc pyrophosphorylase activity was found to be 2- to 3-fold higher in lines producing plump seed than that of shriveled seed, indicating that this enzyme may play an important role in the degree of plumpness of triticale seeds (5). In addition, triticale endosperm APase[2] activity was significantly higher in shriveled seeds at later stages of seed development. The high APase activity might deplete the energy supply for biosynthesis, and degrade substrates for starch accumulation and membrane components for tissue integrity (6). In that study, some slow moving isozymes of endosperm APase separated by PAGE were highly active at the later developmental stages in lines with shriveled seeds, but not in lines producing plump seeds. It was proposed that these slow-moving or less anionic acid phosphatases may play an important role in causing shrivelness in mature seeds (6).

In order to substantiate the role of the APase stated above, in this paper (a) localization of APase was attempted in endosperm cells; (b) the *in vivo* function of the APase was explored by mixing and incubating cell free filtrate of young and older developing seeds of plump and shriveled types and analyzing the resulting activity of some relevant anabolic and hydrolytic enzymes; (c) the most active and least anionic APase isozyme was purified 140- and 230-fold, respectively, from 6-week-old developing seeds of sib-lines that produced plump or shriveled seeds for more than five generations.

The experimental results indicated that (a) APase probably are not totally compartmentalized in vacuoles—the cytosolic and membrane bound APase may deplete substrates and degrade tissue integrity; and (b) the quantitative yield of the purified major APase from plump and shriveled lines and their differences in kinetic properties indicated that the role of APase in shriveled material probably is reducing starch accumulation by depleting endogenous substrate and energy supply.

MATERIALS AND METHODS

Materials. *Plant.* Four lines of winter triticale (X *Triticosecale* Wittmack), 6TA 876 and Red Bobs//Daws/Snoopy-P with plump seed characteristics and 6TB 163 and Red Bobs//Daws/Snoopy-S with shriveled seed conformation (6) were used. Seeds were planted using common cultural practices at the East Farm, Corvallis, OR, in October 1984 and 1985. Spikes were tagged at anthesis, and harvested weekly starting 4 WAA in July and August of 1985 and 1986. Some spikes were used fresh and the rest stored in heavy polyethylene bags at −68°C prior to use.

Chemicals. Column packings were purchased from Pharmacia or BioRad, and chemicals from Sigma or Baker.

Protein Determination. Soluble proteins in dialyzed extracts, eluates in even numbered tubes, and concentrated fractions were determined by the Coomassie blue method using BSA as a standard (4). When the concentration of protein was too dilute, A_{280} was used as an estimation.

Enzyme assays. *p-Nitrophenyl phosphatase* (*pNPPase*). Fifty μl of diluted dialyzed enzyme extract or of column fractions were incubated at 35°C for 10 min in 0.2 ml of 0.1 M Na-acetate buffer containing 9 mM pNPP (pH 4.5). After incubation, 1 ml of 0.5 N NaOH was added to stop the reaction and develop the color.

[1] Oregon Agricultural Experiment Station Technical Paper No. 7435.

[2] Abbreviations: APase, acid phosphatase; A, triticale line 6TA 876—plump form; B, triticale line 6TB 163—shriveled form; P, triticale line Red Bobs//Daws/Snoopy—plump form; S, triticale line Red Bobs//Daws/Snoopy—shriveled form; PMSF, phenylmethyl sulfonyl fluoride; ME, β-mercaptoethanol; PLPase, phosphotidic acid phosphatase; PNP, para-nitrophenyl; pNPP, *p*-nitrophenyl phosphate; pNPPase, *p*-nitrophenyl phosphatase; WAA, weeks after anthesis; GB; grinding buffer; ppt, precipitate; ConA, concanavalin A

The reaction mixtures were then read against a blank at 400 nm (6).

ATP-Hydrolase (ATPase) and Other Phosphatases. Fifty μl diluted enzyme extract or of column eluates were incubated with rapid shaking at 35°C for 10 min in 0.2 ml of 0.1 M K-acetate buffer containing 5 mM ATP or other phosphates (pH 5). The reaction was stopped by adding 1.25 ml of 0.3% ammonium molybdate in 0.2 M Na-acetate (pH 4.0), and 0.1 ml of 1% ascorbic acid in 0.2 M Na-acetate (pH 4.0). After 30 min the blue color of phosphomolybdic acid was read at 700 nm against a reagent blank (20).

ADP-Glc pyrophosphorylase, soluble starch synthase and phosphatidic acid phosphatase were assayed as described previously (6).

