The tissue and substrate degradational enzymes, acid hydrolases, in endosperm were studied in order to discern the mechanism of seed shriveling in triticale (X. Triticosecale Wittmack).

Acid hydrolases in crude extract of 7-week-old developing endosperm of 2 lines each of triticale with shriveled (6TB, RBS) and plump seeds (6TA, RBP) were successfully separated into 5 fractions by DEAE-Sephacel column chromatography. Dramatically difference was observed in the run-off fraction which had a 6-fold higher acid phosphatase activity in shriveled seeds than that in plump ones. Fraction III had much higher acid phosphatase, ATPase and phospholipid phosphatase activity in shriveled seeds than that of plump ones.

A detection method of enzyme activity on polyacrylamide gel was developed for nucleoside phosphatase in order to monitor the efficiency of purification steps. Using the detection method developed, 2 slow-moving isozymes were found in shriveled seeds to be different from plump ones. Nucleoside phosphatase activity was 1.3-
to 2.5-fold higher in shriveled endosperm than in plump ones.

In fraction III, 2 pairs each of corresponding acid phosphatase isozymes on gel from RBP and RBS were partially purified and compared for their kinetic properties. RBP-I and RBS-I, a corresponding pair slower moving on gel (Rm 0.43) and fast-eluted from column (105 mM KCl) were very different in their kinetic properties. Specifically RBS-I had lower Km for p-nitrophenyl phosphate (0.21 mM) than RBP-I (0.57 mM) and 9-fold higher I_{50} for Pi (32 mM) than RBP-I (3.6 mM). RBP-II and RBS-II, another corresponding pair fast-moving on gel (Rm 0.61) and slow-eluted from column (140 mM KCl) had similar properties.

This study confirms past report that higher acid hydrolase activity was found in genetic lines producing shriveled seeds. The kinetic properties of 2 corresponding pairs of isozymes of endosperm acid phosphatase indicated that slow-moving isozymes in shriveled seeds were able to degrade more substrate, energy compound and activator for starch synthesis, and could function well under high endogenous Pi concentration. Thus the in situ functional difference in enzymatic activity may have resulted in morphological variation of seed conformation.
ACID HYDROLASES AND SEED SHRIVELING IN TRITICALE SEEDS

by

Tsan-Piao Lin

A THESIS
submitted to
Oregon State University

in partial fulfillment of
the requirements of the
degree of
Doctor of Philosophy

Completed May 6, 1985
Commencement June 9, 1985
APPROVED:

Redacted for privacy
Professor of Crop Science in Charge of major

Redacted for privacy
Head of Department of Crop Science

Redacted for privacy
Dean of Graduate School

Date thesis is presented May 15, 1985

Typed by Tsan-Piao Lin for Tsan-Piao Lin
ACKNOWLEDGMENTS

My sincere appreciation is offered to Dr Te May Ching, my major professor, for the time she so unselfishly gave to train me in conducting research and to communicate with the English language. Her encouragement and support throughout my graduate program will be unforgettable. I thank Dr Don Grabe, Dr W. David Loomis and Dr Thomas C. Moore for their helpful advice during the writing of my thesis and their service as committee members. Thanks also to Dr Jack R. Stang for serving as Graduate Council Representative on my committee.

I would also like to gratefully acknowledge the financial support which I received from National Science Council, Republic of China for 1981 to 1982 and from Dr Te May Ching's research project for 1983 to 1984.

I would like to express my gratitude to Mr. Ta-Wei Hu, Chairman of Sivilculture Division and Mr. Shuan-Chen Liu, Director of Taiwan Forestry Research Institute in Taipei for their support of my study program at Oregon State University.

I give special thanks to Julie H. Zhu for peeling triticale seeds up to 500 grams for this study, to Robert Turley for his help and friendship all the time and to Phil Shuler for his help in preparing my thesis using computer.

Finally, I am indebted deeply to my wife, Chung-Ly Chen for her understanding, help and encouragement while nursing our baby and taking care of the family.
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INTRODUCTION

Cereal shriveling is a common phenomenon in early-generation hybrids of inter- and intraspecific or intergeneric crosses (2, 3, 16). Only through repeated selection for plumpness and other desired traits, such as disease and insect resistance, drought, cold, and chemical tolerance, etc., can the seed shriveling be bred out or eliminated in the later generations of many hybrids. If the mechanism of seed shriveling can be discerned, cultural or biotechnological remedies may be developed to speed up the crop improvement endeavor so that food supply can keep pace with the ever-increasing world population.

Thus the mechanisms of seed shriveling have received much attention in the past two decades. The shrunken endosperm mutants of corn are low in starch content as well as storage protein, zein (13). These mutants were known to be related to the low ADP-glucose pyrophosphorylase activity in brittle-2 (7), to defective sucrose synthase in shrunken-2 (6), or to high ribonuclease (RNase) activity at the later stages of seed development in double mutant brittle-2; opaque-2 (13). In the last case, the post transcriptional degradation of zein mRNAs' and reduced zein synthesis were coincidental to a rise of RNase activity in the endosperm. The high RNase not only degraded mRNAs' but also influenced the stability of rRNAs' resulting in negligible amounts of zein being synthesized in the double mutant. These studies provided much basic knowledge in correlating phenotypic expression
with the function of genic products from altered genomes, the mutants. The cause of seed shriveling in hybrids still remains an enigma.

The shrunken endosperm in barley mutant (seg) was caused by premature chalazal necrosis resulting in physical blockage of assimilate transport from the phloem into the nucellar projection and endosperm cavity (14). What causes the premature chalazal necrosis was not identified biochemically or genetically.

Triticale \((\text{X} \text{Triticosecale Wittmack})\) is the first man-made hybrid genus between wheat \((\text{Triticum L.})\) and rye \((\text{Secale L.})\), that has superior nutritional qualities to wheat due to its high protein and lysine content and better baking qualities than rye (17). Triticale, however, suffers from many reproductive disorders including kernel shriveling (16). In a comprehensive review of the past 15 years research, Thomas et al. (16) stated that kernel shriveling in triticale is manifested in two ways: 1. Endosperm cells and contents may not grow fast enough to fill the pericarp. 2. Internal regular or irregular cavities are common within the endosperm. In the shriveled seeds, aleurone cells were often disrupted, and the pericarp and seed coat had collapsed to produce a wrinkled appearance in later stages of development. At maturity the starch grains could be pitted but still maintained their shape.

Several possible causes of seed shriveling in triticale have been proposed by research workers:

1. Aberrant nuclei formed in the endosperm during the early coenocytic period by division error is related to seed shriveling. Bennett (1) found that the frequency of a berrants in the coenocytic endosperm was highly positively correlated with the degree of kernel
shriveling. The first sign of abnormality in the kernel development was the appearance of abnormal-looking nuclei in the coenocytic endosperm and the most common type of abnormality was the anaphase bridge at mitosis. Anaphase bridges could result in polyploid nuclei, some of which may repeatedly duplicate without cell division (10). Bennett (1) analyzed the karyotype of bridged chromosomes in the endosperm and found that these were nearly always rye chromosomes. In addition, Darvey (4) found that, with the possible exception of chromosome 2R, all other chromosomes of rye when added individually to wheat have shriveling effects on the kernel. How the anaphase bridge, polyploid nuclei, and rye chromosomes affect structural and biochemical development of the endosperm is unclear, though many papers have been written on the subject.

2. Alpha-amylase activity is correlated highly with the degree of grain shriveling. Klassen et al. (12) observed that lines with the highest alpha-amylase activities of mature grain of triticale were also the ones with the poorest kernel type and lowest grain density. Rao et al. (15) observed a direct relationship between the degree of grain shriveling and the activity of alpha-amylase and peroxidase during seed development of several lines. Many other workers, however, did not find such a relationship. Klassen (11) observed that reduction of alpha-amylase content in mature seed by prior application of cycocel didn't result in an increase of kernel dry weight in shriveled triticale line 6A190. Dedio et al. (5) reported that the release of alpha-amylase within the maturing kernel was quite localized and was associated with a necrotic or degenerating area only. If these lesions were not present, destruc-
tion of starch granules by the enzyme was generally not observed. It appears that tissue destruction is the primary cause of seed shriveling. Thomas et al. (16) concluded from these reports that although premature digestion of starch by this alpha-amylase undoubtedly contributes a little to the depression of dry matter accumulation in the kernel of shriveled triticale lines, it is probably a side effect of shriveling rather than a major cause.

Recently Ching et al. (4) reported that alpha-amylase activity was low in the endosperm throughout the developmental stages, and little difference was found among plump-seeded and shriveled-seeded triticale lines. Furthermore the alpha-amylase activity did not increase at the later stages of seed development when starch accumulation was particularly low in shriveled seeds. Therefore they (2) concluded that the activity of alpha-amylase in triticale endosperm probably has no direct bearing on the starch reduction in developing seeds.

3. Efficiency of nutrient transport to the spike is involved in seed shriveling. Two papers rendered support to this concept. Hill (8) reported that the ability of translocating $^{14}$C-photosynthate from flag leaf to kernel in severely shriveled line of triticale 6A190 decreased after 23 days after anthesis when compared to the plump seeded line 6A250. In addition lines producing shriveled seeds took less labelled sucrose into the endosperm than the lines producing plump seeds (11). Based on other reports, Thomas et al. (16) concluded that there is no consistent evidence that under reasonable growing conditions the ability of the triticale plant to fix, to translocate, or to mobilize photosynthate is a limiting factor which leads to
kernel shrivel.

4. Low ADP-glucose pyrophosphorylase activity and high acid hydrolase activity are the characteristics of triticale lines producing shriveled seeds. Several synthetic enzymes directly related to starch accumulation in the cereal seeds such as starch synthase, ADP-glucose pyrophosphorylase, ADP-glucose sucrose synthase, UDP-glucose pyrophosphorylase, UDP-glucose sucrose synthase, and phosphorylase were assayed simultaneously at different stages during seed development of triticale lines producing plump and shriveled seeds (2) so that their anabolic differences can be compared.