Localization of APase in Endosperm Cells. APase is generally known as vacuolar enzyme in mature plant cells (13). Attempts were made to isolate vacuoles from fresh developing endosperm tissue by the methods of enzyme digestion (15) or mechanical slicing (13) and differential density centrifugation, but both procedures failed to produce the vacuole band. Being a growing storage tissue of mainly starch granules and some protein, the endosperm cells probably do not have large vacuoles; some prevacuoles would be present for the formation of protein bodies, but they might be of different density. Thus the failure was not unexpected. For the purpose of estimation, the location of APase activity was separated with minimal shearing of the tissue (13). One hundred endosperms (about 10 g) were dissected from 6-week-old fresh seeds by removing embryos and peeling off seed coats. The endosperm was sliced by razor blade in 5 ml extraction buffer containing 0.7 M mannitol, 50 mM Tris-HCl, 0.5 mM EDTA, and 14 mM ME (pH 7.5), 5 ml extraction buffer was added, and the slurry filtered through 500 μm nylon mesh. Ten ml extraction buffer was used to wash the nylon cloth and the residue down and slicing was repeated twice. The combined filtrates were centrifuged at 2,000 *g* for 10 min to separate enzymes of cytosol and some broken organelles in the supernatant, and the vacuoles, nuclei, and other organelles in the pellet (13). The pellet was lysed and extracted by 20 ml 0.1 M Na-acetate + 14 mM ME (pH 5.0) and centrifuged at 20,000 *g* for 10 min. The APases in the supernatant were considered to be mainly vacuolar (13); the pellet was further extracted by 20 ml 0.1 M Na-acetate, 0.5% Triton X-100, and 14 mM ME (pH 5.0) and centrifuged at 20,000 *g* for 10 min. The APase in the supernatant were considered to be membrane bound (13). All supernatants were diluted and assayed for protein, pNPPase, and ATPase. All operations were conducted at 0°C except noted.

***In Vivo* Function of Degradative Acid Hydrolases.** It is difficult to discern the *in vivo* function of individual enzymes in living cells; the degradative action of acid hydrolases from endosperm of later developmental stage of shriveled line might be demonstrated in younger material or in endosperm of plump lines. Thus, an experiment was conducted in which 20 endosperms each of fresh seeds collected 4 WAA and 8 WAA of A, B, P, and S lines were sliced in 10 ml buffer containing 0.4 M sucrose, 50 mM Tris-HCl (pH 7.0) and filtered through the nylon mesh. One ml of the cell-free-filtrate from young stage (4 WAA) was mixed with 1 ml of that from older stage (8 WAA) of the same line or different line of seed configuration. The mixtures were incubated at 0°C for 10 min to allow any degradative reactions to occur but at slower rate since the temperature and pH conditions were not optimum. After incubation each mixture was lysed by adding 8 ml of Tris-HCl (50 mM, pH 7.0) and 14 mM ME to extract the soluble enzymes, and the diluted extract was centrifuged at 20,000 *g* for 10 min. The supernatant was dialyzed in the dilution buffer to remove endogenous substrates and reaction products, and then assayed for the activity of ATPase, PLPase, starch synthase, and ADP-Glc PPase. The original filtrates were processed and assayed similarly. All operations were conducted at 0°C unless noted.

Purification. Only P and S sib-lines harvested at 6 WAA were used. All procedures were conducted at 0 to 4°C unless noted.

Step 1—Extraction. Lemma, palea, and embryo were first removed from the frozen seed, and the remaining endosperm with seed coat was collected in a GB containing 0.1 M Na-acetate, 0.2 M KCl, 1.5 mM PMSF (19), and 14 mM ME (pH 5.0) (22). Thirty g of dissected endosperms from approximately 260 plump or 290 shriveled seeds (6) were ground in a mortar with pestle in 40 ml GB plus 5 g each of GB saturated PVPP and XAD-4 Amberite anion exchanger to remove polyphenols. After the slurry was centrifuged at 30,000 *g* for 20 min, the supernatant was collected and the ppt extracted once more. The combined supernatant was precipitated by $(NH_4)_2SO_4$ to 20% saturation and the ppt was removed by centrifugation and discarded since the ppt after solubilization and dialysis contained less than 3, and 1% of total APase and ATPase activity, respectively. To the soluble fraction, more $(NH_4)_2SO_4$ was added with stirring to give 75% saturation. After stirring for 30 min, the ppt was collected by centrifugation at 10,000 *g* for 10 min and dissolved in 15 ml of 10 mM Tris-HCl buffer containing 50 mM KCl, 0.5 mM PMSF, and 14 mM ME (pH 7.5) (buffer A). The preparation was dialyzed against 4 L of buffer A for 18 h. The dialysate was clarified by centrifugation at 20,000 *g* for 10 min to yield fraction 1.