The seed weight and starch accumulation pattern were comparable during the first 4-5 weeks for both seed lines, while a distinct slow down in weight and starch gain was later observed in lines producing shriveled seeds (2). The activity patterns of most of the enzymes tested during seed development were similar in plump and shriveled kernels. The ADP-glucose pyrophosphorylase was the only enzyme that exhibited 2-fold higher activity in plump seeds than shriveled seeds, indicating that ADP-glucose pyrophosphorylase may play an important role in the degree of plumpness of triticale seeds. Comparable experimental results have been repeatedly confirmed during the subsequent four years on two genetic lines each producing plump and shriveled seeds.

The shriveled seed conformation was caused not only by less starch accumulation but also by a two-fold higher hydrolytic enzymic activity than that of plump lines (3). The degradational activities were attributed to acid phosphatases such as p-nitrophenyl phosphatase, alpha-naphthyl phosphatase, glucose-1-phosphatase, ATPase,
ADPase, and phospholipid phosphatase. The activity of these enzymes was specifically increased at later stages of seed development and their activities correlated well with the reduction of seed weight and starch accumulation in lines producing shriveled seeds. The slow moving isozymes of acid phosphatase on polyacrylamide gel, reached highest activity especially at the later stages, were primarily from lines producing shriveled seeds. They concluded that many isozymes of acid phosphatase in shriveled lines not only are needed for normal seed development, cell differentiation, and seed maturation, but some of them also are involved in degradation of tissues and cells resulting in shriveled seed conformation.

To verify the above conclusion and to further explore the mechanism of seed shriveling in order to eliminate problems for rapid improvement of triticale, this study was instigated. The first attempt of this research involved the methodology of separating major isozymes of acid hydrolases in both plump and shriveled seeds. The second step was developing detection methods for hydrolases other than acid phosphatase using endogenous substrate to facilitate the purification of these hydrolases. The third portion summarized the characteristics of two pairs of partially purified acid phosphatase isozymes each in lines producing plump and shriveled seeds. The kinetic properties of these isozymes indicated some functional differences which may result in morphological variations of seed conformation.

Future study should concentrate on purifying these isozymes, comparing their physical structures to determine if they were the products of posttranslational modification or derived from different
genes, making their antibodies for the enzyme quantification in endosperm during development, and for the isolation of their specific mRNAs' from which cDNAs' may synthesized and cloned. The cloned cDNAs' may be used to probe their genetic origin (9), and the genes producing the damaging enzymes may be deleted by biotechnological or chemical means.

Literature Cited


CHAPTER ONE

ACID HYDROLASES IN PLUMP AND SHRIVELED SEEDS OF TRITICALE LINES
ABSTRACT

Endosperm of 7-week-old developing seeds of two lines each of triticale (X Triticosecale Wittmack) with shriveled or plump seed conformation was extracted with buffer containing 0.1 M NaOAc-0.2 M KCl-14 mM ME-0.5% Triton X-100, pH 5. DEAE-sephacel column chromatographic separation of dialyzed extracts resulted in five fractions. The first cationic fraction was 2- and 6-fold higher in protein content and acid phosphatase activity, respectively, in shriveled seeds than that of plump ones. The fourth fraction eluted by 180-230 mM KCl contained comparable protein quantity, but 3.5-fold higher carboxypeptidase activity in shriveled seeds than that of plump ones. Higher adenosine 5'-triphosphate hydrolase and phospholipid phosphatase activity was observed in fraction III (eluted by 120-160 mM KCl) of shriveled seeds than of the plump seeds. These fractions could provide material for further purification and characterization so that the biochemical and molecular mechanism(s) of seed shrivelness in triticale might be discerned.

INTRODUCTION

The shrunken endosperm mutants of corn are known to be related to low ADP-G pyrophosphorylase activity (bt2)(14), to defective sucrose synthase (sh2)(10), or to high ribonuclease activity at the later stages of seed development (bt2;02)(17). One of the shrunken endosperm barley mutants was due to a premature chalazal necrosis causing physical blockage of assimilate transport to endosperm tissue, and the other was
caused by abnormal endosperm development (19). The shriveling of triticale kernels has been attributed to aberrant nuclei formed by mitotic errors in the endosperm during the early coenocytic period, but is not caused by a poor supply of substance to the kernel (23). Recently it was found that endosperm ADP-glucose pyrophosphorylase activity during triticale seed development was 2- to 3-fold higher in lines with plump seed than that of lines producing shriveled seed, indicating that this enzyme may play an important role in the degree of plumpness of triticale seeds (5). In addition, the high activity of acid phosphatases that was observed at later stages of seed development in triticale lines with shriveled seeds might deplete the energy supply for biosynthesis, and degrade substrates for starch accumulation and membrane component for tissue integrity (6). In that study, some slow moving isozymes of endosperm acid phosphatase separated on polyacrylamide gel by electrophoresis (PAGE) were prominent in lines with shriveled seeds, but not in lines producing plump seeds. It was proposed that these less anionic acid phosphatase isozymes may play an important role in causing shrivelness in mature seeds (6).

Prior to characterizing the specific functions of these acid phosphatase isozymes, especially the less anionic forms, in lines producing either plump or shriveled seeds, it is necessary to separate these isozymes into different fractions or even in purified forms.

This report describes the detailed procedures used in separating the acid phosphatase isozymes into several distinct fractions by DEAE-sephacel column chromatography. The results allowed us to characterize the differences in enzyme activity between lines producing plump and shriveled seeds. In addition, one hydrolase, carboxypeptidase, was
observed and found to be particularly high in the endosperm of the lines producing shriveled seeds.

MATERIALS AND METHODS

Plant material

Two genetic lines of winter triticale (x Triticosecale Wittmack), 6TA 876 (6TA) and Red Bobs//Daws/Snoopy-P (RBP) with plump seed characteristic and two lines with shriveled seed conformation, 6TB 163 (6TB) and Red Bobs//Daws/Snoopy-S (RBS) (6), were used. Seeds were planted using common cultural practices at the Hyslop Agronomy Farm, Corvallis, Oregon, in October 1983. Spikes were harvested weekly after anthesis in June and July of 1984. Only the seeds harvested at 6 to 7 weeks after anthesis (WAA) were used in this study since distinctive differences in acid phosphatase isozymes between lines producing plump and shriveled seeds were observed only at the later developmental stages in previous years (6). Spikes were collected in 1% sucrose and 0.1% glycine to provide a continuous simulated supply of photosynthate while being transported to the laboratory. Samples were stored in heavy polyethylene bags in a super freezer (-63°C) prior to use.

Extraction and separation of acid phosphatase isozymes and other hydro-lases

In previous studies, only cell-free enzymes extracted in 50 mM Tris-HCl, pH 7, were assayed (5, 6). For structural degradation, such as the case of tissue necrosis in shriveled seeds, membrane-bound hydro-lases may be involved and the extraction method should be modified. A
Preliminary study of different extraction buffers was conducted by using compounds with known ability to solublize membrane proteins; 0.2 M KCl, 0.5% Triton x-100, 0.2 M KCl plus 0.5% Triton x-100, or 0.25% sodium deoxycholate. These were added into a common buffer for extraction of acid phosphatase, 0.1 M NaOAc and 14 mM mercaptoethanol (ME), pH 5, (1, 8, 9, 15, 16, 20, 22). Results indicated that all 4 additives increased extractable proteins from all 4 lines. The buffer containing Triton and KCl extracted the most protein, reaching 180% of the buffer alone and the average activity of acid phosphatase amounted to 160% of the buffer alone. Sodium deoxycholate extracts exhibited a general reduction of enzyme activities. None of the additives, however, affected the kinds and properties of acid phosphatase isozymes separated on PAGE (6).

Based on the above findings, an extraction buffer (EB) of 0.1 M NaOAc, pH 5, containing 14 mM ME, 0.2 M KCl plus 0.5% Triton x-100 was used. All the procedures were conducted at 0-4°C except as noted.

Ten grams of peeled endosperm were ground in a mortar with pestle in 10 ml of EB, and the homogenate was centrifuged at 30 Kg for 15 min. The precipitate was dispersed in another 10 ml EB and extracted again as above. The commonly used ammonium sulfate precipitation for enzyme purification and desalting was not possible in this instance as the detergent in EB prevented precipitation. Therefore, the crude extract was dialyzed to remove interfering ionic soluble compounds and then fractionated by ion-exchange resins. A previous study (6) indicated that the major difference in plump and shriveled triticale endosperms appeared to be the isozymes of acid phosphatase, and the PAGE separation of these isozymes was based on their anionic charges. Consequently, DEAE-sephacel (Pharmacia) was chosen for this endeavor.
The combined supernatant of the first and second extractions was rapidly dialyzed for 3 hours with 2 changes using Spectrapor 2 (12,000-14,000 MW cutoff) in 1 liter of 10 mM Tris-HCl buffer, pH 7.5, containing 14 mM ME (WB). The dialyzed extract was clarified by centrifuging at 30 Kg for 15 min. Because of the low critical micellar concentration of Triton x-100 (4), the detergent was not substantially removed by the dialysis. An average of 0.42% of Triton was found in the supernatant with only a little precipitate, resulting in little differences between the original and dialyzed extracts as far as protein and acid phosphatase isozymes were concerned (Fig. 1; original, 0 and dialyzed, D).

The cleared supernatant was charged onto a 1.6 x 21 cm DEAE-sephacel column pre-equilibrated with WB. The loaded column was washed with 50 ml WB, and then eluted with 320 ml of a 0.25 M KCl linear gradient in WB. The KCl was chosen because the commonly used NaCl had inhibited some enzyme activities. After the gradient elution, the top several centimeters of gel in columns was tannish in color. The colored substances was not removed by 2 M KCl or 0.1 M NaOH, but can be eluted down by 10 mM Tris-HCl buffer (pH 7.5) containing 0.2 M KCl plus 1% Triton x-100. A total of 110 tubes of 3.5 ml each was collected using a peristaltic pump (P-1, Pharmacia) at a flow rate of 21 ml/h.

Protein determination

Soluble protein in dialyzed extracts and eluates in even numbered tubes was determined by the Coomassie blue method using BSA as a standard (3). Triton x-100 over 0.1% interferes with the assay; thus
all the eluates containing Triton were diluted to 0.1% or less prior to assaying.

**Triton x-100 assay.**

The concentration of Triton x-100 in eluate was read against eluting buffer at 275 nm in a Cary 219 spectrophotometer, then calculated by $E_{1%}^{1cm} = 21 A_{275}$ (12).

**Para-nitrophenyl phosphatase (pNPPase) assay.**

One hundred µl of 10X diluted dialyzed enzyme extract or of column fractions were incubated in 0.2 ml of 0.1 M NaOAc buffer containing 9 mM pNPP, pH 4, for 10 min. at 30°C. After incubation, one ml of 0.5 N NaOH was added to each tube to stop the reaction and develop the color. The reaction mixtures were then read against a blank at 400 nm (6).

**Adenosine 5'-triphosphatase (ATPase) assay.**

Two hundred µl of 10X diluted enzyme extract or of column eluates were incubated with rapid shaking at 30°C for 10 min in 0.2 ml of 0.1 M KOAc buffer containing 5 mM ATP, pH 5. The reaction was stopped by adding 0.5 ml of 20% TCA. After 10 min at 1°C, the reaction mixture was centrifuged at 10 Kg for 5 min and the supernatant was assayed for Pi produced by the enzyme using the Fiske-Subbarow method (8). The $A_{660}$ against a reagent blank was recorded.

**Acid phospholipid phosphatase (PLPase) assay.**

The method used was described by Ching et al. (6). One gm of phosphatidylcholine was dissolved in 10 ml of chloroform:methanol (1:2,
v/v) with constant stirring for 24 hours at room temperature. One ml of the solubilized substrate was suspended in 4 ml of 50 mM NaOAc and 10 mM CaCl$_2$ buffer, pH 5, and this suspension was used as substrate. Four hundred µl of 10X diluted enzyme extract or of column eluates and 0.5 ml substrate suspension were mixed well and incubated with rapid shaking at 30°C for 30 min. The reaction was stopped by adding 1 ml 20% TCA. After centrifuging at 10 Kg for 5 min, the supernatant containing hydrolyzed Pi was analyzed by the Fiske-Subbarow method and the A$_{660}$ against a reagent blank was recorded.

**Carboxypeptidase (CPase) assay.**

In a preliminary study, several different substrates were used to test for protease activity in the crude extract or column eluates: BSA, hemoglobin and casein for endo- and exopeptidase; α-N-benzoyl-DL-Arg-P-nitroanilide and benzoyl-DL-Arg-β-naphthylamide for peptidases; L-leu-P-nitroanilide for aminopeptidase and N-carbobenzoxy-L-phenyl-alanyl-L-alanine for CPase. Only CPase showed measurable activity. This lack of endopeptidases might be inherited from wheat, one of the triticales parents, as the major proteolytic enzyme in developing wheat endosperm was CPase (20) and the activity of CPase increased synchronously with the endosperm development (16). Furthermore, in a comparative study of peptide hydrolase activities in cereals, triticale had a ratio of carboxypeptidase to endopeptidase of 220-260 (25). The procedure of Tulley and Beevers (24) was followed to assay for CPase. Two hundred µl of enzyme preparation were incubated in 0.5 ml of 50 mM NaOAc buffer, pH 4, containing 1 mM N-carbobenzoxy-phenyl-alanyl-L-alanine with rapid shaking at 30°C for 15 min. After incubation, the reaction was stopped
by adding 0.5 ml of 20% TCA and chilled in ice for 10 min. The reaction mixtures were then centrifuged at 10 Kg for 5 min and the supernatant was assayed for free amino acid by using a modified ninhydin method (18). The absorbance at 570 nm against a reagent blank was recorded. All the enzyme assays were duplicated.

Electrophoresis and isozyme detection of acid phosphatase.

Enzyme extracts and eluates in tubes containing peak proteins or activities were electrophoretically separated on a 7.0% polyacrylamide slab gel (Bio-Rad) using Davis' method without stacking gel (7). Acid phosphatase was stained by the method of Scandelios (21) using α-naphthyl acid phosphate as substrate and fast Garnet GBC salt as dye and incubated at 30°C for 1 hour with rapid shaking. The gel was then destained in 50% ethanol for 1 h and fixed in 7% acetic acid. Protein bands were stained with Coomassie blue G 250 (2).

RESULTS AND DISCUSSION

The representative column chromatographic profile of proteins and activities of pNPPase, ATPase, PLPase, and CPase from endosperm extracts of 6TA, RBP, 6TB, and RBS are summarized in Fig. 1-1. Three to 4 separations were run for each material and the quantitative data are listed in Table 1. It is evident from the table that more hydrolase protein was extracted from shriveled than from plump seeds, and the total hydrolase activities were 1.5- to 4-fold higher in shriveled than plump. These results agree with the previous findings in 1982 and 1983 (6).
Because of its non-ionic nature, Triton X-100 in the dialyzed extract was quickly washed down from the column into tubes 6-20 (Fig. 1 insert). The recovery of Triton averaged 96% in the run-off and the elution pattern by the salt gradient was not affected by the Triton x-100 in the extract. The patterns in Fig. 1-1 were reproducible for each line as long as a new column packing was used for each run. Some tenaciously-bound material apparently interfered with the exchange capacity of the resins even though the recovery of protein for each run reached an average of 98%.

The protein profile (Fig. 1-1) clearly shows that five fractions were separated from each extract by the ion-exchanger with the elution system used. The first fraction (I) depicted the most dramatic difference between the plump and shriveled seeds in that the latter had 2- to 2.5-fold more cationic (at pH 7.5) proteins in the run-off than the former (Table 1-1). The pNPPase activity was 3- to 11-fold higher in shriveled than the plump seeds (Table 1-1). ATPase was low in activity in this fraction for all lines, whereas the shriveled seed of 6TB had a 110-fold higher activity of PLPase over that of 6TA. A 2-fold higher activity of CPase was found in shriveled seeds of 6TB and RBS than in plump seeds.

These observations verified the findings with material grown in 1982 and 1983 in that some late-stage specific less anionic (at pH 8.3) APases occurred in large quantities in shriveled endosperm (6). The protein composition of fraction I was quite simple (Fig. 1-2, lower frame). For plump seeds (6TA), only two major bands were observed. One was apparently Triton x-100 bound and remained on the top of gel while the other was located just below (Fig. 1-2, lower frame, tube 8).
Shriveled seeds (RBS) had 3 bands with one on the top of gel and the other two coincident with two slow-moving APase isozymes (Fig. 1-2, upper frame, tube 10). Further purification of this fraction and characterization of purified enzyme should delineate their function in relation to tissue degradation, depletion of energy supply, and prevention of starch accumulation in lines with shriveled seeds.

Protein fraction II was eluted mainly by 0.085 M KCl from the extracts of 6TA and RBP. It is a small protein fraction with moderate activity of acid hydrolases (Table 1-1). There was no comparable fraction II in extracts of 6TB or RBS (Fig. 1-1). The second protein fraction of shriveled endosperm of 6TB and RBS, IIa, was eluted mainly by 0.025 M KCl. These two genetic lines were different in that the IIa fraction of 6TB was small with low activity of acid hydrolases while the IIa of RBS was moderate in quantity and high in pNPPase, ATPase, and PLPase (Figs. 1 and 2, Table 1). Therefore, the second protein fraction (II or IIa) was unique to each line and appeared to be not absolutely related to seed shrivelness. The protein fraction IIIs were rather complicated in their protein composition (Fig. 1-2 lower frame). At least 4 to 5 bands were separated by one dimensional PAGE. Furthermore, the APase isozyme of IIa fraction (Fig. 1-2, upper frame, RBS) was a minor protein band (lower frame) and purification might be difficult due to the small quantity (Table 1-1).

The fraction III eluted by 110-170 mM KCl was the most complicated in protein composition and in number of isozymes of APase (Fig. 1-2). For plump endosperm (6TA), at least 8 protein bands and 3 acid phosphatase isozymes were detectable. An equal number of protein bands and isozymes were observable for shriveled endosperm (RBS). This fraction
also contained the main peaks of ATPase and PLPase for all 4 lines regardless of plump or shriveled endosperms (Fig. 1, Table 1-1). The total activity of ATPase and PLPase, however, was about 2- to 4-fold higher in shriveled endosperms (6TB and RBS) than in plump endosperms (6TA and RBP) (Fig. 1 and Table 1-1). The total activity of pNPPase and CPase was higher in 6TA and 6TB than in RBP and RBS indicating that genetic make-up might be involved in this instance. This is an important fraction as the major degrading enzymes, ATPase, PLPase, and CPase resided in this fraction. Further purification and characterization would be fruitful in exploring the mechanisms of seed shrivelness.

The first portion of fraction IV, as represented by the eluate in tube 64 of 6TA and in tube 70 of RBS (Fig. 1-1), was composed of several protein bands and 5 APase isozymes in plump endosperms (6TA) and 2 minor acid phosphatase isozymes in shriveled endosperms (RBS) (Fig. 1-2). The second portion of fraction IV (tube 72 of 6TA and tube 76 of RBS) contained two minor APase isozymes and 2 major protein bands in the plump endosperm and no APase, but 3 major protein bands, in shriveled endosperm (Fig. 1-2). The two major protein bands might be the two CPase peaks observed in Fig. 1-RBS. An effort has been made to detect CPase on PAGE using several known methods, but failed. Whether the highly active CPase was the major protein component of the second portion of fraction IV in shriveled endosperm awaits future research. The 2- to 5-fold higher total activity of CPase in fraction IV of shriveled materials (Fig. 1-6TB and RBS, Table 1-1) than in the plump material definitely warrants further investigation. Even though these CPases were eluted down with approximately the same salt concentration range, they might be vastly different in their properties and
structures. Only detailed studies would reveal their function and influence on seed shrivelness.

Fraction V appeared to be low in hydrolase activity except that of RBS where CPase and PLPase were moderately high (Fig. 1, Table 1-1). This is a difficult fraction to study as the proteins and enzymes were solubilized only by 0.2 M KCl and 1% Triton and further purification procedures would be too complicated (4, 11) to be of value for the purpose of the present study. Most of all, these proteins might represent tightly bound membrane proteins and appear to be genotype specific as evidenced by the quantitative similarity between 6TA and 6TB as well as RBP and RBS.