Step 2—DEAE-Sephacel Ion-Exchanging Column Chromatography. Fraction 1 was loaded onto a 1.6 × 25 cm DEAE-sephacel column preequalibrated with buffer A. The loaded column was washed with 100 ml buffer A, eluted with 300 ml of a linear gradient 50 to 220 mM KCl in 10 mM Tris-HCl buffer, 0.5 mM PMSF, and 14 mM ME (pH 7.5), and then washed with 50 ml of 1 N NaCl. The flow rate was 0.14 ml min^{-1}, and 5 ml eluate per tube was collected. The eluate in tubes containing the major peak of APase activity (Fig. 1A, shadowed peak) was pooled, $(NH_4)_2SO_4$ added to 75% saturation, and centrifuged to collect the ppt. The ppt was dissolved in 5 ml of 25 mM imidazol containing 0.1 mM PMSF and 14 mM ME (pH 7.4) (buffer B), and dialyzed in 2 L buffer B for 16 h. The dialysate was clarified by centrifugation to yield fraction 2.

Step 3—Isoelectric Focusing Column Chromatography. Fraction 2 was applied on a chromatofocusing (18) column (1.2 × 20 cm) preequilibrated with buffer B. The loaded column was eluted with 320 ml of polybuffer 74-HCl (8 × dilution, containing 0.1 mM PMSF, pH 4.0) at a flow rate of 0.11 ml min^{-1} and the eluate was collected 3 ml per tube. The column was further washed with 50 ml 1 M NaCl and eluate collected. The major peak of APase activity (Fig. 1B, shadowed peak) was precipitated by $(NH_4)_2SO_4$ to 75% saturation and the ppt was dissolved in 3 ml of 0.1 M Na-acetate containing 1 M NaCl, 1 mM $Cacl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.1 mM PMSF (pH 6.0) (buffer C). The solubilized ppt was dialyzed in 1 L buffer C plus 14 mM ME for 8 h, and the dialysate was clarified to yield fraction 3.

Step 4—Concanavalin A-Sepharose 4B (ConA) Affinity Column Chromatography. Fraction 3 was applied on a ConA column (0.8 × 6 cm) prewashed thoroughly with buffer C, washed with buffer C at a flow rate of 0.12 ml min^{-1} till no protein was detected in the wash, and then eluted with buffer C containing 0.2 M methyl-α,D-gluco-pyranoside (7). The eluted Apase activity (Fig. 1C, shadowed peak) was concentrated by Centricon-30 (Amicon Corporation) to about 2 ml and dialyzed against 1 L Tris-HCl buffer (50 mM containing 0.5 mM PMSF and 14 mM ME, pH 7.0) (buffer D) for 4 h. The dialysate constituted fraction 4.

Step 5—Gel Filtration Column Chromatograph. Fraction 4 was applied to a Sephacryl-200 or Bio-gel P60 (superfine) column (1.5 × 90 cm) and eluted by gravity with buffer D at a flow rate of 0.035 ml min^{-1}. The eluate containing APase activity (Fig.

1D, shadowed peak) was concentrated by Centricon-30 and sucrose was added to 20% (w/v) to the purified APase preparation and stored at −70°C for characterization within a period of 3 months. Without the protection of sucrose or ethylene glycol, the preparation gradually loses its activity within 1 month.

Characterization of Purified APase. *pH optimum.* Both pNPPase and ATPase were assayed as described above by incubating 50 to 100 ng enzyme and 1.0 mM substrate in 250 μl of 0.1 M various buffers of different pH value: lactate, pH 3 and 4; acetate, pH 4.5, 4.6, 4.7, 4.8, 4.9, and 5; Mes, pH 5.5, 6, and 6.5; Hepes, pH 7; Tris-HCl, pH 8 and 9.

Substrate Specificity. Fifty to 100 ng of enzyme in 50 μl of buffer D and 5 mM of different substrates in 200 μl 0.1 Na-acetate buffer (pH 5) were incubated at 35°C for 10 min. The phosphoric acid production was determined by the method of Saini and Van Etten (20).

Influence of Effectors on pNPPase and ATPase. Fifty to 100 ng enzyme in 50 μl of buffer D, 100 μl of 0.1 M Na-acetate containing 10 mM pNPP or ATP and 100 μl of different effectors in 0.1 M Na-acetate (pH 4.5 or pH 5.0), respectively, were incubated at 35°C for 10 min. Reaction products of pNP or Pi were estimated as described above.

Effect of Inorganic Phosphate on pNPPase Activity. Fifty to 100 ng of enzyme in 50 μl of buffer D, 100 μl of 10 mM pNPP in 0.1 M Na-acetate (pH 4.5), and 100 μl of different concentrations of NaH_2PO_4 in 0.1 M Na-acetate (pH 4.5) were incubated at 35°C for 10 min. The pNP was read at 400 nm against a reagent blank.

K_m and V_{max} of APase. Fifty to 150 ng of enzyme in 50 μl of buffer D, and 200 μl of 0.1 M Na-acetate (pH 4.5 or 5) (for ATPase) containing 12 concentrations of substrates (pNPP and ATP) varying from 0.1 to 10 mM were incubated at 35°C for 5 min. The products were assayed and Lineweaver-Burk reciprocal plots were used to estimate K_m and V_{max} (21).

Molecular Weight Estimation. Two mg each of mol wt standards (aprotinin, 6.5 kD; Cyt *c*, 12.4 kD; α-chymotrypsimogen A, 24.5 kD; and hemoglobin, 64.5 kD) were dissolved in 1 ml buffer D, applied on a 1.5 × 90 cm, Bio-gel P60-(super fine) column, and eluted by buffer D with gravitation at a flow rate of 0.035 ml min^{-1}. The eluate was collected 3 ml per tube and A_{280} assayed in even numbered tubes. A plot of log (mol wt) *versus* *Ve/Vo* (where *Ve* = elution volume, and *Vo* = void volume) showed a straight line from which the mol wt of an unknown separated under an identical condition was estimated.

SDS-PAGE was also conducted for mol wt determination using Bio-Rad low mol wt standards (3).

Carbohydrate Content. Sugar content in APase was estimated by the anthrone method (2) using 2 to 4 μg of enzyme per assay.

Isoelectric Point. Based on chromatofocusing results, the isoelectric point of purified APase was estimated (18).

RESULTS AND DISCUSSION

Localization of APases in Developing Endosperm. The data in Table I roughly indicate that a major portion of APase activity was located in cytosol and some broken organelles, organellar APase activity composed only 20 to 22% of the total, and membrane bound about 16 to 19%. It is known that during the formation of vacuoles in meristematic cells, hydrolases are located in cytosol around the prevacuoles and are sequestered into the vacuolar cavity (13). Therefore, the high cytosolic content of APase activity is possible in this developing storage tissue. Concrete evidence, however, will be needed. The data in Table I showed little enrichment of APase activity in organellar fraction based on the specific activity of APases indicating that vacuoles or prevacuoles, if present, were small in quantity. Based on these data it can be concluded that APases were not compartmentalized in vacuoles like that of mature vegetative cells and they are fully capable of depleting starch substrates and energy supplies in the cytosol and organelles.

***In Vivo* Function of Degradative Acid Phosphatases from Endosperm of Older Shriveled Seeds.** The data in Table II showed that the activity of degradative hydrolases, PLPase and ATPase, was significantly enhanced in the mixture of young and old shriveled materials (data in lower half of Table II) indicating that membrane degradation by the enzymes in the shriveled tissue might have occurred during the incubation. Synthetic enzymes for starch accumulation, soluble starch synthase, and ADP-Glc PPase, on the other hand, were significantly reduced. This is probably due to the same membrane deterioration reaction of the shriveled material that released ATPase and hydrolyzed the ATP being assayed. Older stage of plump lines, on the other hand, doesn't appear to be degradative since little significant differences were observed (data in upper half of Table II). The results definitely support the conclusions made in previous paper (6).