The experimental results provide leads for future study into the mechanism of seed shrivelness. Fraction I would be good for purification and characterization of CPase and APase, fraction III (eluted by 120-160 mM KCl) for ATPase and PLPase, and fraction IV for CPase. The purified enzymes may be used as probes for molecular biological studies to discern whether the seed shrivelness is a direct gene expression, or chromosomal deletion (1, 13), or can be attributed to post translational modification (11).
Table 1-1. Distribution of protein and activity of pNPPase, ATPase, PLPase, and CPase in different fractions separated by DEAE-sephacel column chromatography from endosperm (10 gm) extracts of two genetic lines each of plump (6TA, RBP) and shriveled (6TB, RBS) seeds collected 6 to 7 weeks after anthesis.

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<td>1.87</td>
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°Percent of total.

*The average of 3 runs with a coefficient of variation <20%.
Fig. 1-1 Typical DEAE-sephacel chromatographic separations of dialyzed crude extracts of four triticale lines. For each run, extract of 10 gm endosperm containing 40-60 mg protein was applied to a 1.6 x 21 cm column pre-equilibrated with 10 mM Tris-HCl and 14 mM ME (wash buffer), pH 7.5. The column was washed by the same buffer, eluted by a KCl gradient up to 0.25 M in the wash buffer, then was washed with 0.2 M KCl and 1% Triton x-100 in the wash buffer. A total of 110 tubes of 3.5 ml were collected and the even numbered tubes were assayed for pNPPase (--+-+---), CPase (--- --- ---), ATPase (-- --- ---), PLPase (--- --- ---), and protein (---- ---- ----). The concentration of salt gradient was measured by the conductivity of eluates from several blank runs.
Figure 1-1

TUBE NUMBER
Fig. 1-2 Polyacrylamide electrophoretic separation of acid phosphatase isozymes (upper) and protein patterns (lower) of original (O), and dialyzed (D) extracts and eluates in column chromatographic peak tubes of 6TA (plump) (left) and RBS (shriveled) (right). Each slot was charged 100 μl of 10 x diluted extract or eluate from various tubes.
LITERATURE CITED


CHAPTER TWO

ACID NUCLEOSIDE PHOSPHATASES IN SHRIVELED AND PLUMP TRITICALE SEEDS
ABSTRACT

Two slow-moving and highly active isozymes of endosperm acid nucleoside phosphatases in 7-week-old developing shriveled seeds were observed to be different from those in plump seeds of triticale (X_Triticosecale Wittmack). The difference in isozyme patterns provides the possibility of exploring further the mechanism of shrivelness by studying the characteristics of differing isozymes. The crude extract of these enzymes had an activity ratio between shriveled and plump seeds of 1.3 to 2.5. This indicates the possible participation of these enzymes in reducing energy supply in shriveled seeds. The endogenous Pi concentration was found to be 38 to 50 mM in plump seeds and 133 to 173 mM in shriveled seeds. This difference may inhibit the synthesis of ADP-glucose and starch and contribute to shrivelness. A simple detection method was developed for ATPase, ADPase, UTPase, and CTPase on polyacrylamide gel.

INTRODUCTION

Shrivelness and low starch content in triticale seeds are related to the low activity of ADP-glucose pyrophosphorylase (ADP-GPPase)(3), and the higher activity of acid hydrolases (4). At later stages of seed development, the activity of endosperm acid hydrolases including p-nitrophenyl phosphatase (pNPPase), adenosine 5'-triphosphatase (ATPase), adenosine 5'-diphosphatase (ADPase), glucose 1-phosphatase (G1Pase), phosphotidic phosphatase (PLPase)(4) and carboxypeptidase (CPase) (unpublished data) increased specifically in
shriveled seeds. Several more basic acid phosphatase (APase) isozymes were specifically implicated as shrivelness related degradational enzymes (4). This study was conducted to determine if all acid phosphatases in the endosperm of triticale lines producing plump seeds are different from those of shriveled seeds. A staining method was developed for detecting ATPase, ADPase, adenosine 5'-monophosphatase (AMPase), cytosine 5'-triphosphatase (CTPase), and uridine 5'-triphosphatase (UTPase) on polyacrylamide gel (PAG) in crude extracts.

MATERIALS AND METHODS

Plant materials

Two Winter triticale lines with plump seed (6TA 876 (6TA) and Red Bobs//Daws/Snoopy-p (RBP)) and two with shriveled seed (6TB 163 (6TB) and Red Bobs//Daws/Snoopy-s (RBS)) conformation (3) were used. Seeds were planted in Corvallis, OR in October 1983. In June of 1984 samples were collected and stored as previously described (3).

Endosperm fresh weight and water content

After samples were collected from the field, ten seeds were removed from the mid-portion of each 10 spikes. The pericarp and embryo were peeled off on moist paper over ice. The endosperm was weighed for fresh weight, dried at 85 C for 24 h, and weighed again for dry weight. The difference between the fresh and dry weight was used to calculate the water content.
Preparation of enzyme extracts

All procedures were conducted at 0 to 5 C, except as specially noted. Two seeds from each of 10 spikes collected 7 WAA were dissected to obtain endosperms. The twenty endosperms were ground in a mortar with pestle in 5 ml of extraction buffer (EB), containing 0.1M NaOAc buffer, 14 mM ME, 0.5% Triton X-100, pH 5. The slurry was centrifuged at 30 Kg for 15 min. The supernatant was used for enzyme assay and for PAGE separation.

Assay of pNPPase

As a comparison to the material harvested from other years, pNPPase was assayed as previously described (4).

Assay of acid NSPase and other phosphatases

Enzyme extract (50 ul) was added to 0.2 ml reaction solution of 0.1M KOAc, pH 5, containing 3 mM substrate and 14 mM ME and incubated with rapid shaking for 0 and 10 min at 30 C. The reaction was stopped by adding 0.5 ml of ice cold 20% TCA; after 10 min, the reaction mixture was centrifuged at 10 Kg for 5 min and the supernatant was assayed for Pi released by the enzyme using the Fiske-Subbarow method (4). The 0 and 10 min incubated samples were read against the reagent blank at 660 nm, and the difference was used to calculate the enzyme activity. The 0 times A_660 was used to calculate the endogenous Pi concentration in endosperm by the formula: umole endosperm^{-1} / (g FW endosperm^{-1} - g DW endosperm^{-1}). Under our assay condition, one absorbance unit at 660 nm equaled 0.69 umole of Pi in a total 2.5 ml reaction mixture. In addition to ATP, other
substrates: ADP, AMP, UTP, CTP, GTP, G1P and 3PGA were assayed similarly. Each assay was replicated four times.

Electrophoresis and acid NSPase detection

The fresh enzyme extract containing 30 to 35 ug protein was electrophoretically separated on 7% polyacrylamide gel using Davis' method (3). The gels were incubated in reaction solutions containing different substrates. A computer search revealed that three methods had been developed to detect ATPase on gel: (A) immersing the reacted gel in a color developing reagent of ammonium molybdate which combines with Pi released by the enzyme to form a blue color (8); (B) incubating the gel in 100 mM Tris, pH 8, containing 1 mM ATP and 5 mM CaCl2, and detecting the liberated Pi by the formation of white 3Ca₃(PO₄)₂.Ca(OH)₂ (10); (C) incubating the gel in 50 mM Tris-acetate, pH 4.5, containing 2 mM each of ATP and Pb(NO₃)₂ for 1-2 h to produce lead phosphate with subsequent substitution of sulfide for phosphate to form brown lead sulfide bands(1). The blue color formed by phosphomolybdate in method (A) is soluble in water and the bands diffuse quickly. The white calcium phosphate formed under alkaline condition in method (B) is soluble in acidic solution. Lead phosphate is a white precipitate insoluble in water and acid solutions of pH 4-5. Therefore, the first two methods were not suitable for detecting acid NSPase and method (C) was then adopted and simplified by not converting to lead sulfide. The enzyme bands were poorly stained by using the incubation solution described in (1). A further experiment was conducted to develop a suitable procedure for detecting acid NSPase. After electrophoresis, released tube gels were preincubated
in 3 mM ATP or other phosphate esters in the following solutions: (a) 50 mM KOAc buffer, pH5; (b) 0.1 M KOAc buffer, pH5; (c) 0.1 M KOAc buffer, pH5, containing 2 mM Mg(OAc)$_2$; (d) 0.1 M KOAc buffer, pH5, containing 2 mM CaCl$_2$; (e) 0.1 M NaOAc buffer, pH5; or (f) 0.2 M KOAc buffer, pH5. for 10 min in a 35 C water bath with shaking. Then 3 mM Pb(OAc)$_2$ was added to each tube, mixed well and continued incubation to observe color development. Within 10 min, sharp enzyme bands were evident in the 0.2 M KOAc solution only and the incubation was terminated by immersing the gel in water. After a 20 min incubation, the gel in solution (b) showed clearer banding patterns than the other incubation media. After 40 min most gels developed a white precipitate of lead phosphate at the enzyme bands and in the incubation solution. The gel in solution (a) showed weak enzyme bands but a lot of white precipitate in the incubation solution. Results of these tests indicate that: First, divalent cations such as Mg$^{2+}$ and Ca$^{2+}$ were not necessary for the acid NSPase activity and color development. Second, K$^+$ may be a better cofactor for the enzyme reaction than Na$^+$ since solution b was more efficient in staining the Pi-releasing enzyme than solution e. Third, the higher the KOAc buffer concentration (up to 0.2 M) the quicker the enzyme reaction and sharper the color banding was observed. In addition, the 0.2 M KOAc buffer solution showed little or no white precipitate in the reaction solution when the Pb(OAc)$_2$ was added into the preincubation medium. Perhaps the high osmotic concentration or ionic strength in the reaction solution prevented the outward diffusion of the reaction product, Pi, into the incubation solution and only inward diffusion of Pb was allowed during the 10 min staining time. Since the electro-
phoretic buffer contained 5 mM Tris and 38 mM glycine (Davis' method) and the gel would be in equilibrium with the buffer at the end of the run, the 10 min pre-incubation with substrate in 0.2 M KOAc may have increased the concentration of gel matrix. The difference may be significant enough and inward diffusion resulted. Thus in situ white bands were quickly and exclusively formed at the enzyme bands in gel. Therefore, 0.2 M KOAc buffer, pH5, and 3 mM Pb(OAc)$_2$ were selected for the enzyme detection and a total time of 20 min was the optimum for incubation and staining for sharp bands. After incubation, the gels were rinsed in several changes of water. In water the white precipitate can stay in situ for many days without diffusion of the bands.