Purification of APase. The results of a typical purification procedure of APase are summarized in Table III. The coefficient of variation among the results of six runs each was 17% for plump and 24% for shriveled material both of which are acceptable for biological materials. At the final step, the enzyme was purified about 140-fold and 230-fold, respectively, for plump and shriveled endosperm, over the 20 to 75% ammonium sulfate precipitated crude extract, with an average specific activity of 94 and 153 μmol pNP produced mg^{-1} min^{-1}, respectively. The overall average yield was 0.073% protein for plump material and 0.096% for shriveled. At per seed basis, the plump endosperm contained 438 ng of the APase and the shriveled 758 ng. This differential yield indicates that 2-fold more APase exists in shriveled endosperm of a sib-line which yields plump seeds. This suggests that the higher content of this hydrolase probably could decrease the level of anabolically needed phosphate esters and ATP in this tissue and impair the accumulation of starch result-

Table I. *Localization of APases in Endosperm of 6-week-old Developing Seeds of Plump and Shriveled Line of Red bobs//Daws/Snoopy*

The data are the average of two runs; the variation between runs was less than 21% of the average.

	Plump			Shriveled		
	Protein	pNPPase	ATPase	Protein	pNPPase	ATPase
	μg	*nmol min^{-1}*		μg	*nmol min^{-1}*	
Total endosperm^{-1}	405	214	176	437	426	234
Cytosol						
% total	65.7	59.2	64.1	63.3	68.2	60.3
Organelle						
% total	15.2	21.9	20.0	20.1	21.8	22.4
Membrane						
% total	19.1	18.9	15.9	16.6	18.4	18.7

Table II. *Percent of Observed Enzyme Activity in Incubated Equal Mixture over the Average Activities of Individual Extracts of Young (4 WAA) and Old (8 WAA) Seeds of Plump (A, P) and Shriveled (B, S) Triticale Lines*

Mixture	Experiment	PLPase	ATPase	Starch Synthase	ADP-Glc PPase
			With old seeds of plump lines		
A4/A8 / A4 + A8	1	95	122	118	94
	2	94	108	109	93
	3	89	100	99	96
P4/P8 / P4 + P8	1	90	105	118	86
	2	88	113	105	93
	3	94	135	98	95
B4/A8 / B4 + A8	1	109	118	106	98
	2	96	121	124	100
	3	107	112	100	103
S4/P8 / S4 + P8	1	100	152	103	101
	2	102	167	112	89
	3	104	189	107	114
Mean ± SD		97 ± 7	128 ± 27	108 ± 8	97 ± 7
			With old seeds of shriveled lines		
A4/B8 / A4 + B8	1	146	241	86	68
	2	140	243	89	86
	3	182	206	87	77
P4/S8 / P4 + S8	1	121	289	85	62
	2	125	262	83	63
	3	126	244	84	68
B4/B8 / B4 + B8	1	143	229	94	74
	2	157	225	80	85
	3	142	238	75	64
S4/S8 / S4 + S8	1	143	286	92	65
	2	157	296	84	70
	3	142	213	76	76
Mean ± SD		149 ± 22*	248 ± 30*	85 ± 6*	70 ± 9*

* Significantly different at 5% from cell-free-filtrates of younger staged seeds incubated with filtrates of older staged seeds of plump lines.

Table III. *Purification of Acid Phosphatase from 30 g of Endosperms of 6-week-old Plump and Shriveled Triticale Seeds*

Fraction	Total Protein		Total APase		Specific Activity		Purification	
	P[a]	S[a]	P	S	P	S	P	S
	mg		A_{400} min^{-1}		A_{400} mg^{-1} min^{-1}		*fold*	
Crude extract	157	229	1593	2493				
I. 20–75% ammonium sulfate ppt	130	174	1280	2201	9.8	12.6	1	1
II. DEAE-Sephacel	28.1	35.2	524	986	18.6	28.0	1.9	2.2
III. Chromatofocusing	4.5	7.1	302	890	71.9	125	7.3	9.9
IV. ConA-sepharose	2.0	3.4	280	785	140	230	14.2	18.3
V. Bio-Gel P60	0.115	0.220	205	640	1783	2909	141	232

[a] P, plump; S, shriveled.

ing in a shriveled conformation.

The chromatographic profiles of the enzyme extracted from shriveled seeds separated by the four columns are presented in Figure 1. Three peaks of APase activity were separated by DEAE-Sephacel column with the first or the least anionic one being the major peak (Fig. 1A, shadowed peak). Thus, the first peak was collected, concentrated, and applied on a chromatofocusing column from which one major peak was obtained (Fig. 1B, shadowed). The concentrated eluate of this peak contained carbohydrate by the anthrone test, and a ConA column was used for further separation that yielded an APase activity peak (Fig. 1C, shadowed). Further fractionation by Bio-gel column resulted in one major peak of the APase activity (Fig. 1D, shadowed). Based on the elution pattern of mol wt standards separated on the same Bio-gel column (Fig. 1D, □——□), the apparent M_r of the purified APase was 45.7. Using a Sephacryl S-200 column, the M_r of the enzyme was 46.4. Ten percent SDS-PAGE of the purified APase yielded two bands of M_r = 66.2 and 45.2. It is

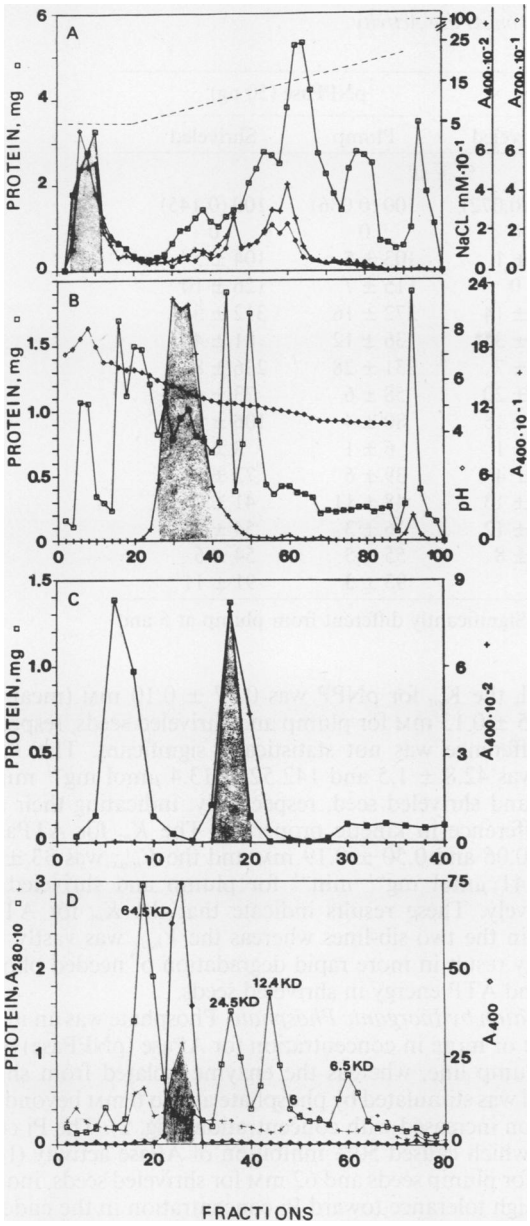

FIG. 1. Elution profiles of APase extract of 30 g shriveled endosperm at each purification step. A, DEAE-Sephacel chromatographic profile; B, chromatofocusing profile; C, Con A-Sepharose 4B affinity column chromatographic profile; D, Bio-gel P60 gel filtration chromatographic profile. Molecular mass standards (aprotinin, 6.5 kD; Cyt *c* 12.4 kD; α-chymotrypsimogen A, 24.5 kD; and hemoglobin, 64.5 kD) were eluted under identical conditions for the calculation of unknown (see "Materials and Methods" for details).

difficult to explain the 66.2 kD band observed on SDS-PAGE gel; the band may be an artifact of boiling with high concentration of glycerol and DTT prior to electrophoresis. Further purification of the isolated APase with a 0.8 × 120 cm column of Bio-gel P60 did not remove the 66.2 kD band on SDS-PAGE, neither did a hydroxylapatite column. Three bands with Apase activity and comparable protein with Rm 0.15, 0.20, and 0.22 were separated from the purified enzyme by Tris HCl-glycine (pH 8.3) on 7.5% polyacrylamide gel. Based on densitometer readings of APase stained gel, the activity ratio of 1:666:720 was observed for the three bands indicating the last two were active ones. It is known that minor charge heterogeneity of purified enzymes often occur and result in several bands after PAGE (7);

Table IV. *pH Optimum of APase (50 ng) Isolated from Triticale Endosperm*

Data are means ± SE of two replicated assays each of two preparations.