RESULTS AND DISCUSSIONS

The activity of NSPase in crude endosperm extracts and the estimated endogenous concentration of inorganic phosphate of the four triticale lines are summarized in Table 2-1. Because of their close genetic pedigree (3) but different seed conformation (4), the activity ratio in pairs of shriveled to plump was calculated separately as 6TB/6TA and RBS/RBP. It is evident that shriveled endosperm collected at 7 to 8 weeks after anthesis contained more acid hydrolase activity than in plump ones as indicated by their activity ratio of 1.3 to 2.5. The ratios were also observed in the same lines harvested in 1982 and 1983 (4). The activity rate, nmole min$^{-1}$ endosperm$^{-1}$, in material harvested in 1984 (table 1) was on the average two orders magnitude higher than 1982 and 1983 (4) indicating that the pH 7.0
used for the latter may not have extracted all the acid phosphatases as completely as at pH 5 plus 0.5% Triton used in this study. Nevertheless, the higher phosphatase affinity toward the 8 substrates was uniformly expressed by the lines with shriveled seeds. These acid NSPases generally exhibited a comparable activity toward nucleoside triphosphates and ADP, a lesser affinity for 3PGA, and a low affinity toward AMP (Table 2-1).

ATP serves as energy for anabolic activities and for cellular maintenance. Other nucleotides serve as carriers for many synthetic functions. In addition, ATP and UTP are the substrates for the synthesis of ADP-G and UDP-G by ADP-glucose pyrophosphorylase (ADP-G PPase) and UDP-glucose pyrophosphorylase (UDP-G PPase), respectively, and in turn the products serve as substrates for starch synthesis in triticale (3). It is known that 3PGA is the effector of starch synthesis in cereal seeds (6). The stage specific high activity of nucleosidases and other phosphatases, and the differing isozymes in shriveled seed apparently resulted in reduced dry weight and less starch accumulation observed in this and previous reports (3, 4).

Based on the endosperm Pi content in extracts, 38 to 50 mM Pi was detected in plump endosperm of triticale lines (6TA, RBP) (Table 2-1). This concentration approximates the magnitude found in wheat endosperm of 82 mM at 40 DAA (days after anthesis) (9), but was higher than that in dicots, e.g. 6.3 mM at 40 DAA in peas (7) and 20 mM in rape seed at 50 DAA (2). The higher level of Pi in shriveled seed was probably attributed to the 1.3-2.5 fold higher activity of acid NSPase and other phosphatases (4). The calculated molar concentration of Pi in endosperm of shriveled seed lines was 3.5 fold higher than
in plump seeds. If the 133-173 mM Pi (6TB and RBS, respectively, Table 2-1) were uniformly distributed in the endosperm cytosol, the high Pi concentration probably would inhibit the activity of ADP-G PPase thereby reducing the supply of substrate for starch synthase, and causing seed shrivelness. This speculation is supported by the fact that purified corn endosperm ADP-G ppase activity was inhibited as much as 50% (I₀.₅) by 3 mM Pi in the absence of 3PGA and in the presence of 10 mM 3PGA the I₀.₅ was increased to 10 mM Pi (5,6). The specific role of 3PGA as an activator in the reaction of triticale endosperm ADP-G PPase was not well established; however, its stimulatory effect, was observed in the cell free extracts of endosperm collected at 7 to 9 WAA (4). The high 3-PGA phosphatase (3PGAPase) activity observed in shriveled endosperm (Table 2-1) would reduce its endogenous concentration, result in low ADP-G and starch synthesis, and contribute to seed shrivelness.

By using the staining method for phosphate-releasing enzymes described in the methods section, the isozyme patterns of ATPase, ADPase, CTPase and UTPase for each line of triticale were clearly defined (Fig.2-1). The stain of AMPase and 3PGAPase was weak by the procedure described and further improvement will be needed. The lines with plump seeds (6TA, RBP) exhibited the same isozyme patterns for ATPase, ADPase, UTPase and CTPase (Fig 2-1) in that both had 4 isozymes with band 3 and 4 being dominant. On the other hand, line 6TB with shriveled seed conformation had a different isozyme pattern with band 5 and 6 being prominent. RBS, another shriveled line, had bands 5 and 6 as in 6TB but with an additional band 3. Band 1 and 2 appear to be common for every line, and may be involved with normal
seed development. Bands 4, 5 and 6 should be the key enzymes for future purification and characterization in order to explore the molecular mechanism of seed shriveling.
Table 2-1. Acid phosphatase activities and endogenous inorganic phosphate concentration in two lines each producing plump (6TA, RBP) and shriveled seeds (6TB,RBS) of triticale collected 7 WAA.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>6TA</th>
<th>6TB</th>
<th>6TB/6TA</th>
<th>RBP</th>
<th>RBS</th>
<th>RBS/RBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNPP</td>
<td>210</td>
<td>266</td>
<td>1.26</td>
<td>182</td>
<td>271</td>
<td>1.49</td>
</tr>
<tr>
<td>ATP</td>
<td>186</td>
<td>287</td>
<td>1.54</td>
<td>161</td>
<td>290</td>
<td>1.80</td>
</tr>
<tr>
<td>GTP</td>
<td>204</td>
<td>262</td>
<td>1.28</td>
<td>147</td>
<td>330</td>
<td>2.24</td>
</tr>
<tr>
<td>CTP</td>
<td>206</td>
<td>302</td>
<td>1.47</td>
<td>167</td>
<td>277</td>
<td>1.66</td>
</tr>
<tr>
<td>UTP</td>
<td>210</td>
<td>281</td>
<td>1.34</td>
<td>157</td>
<td>270</td>
<td>1.71</td>
</tr>
<tr>
<td>ADP</td>
<td>189</td>
<td>278</td>
<td>1.47</td>
<td>226</td>
<td>316</td>
<td>1.39</td>
</tr>
<tr>
<td>AMP</td>
<td>41</td>
<td>60</td>
<td>1.46</td>
<td>59</td>
<td>94</td>
<td>1.59</td>
</tr>
<tr>
<td>3PGA</td>
<td>124</td>
<td>224</td>
<td>1.81</td>
<td>69</td>
<td>175</td>
<td>2.53</td>
</tr>
</tbody>
</table>

Endogenous Pi concentration endosperm⁻¹

<table>
<thead>
<tr>
<th></th>
<th>umole</th>
<th>mM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNPP</td>
<td>1.7</td>
<td>50</td>
</tr>
<tr>
<td>ATP</td>
<td>3.3</td>
<td>173</td>
</tr>
<tr>
<td>GTP</td>
<td>1.9</td>
<td>3.5</td>
</tr>
<tr>
<td>CTP</td>
<td>1.4</td>
<td>38</td>
</tr>
<tr>
<td>UTP</td>
<td>2.8</td>
<td>133</td>
</tr>
<tr>
<td>ADP</td>
<td>2.0</td>
<td>.5</td>
</tr>
</tbody>
</table>

+ Average of two replicated extractions of 4 assays, coefficient of variation § 20%.

* Average fresh weight per endosperm of 6TA, 6TB, RBP, RBS at 7 WAA was 76, 72, 68 and 58 mg, respectively. Average water content was 45, 26, 55 and 37%, respectively.
Figure 2-1. Isozymes of ATPase, ADPase, CTPase and UTPase in endosperm extracts of 7-week-old seed from 2 triticale lines each producing plump (6TA, RBP) or shriveled seed (6TB, RBS). Gels were charged with 30-35 ug of soluble protein (equivalent to 0.5 endosperm) and electrophoresed according Davis' method and stained by the method described in this paper. The electrophoresis time was increased by 30 min after the dye front had reached the end of tube in order to ascertain the relative position of the isozymes.
LITERATURE CITED


CHAPTER THREE

PHYSICAL AND KINETIC STUDIES OF PARTIALLY PURIFIED ACID PHOSPHATASES
FROM TRITICALE LINES PRODUCING PLUMP AND SHRIVELED SEEDS
ABSTRACT

Two endosperm acid phosphatases (EC 3.1.3.2.) each from triticale (XTricitosecale Wittmack) lines producing plump seeds (RBP) and shriveled seeds (RBS) were partially purified to study their physical and kinetic properties. The acid phosphatases designated as RBP-I and RBS-I were the corresponding isozymes moving slower on polyacrylamide gel (Rm 0.32) and eluted by 105 mM KCl from DEAE-Sephacel column; RBP-II and RBS-II were another corresponding isozymes moving faster on the gel (Rm 0.48), and eluted by 140 mM KCl from the anion exchanger column. The molecular weight of these native enzymes from RBP and RBS were estimated by gel filtration to be about 26,000 to 49,000. By chromatofocusing chromatography the isoelectric point of RBP-I, RBP-II, RBS-I and RBS-II were 5.3, 5.0, 4.7, and 4.3, respectively. RBP-II and RBS-II had the same pH optimum, heat stability, Km for p-nitrophynolphosphate (0.45 mM), and similar I_{50} for inorganic phosphate (2.8 mM and 4.4 mM respectively). On the other hand, RBP-I and RBS-I were very different in many of their kinetic properties. Specifically RBS-I has lower Km for p-nitrophenyl phosphate (0.21 mM) than RBP-I (0.57 mM) and nine times higher I_{50} for inorganic phosphate (32 mM) than RBP-I (3.6 mM). These results indicated that RBS-I, one of the many slow moving isozymes on polyacrylamide gel that may contribute in seed shriveling of triticale line RBS.
INTRODUCTION

APase, which catalyzes the hydrolysis of many phosphate esters, was found to be the major acid hydrolase involved in seed shriveling in triticale line producing shriveled seeds (3, 6). Eight discernable isozymes of APase could be observed on PAG from triticale endosperms. Five slow moving ones on PAG appeared to be related to the seed shrivelness in triticale (3). In order to explore the molecular mechanisms of seed shriveling, these slow moving isozymes of APase should be purified and their properties delineated. In this study, two easily separated APases have been partially purified and characterized from 7-week-old developing endosperm of triticale lines producing plump seeds (RBP) and shriveled seeds (RBS). The data provided some possible explanations in the tissue degradation, and the decline of starch accumulation by the endosperm APases in the line producing shriveled seeds.