pH	PNPPase		ATPase	
	Plump	Shriveled	Plump	Shriveled
		%		
3.0	14 ± 4	5 ± 4	24 ± 10	9 ± 3
4.0	46 ± 12	41 ± 5	49 ± 11	44 ± 7
4.5	100 (0.034)[a]	100 (0.142)	88 ± 12	100 (0.038)
4.7	110 ± 8	112 ± 7		
4.8	127 ± 5	121 ± 6	88 ± 10	82 ± 8
4.9	101 ± 3	98 ± 5		
5.0	78 ± 4	88 ± 5	90 ± 10	62 ± 6
5.5	57 ± 7	63 ± 4	100 (0.013)	54 ± 5
6.0	38 ± 5	36 ± 7	87 ± 7	46 ± 3
6.5	27 ± 6	26 ± 6	51 ± 10	38 ± 3
7.0	14 ± 5	18 ± 3	63 ± 11	24 ± 3
8.0	4 ± 2	4 ± 3	20 ± 4	2 ± 1
9.0	3 ± 2	2 ± 1	15 ± 4	3 ± 1

[a] (A_{400} min^{-1}) for pNPPase or (A_{700} min^{-1}) for ATPase.

Table V. *Substrate Specificity of APase (100 ng per assay) Isolated from Plump and Shriveled Endosperm of Triticale*

Data are means ± SE of two assays of each two preparations.

Substrate	Plump	Shriveled
	%	
AMP	4 ± 2	2 ± 1
ADP	19 ± 3	33 ± 4
ATP	62 ± 6	64 ± 12
GTP	74 ± 20	69 ± 13
UTP	62 ± 14	64 ± 5
GIP	0	6 ± 3**
3PGA	6 ± 2	7 ± 2
PPi	86 ± 6	84 ± 7
NADP	5 ± 2	29 ± 4**
NAP	12 ± 3	11 ± 4
pNPP	100 (0.052)[a]	100 (0.130)

** Significantly different at 1% probability level. [a] (A_{700} min^{-1}).

the three close activity bands observed on polyacrylamide gel of this APase may be caused by the minor charge heterogeneity. Similar results were observed in a purified sunflower seed APase in that two distinct bands of activity as well as Coomassie blue staining were separated by PAGE (16).

The APase extracted from plump seeds exhibited identical characteristics for separation (Table III) and the activity profiles were similar to Figure 1, except that the first peak in Figure 1A was 50% smaller. Even the results of PAGE and SDS-PAGE of isolated APase were comparable.

Properties of Purified APase. The pH optimum of the isolated APase was 4.8 for pNPP (Table IV) for both materials and for ATP in plump material was 5.5 and 4.5 for shriveled material. The isoelectric point was pH 5.9 for APase isolated from both materials (Fig. 1B and other runs of purified enzymes not shown). The carbohydrate content was estimated for plump APase 12.3 ± 0.3% and for shriveled 12.1 ± 0.2%.

The substrate specificity of the purified APase for the two materials was nonspecific (Table V) as other APase isolated from many organisms (1, 8, 11, 20, 22, 23, 25). The relative activity in reference to pNPPase of the two materials toward different substrates was similar except GlP and NADP which were more favored by the enzyme isolated from the shriveled endosperm. This higher affinity may have significant consequences *in situ* as GlP is the substrate for ADP-Glc PPase which provides ADP-

Table VI. *Influence of Various Effectors on APase Activity*

Data are means ± SE of two assays each of two preparations.

Effectors	Final Concentration	ATPase (100 ng)		pNPPase (50 ng)	
		Plump	Shriveled	Plump	Shriveled
	mM	% ± SE			
None		100 (0.030)[a]	100 (0.072)	100 (0.036)	100 (0.145)
Molybdate	0.1	0	0	0	0
Citrate	40	70 ± 12	95 ± 1	103 ± 5	104 ± 5
EDTA	10	0	0	115 ± 7	126 ± 10
$MgCl_2$	10	115 ± 1	127 ± 14	272 ± 16	312 ± 5
$Pb(Acetate)_2$	10	24 ± 8	88 ± 3**	36 ± 12	81 ± 4*
$CaCl_2$	10	137 ± 6	152 ± 7	231 ± 28	236 ± 8
$HgCl_2$	1	107 ± 18	142 ± 20	58 ± 6	73 ± 4
Tartrate	10	139 ± 11	149 ± 15	80 ± 4	106 ± 5
NaF	5	9 ± 3	16 ± 1	6 ± 1	8 ± 4
FeS_4	1	16 ± 5	8 ± 4	39 ± 6	72 ± 5*
Ascorbate	10	175 ± 18	209 ± 13	48 ± 11	41 ± 7
$CuSO_4$	1	112 ± 21	150 ± 12	16 ± 3	54 ± 8**
$ZnSO_4$	1	142 ± 11	150 ± 8	55 ± 5	54 ± 6
$HSCH_2CH_2OH$	14			93 ± 3	91 ± 11

[a] (A_{400} min^{-1}) for pNPPase, (A_{700} min^{-1}) for ATPase. *, ** Significantly different from plump at 5 and 1% level probability.

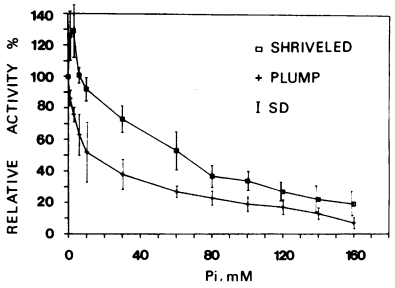

FIG. 2. Effect of inorganic phosphate concentration on the relative activity of pNPPase isolated from plump and shriveled triticale seeds (see "Materials and Methods" for details).

glucose for the synthesis of starch for the filling of endosperm tissue. If GIP is depleted by the more active APase in shriveled seeds, the seed will be less filled than plump seeds from which a less active APase was isolated. More active hydrolysis of NADP by the APase isolated from shriveled seed may not have direct impact on starch synthesis and seed filling, but indirectly this may affect anabolic activities in the developing seeds and, thus, the degree of seed plumpness.

Influence of Various Effectors on APase Activity. The results in Table VI indicate several points. First, the APase isolated from shriveled material was much less inhibited by Pb^{2+} than that from plump material for both ATPase and pNPPase activity. Second, EDTA inhibited ATPase activity, but not pNPPase activity in the APase isolated from both materials probably because of the cation requirement by ATPase. Ascorbate stimulated ATPase activity for both plump and shriveled material as for ATPase of chicken liver lysosomes (14), although ascorbate suppressed pNPPase activity. Third, molybdate, F^-, and Fe^{2+} inhibited the activity of both ATPase and pNPPase from both materials, whereas Mg^{2+} and Ca^{2+} increased activity of both enzymes from both materials. Fourth, Hg^{2+}, Cu^{2+}, Zn^{2+} enhanced ATPase activity and suppressed pNPPase activity from both materials. These data indicate that the purified APase exhibit characteristics of both ATPase and pNPPase as found in various plant and animal materials (7, 8, 14, 20, 22, 23, 25). Small differences, however, were observed in the plump and shriveled materials.

K_m and V_{max}. Based on four preparations of APase from each material, the K_m for pNPP was 0.57 ± 0.10 mM (mean + SD) and 0.35 ± 0.13 mM for plump and shriveled seeds, respectively. This difference was not statistically significant. The V_{max} for pNPP was 42.8 ± 1.5 and 142.52 ± 13.4 μmol mg^{-1} min^{-1} for plump and shriveled seed, respectively, indicating their significant difference in kinetic properties. The K_m for ATPase was 0.32 ± 0.06 and 0.50 ± 0.19 mM and the V_{max} was 33 ± 7 and 175 ± 41 μmol mg^{-1} min^{-1} for plump and shriveled seeds, respectively. These results indicate that the K_m for ATP was similar in the two sib-lines whereas the V_{max} was vastly varied; that may result in more rapid degradation of needed phosphate esters and ATP energy in shriveled seeds.

Inhibition by Inorganic Phosphate. Phosphate was an inhibitor at 1 mM or more in concentration for APase (pNPPase) isolated from plump line, whereas the enzyme isolated from shriveled material was stimulated by phosphate at 1 to 6 mM beyond which inhibition increased with concentration (Fig. 2). The Pi concentration which caused 50% inhibition of APase activity (I_{50}) was 11 mM for plump seeds and 62 mM for shriveled seeds, indicating a very high tolerance toward Pi concentration in the endosperm tissue of the APase in shriveled seeds. The content of Pi in the endosperm of P at 6 WAA was found to be 1.5 ± 0.5 μmol $endosperm^{-1}$; with an average fresh weight of 101 mg and water content of 60%, the Pi concentration was 25 mM. The Pi concentration in S was 47 mM (2.8 ± 0.6 μmol Pi, 96 mg fresh weight, and water content 60%). The APase, therefore, was about 60% inhibited *in situ* in plump and only 35% inhibited in shriveled seeds. This differential inhibition further results in more nucleotides (potential energy supplies) and phosphate esters (substrates) being degraded in shriveled than in plump seeds.

While the quantitative and kinetic differences of this nonspecific APase in plump and shriveled lines are sufficient to explain the reduction of energy supply and substrates in causing seed shrivelness, the mechanism(s) of tissue degradation, however, will await future studies.

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