MATERIALS AND METHODS

Plant Materials

Two Winter triticale lines, one producing plump seeds (Red Bobs// Daws/Snoopy-p(RBP)), and the other shriveled seeds (Red Bobs// Daws/Snoopy-s(RBS)), were used. Seeds were planted in Corvallis, OR in October 1983. In June of 1984 samples were collected and stored in a superfreezer at -70 C until used.

Enzyme Assay
Enzyme activity was routinely estimated by using pNPP as substrate. The standard reaction mixture contained 0.2 ml of 0.1 M KOAc buffer (pH 4.5) or 0.1 M MES buffer (pH 6.0) containing 7.6 mM pNPP and 50 ul enzyme preparation. After incubation for 10 min at 35 C, the reaction was stopped by adding 1 ml of 0.5 N NaOH, then the A at 400 nm was measured against the buffer blank. One unit of enzyme activity was defined as the amount which liberated 1 umole of pNPP per min. A molar extinction coefficient of 18,000 M$^{-1}$cm$^{-1}$ was used in the calculation. When inorganic phosphate released was measured, the incubation mixture was identical in quantitative aspects with that of above assay except different substrates were used. The reaction was stopped by adding 0.5 ml cold 20% TCA. The mixture was stand on ice for 10 min and centrifuged at 16,000g for 5 min, after which the supernatant was assayed for Pi, released by the enzyme, using the Fiske-Subbarow method (3). The 0 and 10 min incubated samples were read against the reagent blank at 660 nm, and the difference was used to calculate the enzyme activity. Under our assay condition, one absorbance unit at 660 nm equaled 207 nmole of Pi in a total 0.75 ml reaction mixture.

Protein Determination

Protein concentration was estimated by the method of Bradford (2) using bovine serum albumin as the standard.

Molecular Weight Estimation

A Sephacryl S-300 column (1.5 X 53 cm) was calibrated using phosphorylase a (495,000 dalton), pyruvate kinase (237,000), glucose-
6-phosphate dehydrogenase (128,000) and cytochrome c (13,200) as the MW markers. The void volume of the column was determined using blue dextran 2000. The MW of the unknown protein was estimated following the method of Andrews (1).

**Electrophoresis and Detection of Acid Phosphatase**

Enzymes were subjected to electrophoresis in a 7.5% PAG (Bio-Rad) using Davis' method (4). Acid phosphatase was stained by the method of Scandalios (12) using alpha-naphthyl acid phosphate as substrate and fast Garnet GBC salt as dye and incubated at 30°C for 30 min. The gel was then destained in 50% ethanol for 30 min and fixed in 7% acetic acid. Protein bands were stained by Coomassie blue G 250.

**Action of Effector**

Fifty ul of partially purified enzyme containing 1 ug protein was first mixed with 30 ul of stock solution of a specific compound (this was prepared so that a final concentration of that specific compound in incubation mixture reached as listed in Table 3-3) and then 220 ul of 7.6 mM pNPP in 0.1 M MES buffer pH 6.0 or acetate buffer, pH 4.5 was added. After incubation at 35°C for 10 min, the reaction was stopped by adding 1 ml 0.5 N NaOH, then absorbance at 400 nm was measured against a respective substrate blank.

**pH optimum**

The pNPP-hydrolyzing activity at various pH was determined under the standard assay condition using 0.1 M buffer of different pH
value: pH 3-4, lactate; pH 4.5-5, acetate; pH 5.5-6, MES; pH 6.5-7, HEPES; pH 8-9, Tris-HCl.

Substrate Specificity

The rates of hydrolyzing several substrates catalyzed by the partially purified APase were compared with the rates of hydrolyzing pNPP, a reference substrate. The substrates were uniformly tested at 5 mM concentration. The release of pNP or inorganic phosphate was measured as described in "enzyme assay" section.

Michaelis constant

The Km and Vmax for pNPP were determined from Lineweaver-Burk reciprocal plots (13).

Effect of Inorganic Phosphate

Pi was tested over a range of 10 concentrations. The incubation mixture contained 50 ul enzyme (1 ug protein), and 0.2 ml of 7.6 mM pNPP in 0.1 M MES buffer pH 6.0 (or 0.1 M acetate buffer, pH 4.5) containing specific concentration of Pi. The assay was conducted under the standard condition. A plot of log (% inhibition) versus log (inhibitor concentration) yielded a straight line from which the inorganic phosphate concentration required to inhibit the activity of APase by 50 % was determined (11).

Isolation and Purification of Acid Phosphatases

All procedures, unless otherwise noted, were carried out at 0 to 4 C.
Step 1. Crude Extract  Peeled endosperm of 7-week-old developing triticale seeds and 1/3 weight of buffer saturated insoluble polyvinyl pyrrilidone (PVPP) and polystyrene-divinylbenzene (XAD-4) (1:1 by weight) were homogenized in 10 mM KOAc, pH 5.0, containing 0.2 M KCl and 14 mM ME in a mortar with pestle. The homogenate was centrifuged at 30,000 g for 20 min. The extraction was repeated 2 times, and the resulting supernatants combined (Fraction 1).

Step 2. \((\text{NH}_4)_2\text{SO}_4\) Precipitation.  To Fraction 1, solid ammonium sulfate (AS) was slowly added with stirring to give 25% saturation. After standing for 1 hour, the resulting precipitate was collected by centrifugation at 16,000 g for 15 min and discarded. Solid ammonium sulfate was added to the supernatant to give a final concentration of 65% saturation. After standing for 3 hours, the sample was centrifuged as before and the resulting precipitate was dissolved in 10 mM Tris-HCl buffer, pH 7.5, containing 50 mM KCl and 14 mM ME (buffer A). This preparation was dialyzed overnight against buffer A. The dialysate was clarified by centrifugation at 16,000 g for 15 min to yield the Fraction 2.

Step 3. DEAE-Sephacel Column Chromatography  Fraction 2 was applied to a DEAE-Sephacel column (1.5 x 21 cm) previously equilibrated with buffer A. The column was washed with the same buffer until the protein in eluate was negligible. The wash was discarded. Then the charged column was eluted with 300 ml of a linear gradient of KCl 50 mM - 260 mM in 10 mM Tris-HCl buffer, pH 7.5, containing 14 mM ME. The APase were resolved into 3 major peaks by the linear KCl gradient for both RBP (Fig. 3-1, upper) and RBS (Fig.3-2). First two peaks of RBP were combined, concentrated by
 ultrafiltration, and resolved by chromatofocusing below (Fig. 3-1, lower). Peaks of RBS were collected separately, concentrated by ultrafiltration, and further purified by chromatofocusing below (Fig. 3-3). The two corresponding APase activity peaks eluted down from DEAE-Sephacel column by 105 mM KCl and 140 mM KCl were designated as I and II.

**Step 4. Chromatofocusing Column Chromatography** Each of the two peak fractions was applied separately to a column filled with 15 ml (1 X 20 cm) of Polybuffer exchanger (PBE 94, a basic ion exchanger resin) (Pharmacia). Column resins and protein solution were equilibrated with 0.025 M histidine-HCl buffer, pH 6.2 (start buffer). Following sample application, eluent buffer (Polybuffer 74 diluted 10 times with distilled water, pH 4.0, containing 14 mM ME) was pumped through the column to generate a pH gradient from pH 6 to 4 (Pharmacia Fine Chemicals 1980) and eluate was collected, 1.5 ml/tube. Contents of alternate tube were checked for protein, APase activity and pH value.

**RESULTS AND DISCUSSION**

The principle aim of this study was to compare the physical and kinetic properties of different acid phosphatase isozymes from triticale genetic line producing plump seeds (RBP) and shriveled seeds (RBS). Thus the representative isozymes were purified to the extent that their properties could be studied.

A summary of various steps employed in the purification of endosperm acid phosphatases from both RBP and RBS are shown in Table
3-1 and 3-2. None of these APase was absolute homogeneous, but only few protein bands were observed in SDS-PAG for the partially purified APase isozymes and the major protein bands were comparable in Rm with activity bands.

The specific activity of different isozymes had been increased from 5 to 36 times in the partially purified APases. The combination of PVPP and XAD-4 was added in step 1 to remove phenolic compounds that complex with enzymes and cause difficulty in enzyme purification work (8). In the absence of PVPP and XAD-4, the enzyme solution in later stage of purification, especially after ultrafiltration, exhibited a dark grayish color and had a $A_{260}/A_{280}$ of 1.1 indicating high content of phenolic compound (8). AS at 25% saturation removed more than 20% of the total protein and only 10% of the total activity. APase activity left in the supernatant of AS 60% saturation supernatant was less than 10%.

By checking the partially purified isozyme activity stained on PAG (Figure 3-4), RBP-I and RBS-I were corresponding isozyme in Rm at 0.32 and RBP-II and RBS-II were comparable in Rm at 0.48. Isozyme I was eluted down from DEAE-Sephacel column by 105 mM KCl, whereas isozyme II by 140 mM KCl. Only these 2 pairs of isozymes were used for further comparative characterization as other isozymes were not corresponding pair or too complicate in protein components.

Molecular Weight and Isoelectric Point The apparent MWs of native APases were estimated to be between 26,000 to 49,000 (Figure 3-5) by gel filtration on Sephacryl S-300 column. These low MWs probably indicated that APases in triticale endosperm were not in polymeric structures. APase of low MW probably is a characteristics
of the Graminae family. Purified APase from Poa pratensis had MW of 33,000 (9), from wheat germ 55,000 (14), and from rice grains 68,000 (15). Even though RBP-I and RBP-II as well as RBS-I and RBS-II were quite similar in MW but they were distinctly separated on PAG indicating that they were quite different in charges.

RBS-I and RBP-I each contained 2 bands on PAG (Fig.3-4) that were not separated by the purification procedure used, but could be resolved partially by the gel filtration column into overlapping fractions. Their peak tubes were used for the MWs estimation as indicated in Figure 3-5. RBS-I(f), the fast moving isozyme on PAG had MW 49,000. RBS-I(s), the slow moving isozyme on gel, had MW 26,000. RBP-I(f) had MW 43,000 and RBP-I(s) 30,000. Because of the small quantities obtained at this step of purification, RBS-I(f) and RBS-I(s) as well as RBP-I(f) and RBP-I(s) were combined and characterized together.

The isoelectric point was determined by chromatofocusing column chromatography (Figure 3-1 and 3-3). Isozymes of RBS generally had lower isoelectric points than those of RBP. RBS-I and RBS-II had isoelectric point (pI) of 4.8 and 4.3, respectively. RBP-I, and RBP-II had isoelectric points of 5.3 and 5.0, respectively.

**pH Optimum** All the APase isozymes exhibited their pH optimum in the acidic condition. RBP-I and RBP-II both reached maximum activity at pH 6, RBS-II at pH 6.5, and RBS-I at 4.5 to 5.5 (Figure 3-6).

**Action of Effectors** These isozymes of APase preparations were compared with respect to their sensitivities to various compounds. The enzymes were mixed first with these compounds
respectively and then the activity was assayed (Table 3-3).

APases were almost completely inhibited by 0.1 mM molybdate and 10 mM inorganic phosphate except RBS-I which was inhibited by 60%. RBP-II was clearly distinguished from other APases by its high sensitivity to inhibition by CuSO₄. All the APases were not activated by metal ions such as Mg²⁺ and Ca²⁺ nor influenced by the metal chelator EDTA.

**Substrate Specificity**
The substrate specificity of these isozymes of APase in RBP and RBS were compared (Table 3-4). pNPP is the best substrate for RBS but not for RBP. Generally all these isozymes of APase showed high affinity for physiological substrates such as ATP, ADP and UTP, and low affinity for 3PGA and AMP. G1P, another physiological substrate, also was an effective substrate for these isozymes especially RBP, even though the crude APase activity toward substrate G1P was reported to be quite low or nondetectable (3, 7). The partially purified APase showed no affinity toward lecithin, which is a substrate for acid phospholipid phosphatase (PLPase). This indicates that these partially purified isozymes of APase probably were not the ones hydrolyzing phospholipid phosphates. Even though their activity could be eluted down from DEAE-Sephacel column by similar ionic strength of KCl for both RBP and RBS (6), the purification steps were based on pNPPase thus PLPase was discriminatedly excluded.

**Thermal Stability**
The thermal stability of APase isozymes in RBP and RBS was determined by incubating the enzyme in 10 mM Tris-HCl buffer, pH 7.5 for 15 min at the specific temperature. After cooling on ice, the remaining activity of the enzyme was assayed. The results
(Figure 3-7) showed that the enzyme was stable for at least 15 min at 55-60 C. RBP-II and RBS-II have the same thermal stability. But the other pair, RBP-I was very stable up to 65 C, however RBS-I had only 50% of the activity left after 15 min at 65 C.

**Km and Vmax** Kinetic studies were conducted using the partially purified isozymes obtained from both RBP and RBS. The chromatofocused eluets were used. The data were summarized in Table 3-5.

The Lineweaver-Burk reciprocal plots were presented in Figure 3-8 and 3-9. RBP-II and RBS-II have similar Km, but 10 times higher of Vmax for RBS-II, indicating much higher rate of degrading in shriveled seeds. On the other hand, RBP-I and RBS-I are similar in their Vmax but very different in their Km. This lower Km (0.21 mM) in RBS-I allows this isozyme to degrade the phosphate esters at lower concentration in situ. Thus this isozyme may reduce endogenous substrates and energy compounds much more efficiently than that of RBP-I.

**Inhibition of Inorganic Phosphate** The partially purified APase preparations for both RBP and RBS were compared with respect to their sensitivities to physiological inhibitor, inorganic phosphate (Table 3-6). The results showed that the RBP-II and RBS-II having $I_{50}$ for Pi at the same order of magnitude, though the isozyme from the shriveled seeds being 60% higher in concentration. The other pair, RBP-I and RBS-I showed nine times difference of the Pi concentration needed to cause 50% inhibition. These results indicate that RBS-I could function well at Pi concentration of 30 mM, at which RBP-I would be completely inhibited. The Pi concentration in plump seeds at later stage of seed development was found to be 38 and 50 mM, and 133
and 173 mM in shriveled seeds (Chapter 2; 7). If RBS-I were the only APase in shiveled seeds, no phosphate esters would be hydrolyzed and the starch accumulation probably would not be halted. Since there are many APases, the in situ implication of $I_{50}$ by Pi is not clear at the moment.

In the previous study many fast moving APase isozymes, including RBP-II and RBS-II, were found to be common to lines producing plump seeds as well as shrieveled seeds, and they may be involved in normal seed development, cell differentiation and seed maturation. The slow moving isozymes on PAG, including RBS-I and RBP-1, were specifically high in activity at later stages of seed development in lines producing shrieveled seeds (3). With their other hydrolytic actions (7), they may be the major contributors to seed shrivelying in triticale. This speculation is substantiated by the results of this study. RBP-II and RBS-II have the similarity in molecular mobility on PAG, pH optimum, inhibition by molybdate and Pi, pattern of thermal stability, $K_m$ for pNPP, and $I_{50}$ for Pi. On the other hand, RBP-I has pH optimum at pH 6 but RBS-I between pH 4.5 to 5.5; RBP-I was inhibited much more by molybdate at 0.1 mM and phosphate at 10 mM than that of RBS-I; RBP-I is heat stable up to 65 C but RBS-I only to 55 C; RBP-I has $K_m$ value of 0.57 mM for pNPP, but RBS-I only 0.21 mM; the concentration of inorganic phosphate needed to cause 50% inhibition of the RBS-I is nine times higher than of RBP-I. These differences observed between RBP-I and RBS-I indicated that RBS-I has higher affinity for phosphate esters to degrade more or faster of high energy compounds (ATP, ADP), to hydrolyze more primary substrates for starch synthesis (G1P, ATP, UTP), and to remove the effector for
ADP-glucose pyrophosphorylase (3PGA). In addition to these, RBS-I is able to function at high inorganic phosphate environment which is commonly existing in lines producing shriveled seeds (7), whereas RBP-II might be constantly inhibited in situ and little tissue damage could be resulted.

In this study, the kinetic properties of only one each of the slow and fast moving isozymes of APase were compared between the plump and shriveled seeds. There are many others should be purified and characterized in order to explore the biochemical mechanisms of seed shrivelness in triticale.

Because of the small quantities obtained (Table 3-2), the purity of these isozymes were not vigorously determined. By SDS-PAGE and Coomassie blue stain, they appeared to have the major protein band coincident with the activity band. Further purification will be needed to verify the properties, and for molecular biological studies to discern whether these isozymes are the expression of structural genes or they are originated from post translational modification of a single polypeptide chain (5).
Table 3-1. Results of purification steps on acid phosphatases from 7-week-old endosperm of RBP.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol (ml)</th>
<th>Total Activity (units)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (units/mg)</th>
<th>Purification -fold</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. crude 1 extract</td>
<td>38.7</td>
<td>5156</td>
<td>62.8</td>
<td>82.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. AS 25-60%</td>
<td>18.2</td>
<td>4124</td>
<td>55.0</td>
<td>75.0</td>
<td>0.9</td>
<td>80</td>
</tr>
<tr>
<td>3. DEAE-Sephacel I</td>
<td>18.5</td>
<td>1300</td>
<td>8.8</td>
<td>148</td>
<td>1.8</td>
<td>25</td>
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<tr>
<td>4. DEAE-Sephacel II</td>
<td>I</td>
<td>12.5</td>
<td>750</td>
<td>4.9</td>
<td>153</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>10.8</td>
<td>292</td>
<td>1.3</td>
<td>225</td>
<td>2.7</td>
</tr>
<tr>
<td>5. Chromatofocusing (pH 7-4)</td>
<td>I</td>
<td>13</td>
<td>261</td>
<td>.568</td>
<td>459</td>
<td>5.6</td>
</tr>
</tbody>
</table>

1. 15 gm peeled endosperm.
2. Activity assayed at pH 6.0.
### Table 3-2. Results of purification steps on APase from 7-week-old endosperm of RBS.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>vol ml</th>
<th>total activity units</th>
<th>total protein mg</th>
<th>specific activity units/mg</th>
<th>purification fold</th>
<th>yield %</th>
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<tr>
<td>1.crudef</td>
<td>46</td>
<td>15670²</td>
<td>102.6</td>
<td>153</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.AS(20-65%)</td>
<td>45</td>
<td>10563²</td>
<td>39.7</td>
<td>266</td>
<td>1.74</td>
<td>67.4</td>
</tr>
<tr>
<td>3.DEAE-Sephacel</td>
<td></td>
<td></td>
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<tr>
<td>I</td>
<td>12.7</td>
<td>503³</td>
<td>2.2</td>
<td>228</td>
<td>1.32</td>
<td>3.2</td>
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<td>16</td>
<td>2928²</td>
<td>5.0</td>
<td>586</td>
<td>3.83</td>
<td>18.7</td>
</tr>
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<td>4.Chromatofocusing 1 (pH 7-4).</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>13.5</td>
<td>421³</td>
<td>3.5</td>
<td>120</td>
<td>0.8</td>
<td>2.7</td>
</tr>
<tr>
<td>II</td>
<td>25</td>
<td>1222²</td>
<td>.97</td>
<td>1262</td>
<td>8.3</td>
<td>7.8</td>
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<td>5.Chromatofocusing 2 (pH 6-4).</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>I</td>
<td>18</td>
<td>136³</td>
<td>.157</td>
<td>866</td>
<td>5.6</td>
<td>0.8</td>
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<tr>
<td>II</td>
<td>20</td>
<td>625²</td>
<td>.113</td>
<td>5531</td>
<td>36.2</td>
<td>4.0</td>
</tr>
</tbody>
</table>

1. 15 gm peeled endosperm.
2. Assay at pH 6.0.
3. Assay at pH 4.5.
Table 3-3. The effect of various compounds on the activity of two isozymes of APase from plump (RBP) and shriveled seeds (RBS) of triticale lines using pNPP as substrate.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Final Concentration mM</th>
<th>RBP-I % control</th>
<th>RBP-II % control</th>
<th>RBS-I % control</th>
<th>RBS-II % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>molybdate</td>
<td>0.1</td>
<td>4</td>
<td>5</td>
<td>38</td>
<td>9</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>10</td>
<td>9</td>
<td>15</td>
<td>34</td>
<td>11</td>
</tr>
<tr>
<td>citrate</td>
<td>40</td>
<td>73</td>
<td>111</td>
<td>98</td>
<td>68</td>
</tr>
<tr>
<td>EDTA</td>
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<td>100</td>
<td>107</td>
<td>121</td>
<td>104</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1</td>
<td>80</td>
<td>97</td>
<td>119</td>
<td>105</td>
</tr>
<tr>
<td>Pb(OAc)₂</td>
<td>1</td>
<td>100</td>
<td>92</td>
<td>67</td>
<td>81</td>
</tr>
<tr>
<td>CaCl₂</td>
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<td>104</td>
<td>113</td>
<td>112</td>
<td>102</td>
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<tr>
<td>HgCl₂</td>
<td>0.1</td>
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<td>93</td>
<td>98</td>
<td>87</td>
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<tr>
<td>Na tartrate</td>
<td>1</td>
<td>79</td>
<td>114</td>
<td>117</td>
<td>111</td>
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<tr>
<td>NaF</td>
<td>5</td>
<td>65</td>
<td>97</td>
<td>44</td>
<td>53</td>
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<tr>
<td>FeSO₄</td>
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<td>98</td>
<td>123</td>
<td>146</td>
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<tr>
<td>Ascorbate</td>
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<td>54</td>
<td>116</td>
<td>112</td>
<td>113</td>
</tr>
<tr>
<td>CuSO₄</td>
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<td>91</td>
<td>3</td>
<td>66</td>
<td>95</td>
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<tr>
<td>ZnSO₄</td>
<td>1</td>
<td>66</td>
<td>53</td>
<td>98</td>
<td>110</td>
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</table>
Table 3-4. Substrate specificity of two isozymes of APase from plump (RBP) and shriveled seeds (RBS) of triticale lines. The substrates were tested at 5 mM concentration. The rate of pNP released was 2.5, 1.6, 1.9 and 16.5 nmole/min/ug protein for RBP-I, RBP-II, RBS-I and RBS-II, respectively. These rates were taken as 100 and the other values are indicated as a percentage of this value. (assayed in 0.1 M MES buffer, pH 6.0)

<table>
<thead>
<tr>
<th>substrate</th>
<th>RBP-I</th>
<th>RBP-II</th>
<th>RBS-I</th>
<th>RBS-II</th>
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</thead>
<tbody>
<tr>
<td>pNPP</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NAP</td>
<td>50</td>
<td>12</td>
<td>94</td>
<td>56</td>
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<tr>
<td>lecithin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ATP</td>
<td>128</td>
<td>208</td>
<td>72</td>
<td>74</td>
</tr>
<tr>
<td>ADP</td>
<td>190</td>
<td>111</td>
<td>81</td>
<td>88</td>
</tr>
<tr>
<td>AMP</td>
<td>18</td>
<td>8</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>UTP</td>
<td>118</td>
<td>78</td>
<td>64</td>
<td>72</td>
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<tr>
<td>PGA</td>
<td>38</td>
<td>10</td>
<td>33</td>
<td>9</td>
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<tr>
<td>G1P</td>
<td>115</td>
<td>144</td>
<td>23</td>
<td>34</td>
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</tbody>
</table>
Table 3-5. Kinetic parameters of two isozymes of APase from plump (RBP) and shriveled (RBS) seeds of triticale lines. (pNPP as the substrate)

<table>
<thead>
<tr>
<th></th>
<th>RBP-I&lt;sup&gt;1&lt;/sup&gt;</th>
<th>RBP-II&lt;sup&gt;1&lt;/sup&gt;</th>
<th>RBS-I&lt;sup&gt;2&lt;/sup&gt;</th>
<th>RBS-II&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km (mM)</td>
<td>0.57</td>
<td>0.45</td>
<td>0.21</td>
<td>0.45</td>
</tr>
<tr>
<td>Vmax (umole/min.mg)</td>
<td>2.70</td>
<td>1.25</td>
<td>2.86</td>
<td>15.1</td>
</tr>
</tbody>
</table>

1. Assayed at pH 6.0.  2. Assayed at pH 4.5.
Table 3-6. The effect of inorganic phosphate on the activity of two each of partially purified APase from RBP and RBS, using pNPP as substrate.

<table>
<thead>
<tr>
<th>RBP-I$^1$</th>
<th>RBP-II$^1$</th>
<th>RBS-I$^2$</th>
<th>RBS-II$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6</td>
<td>2.8</td>
<td>32</td>
<td>4.4</td>
</tr>
</tbody>
</table>

I$_{50}$ of Pi in mM

1. Assayed at pH 6.0.
2. Assayed at pH 4.5.
Figure 3-1. DEAE-Sepharose chromatographic profile (upper) and chromatofocusing profile (lower) of APase activity and protein from RBP. The flow rate for DEAE-Sepharose column was 3 ml per 10 min and 1.5 ml per 10 min for CF column.
Figure 3-2. DEAE-Sephacel chromatographic profile of APase activity and protein content of RBS extract.
Figure 3-3. Chromatofocusing profile of APase activity and protein content of RBS extract separated by DEAE-Sephacel.
Figure 3-4. Polyacrylamide gel electrophoresis of total isozymes of endosperm APase from RBP and RBS, and selected partially purified isozymes.
Figure 3-5. Molecular weight estimation of APase isozymes from triticale endosperm on Sephacryl S-300 column (1.5 X 53 cm). Molecular weight markers: 1. phosphorylase a (495,000); 2. pyruvate kinase (237,000); 3. glucose 6 phosphate dehydrogenase (128,000); 4. cytochrome c (13,200).
Figure 3-6. The pH optimum of APase isozymes activity by using pNPP as the substrate.
Figure 3-7. Thermal stability of APase isozymes of RBP and RBS. The enzyme was preincubated for 15 min at different temperatures and cooled on ice. The activity was then measured under the standard assay conditions.
Figure 3-8. Lineweaver-Burk reciprocal plots of APase activity of RBP as a function of pNPP concentrations. The reaction condition was described in "materials and methods" except that the concentration of pNPP was varied as indicated.
Figure 3-9. Lineweaver-Burk reciprocal plots of APase activity of RBS as a function of pNPP concentrations. The reaction condition was described in "materials and methods" except that the concentration of pNPP was varied as indicated.
LITERATURE CITED


CONCLUSION

The endosperm acid hydrolases were found previously to be related to seed shriveling in the man-made hybrid of wheat and rye, triticale. In order to explore the mechanism of seed shriveling, isolation, purification and characterization of these acid hydrolases were attempted in this study.

Crude extracts of 7-week old developing endosperms from two each of triticale genetic lines producing plump (6TA, RBP) and shriveled seeds (6TB, RBS) were successfully separated into five fractions of acid hydrolases by DEAE-Sephacel column chromatography.

The run off fraction was 6-fold higher in acid phosphatase activity in shriveled seeds than in plump seeds. This group of acid phosphatases contained several isozymes with slower mobility (Rm 0.20 to 0.40) on polyacrylamide gel. The results confirmed a previous report (Ching et al. 1984) that these isozymes reached highest activity especially at the later stages of seed development in lines producing shriveled kernels. Complete removal of Triton X-100 was difficult, and the protein components were too complicated to purify for this fraction.

Fraction II exhibited low acid hydrolase activity for each genetic line except RBS in which high acid phosphatase activity was found. This fraction was unique to each line and appeared to be not absolutely related to seed shriveling. Thus further study was not attempted.

Fraction III had higher ATPase and phospholipid phosphatase activity in shriveled seeds than in plump ones. In order to monitor
the efficiency of purification steps, a polyacrylamide gel detection method was developed for ATPase detection. This fraction also contained acid phosphatase, and was chosen for further purification.

The fraction IV contained 3.5-fold higher carboxypeptidase activity in shriveled seeds than in plump ones. This indicated that more soluble metabolically active proteins probably were degraded by the carboxypeptidase in lines producing shriveled seeds. Even though the protein quantity was high in this fraction, the specific enzyme quantity was too low to do purification study.

Fraction V was mainly membrane proteins and not related to seed shriveling, thus it was not studied further.

Two pairs of corresponding acid phosphatase isozymes each from RBP and RBS were partially purified and were compared for their physical and kinetic properties. RBP-II and RBS-II, corresponding pair and fast moving on gel had similar kinetic properties. RBP-I and RBS-I, another corresponding pair and slower moving on gel, were quite different. RBS-I had lower Km for p-nitrophenyl phosphate than for plump seed (RBP-I) so the enzyme in shriveled seeds was able to degrade more substrates for starch synthesis (ATP, UTP, glucose-1-phosphate), to hydrolyze more energy compounds (ATP, ADP), and to reduce the effector for ADP-glucose pyrophosphorylase (3PGA). In addition, the slow moving acid phosphatase had a higher I_{50} for Pi so the acid phosphatase could function well under higher endogenous Pi concentration, whereas the enzyme from plump seeds was completely inhibited. These in situ functional differences may have resulted in morphological variation in seed conformation.

Purified RBS-I and RBP-I will, however, be needed for
determining their physical structures, making antibodies for the enzyme quantification in endosperm and for isolation of their specific mRNAs' from which cDNAs may be synthesized and cloned. The cloned cDNAs' may be used to probe their genomic origin to determine whether these enzymes are the expression of different structural genes or they are the products of post translational modification of a single polypeptide chain so that the mechanism of seed shriveling in triticale could be discerned.
BIBLIOGRAPHY


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Lin, TP, TM Ching, RJ Metzger 1985b Acid nucleoside phosphatases in shriveled and plump triticale seeds. (submitted to Plant Physiology).


