GIBBERELLIC ACID-STIMULATED SYNTHESIS OF A PARTICULAR ISOZYME OF ACID PHOSPHATASE IN WHEAT SEED

by

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General introduction

Induction of hydrolytic enzymes by gibberellic acid (GA_3) in aleurone layer tissue of gramineous plant seeds is one of the interesting problems in germination physiology and has been studied actively.

Relation between GA_3 and seed germination was first reported for mobilization of storage materials in barley endosperm (Paleg 1960, Yomo 1960). Later on, it was established that GA_3 induces de novo synthesis of α-amylase (Filner and Varner 1967), ribonuclease and β-1,3-glucanase (Bennet and Chrispeels 1972) and protease (Jacobsen and Varner 1976) in barley aleurone layers. The activities of other kinds of enzymes are also known to be increased by GA_3 application. For instance, acid phosphatase in barley aleurone layers is enhanced by GA_3 treatment (Pollard and Singh 1968, Jones 1969). Although GA_3-induced synthesis of α-amylase in barley aleurone layers has been well studied at the molecular level, little has been reported about the mode of GA_3 action in the activity enhancement of enzymes like acid phosphatase which is detectable in this tissue even before GA_3 treatment. The purpose of this thesis is to broaden our knowledge of the mode of hormonal action by elucidating biochemical events during GA_3-induced change in acid phosphatase activity in wheat seed and release from it.

In Chapter I, the author demonstrates that a measurable

amount of acid phosphatase occurs in dry wheat seeds and that GA_3 induces a considerable enhancement of the enzyme activity in embryoless wheat half-seeds and its release into the incubation medium. The results of fractionation experiments of acid phosphatase preparation from half-seeds unincubated and incubated in the presence or absence of GA_3, and some basic properties of each enzyme fraction and molecular forms in it are also discussed.

If these molecular forms of acid phosphatase behave differrently from one another in response to GA_3, this enzyme would be a suitable object to study the mode of enzyme secretion from aleurone layers as well as that of enzyme synthesis or activation. Effects of RNA and protein synthesis inhibitors and abscisic acid known as an anti-gibberellin on GA_3-induced activity enhancement of acid phosphatase in wheat half-seeds and the enzyme release into the incubation medium are dealt in Chapter II.

Histochemical investigation carried out by Gahan et al. (1979) indicates that acid phosphatase is localized mainly in the cytoplasm of aleurone cells of unimbibed wheat seeds. In Chapter III, localization of acid phosphatase in wheat aleurone tissue is re-examined cytochemically and, on the other hand, possible effects of GA_3 on the change of the enzyme activity associated with particular fractions are studied using a density gradient centrifugation.

Studies in the previous chapters suggest that GA_3 stimulates the acidic fraction of acid phosphatase. However, it is still not clear whether the enzyme is activated or synthesized de novo in response to GA_3. In Chapter IV, by use of gel isoelectrofocusing technique, the author reveals the occurence of more than 9 isozymes of acid phosphatase in wheat endosperm and the GA_3-induced increase in activity of a particular isozyme with isoelectric pH of 4.0 (pI 4 isozyme). To employ for examining whether this enzyme is activated or synthesized de novo on treatment with GA_3, improvement of the procedure for isolation of pI 4 isozyme is attempted in Chapter V.

The author reports, in the Chapter VI, the evidence suggesting that GA_3 stimulates de novo synthesis of pI 4 isozyme of acid phosphatase but not affects RNA synthesis responsible for this isozyme.

CHAPTER I

ACTIVATION AND RELEASE OF ACID PHOSPHATASE IN WHEAT HALF-SEEDS TREATED WITH GIBBERELLIC ACID

Chapter I

Activation and release of acid phosphatase in wheat half-seeds treated with gibberellic acid

Abstract

Incubation of wheat half-seed with gibberellic acid (GA_3) resulted in an increase in the total acid phosphatase activity by about twice the control without GA_3. The bulk of acid phosphatase activity increased in response to GA_3 was found in the incubation medium. Acid phosphatase in half-seed was separated into two main fractions with CM-cellulose column. The first and second fractions eluted at 18 mM and 145 mM NaCl, respectively, were distinct in their relative activity toward various substrates and optimum pH. Both the fractions were released into the incubation medium by GA_3 treatment. The similar fractions from released acid phosphatase consisted of one or two molecular forms separable by polyacrylamide gel electrophoresis. From experiments of [^{3}H]-leucine incorporation into these molecular forms of acid phosphatase, some particular forms of the enzyme seemed to be newly synthesized and released into the medium in response to GA_3.

Introduction

It has been established that gibberellic acid (GA_3) induced the de novo synthesis of α-amylase (Filner and Varner 1967), ribonuclease and β-1,3-glucanase (Bennet and Chrispeels 1972), and protease (Jacobsen and Varner 1967) in barley aleurone layer. The activities of other enzymes, e.g., acid phosphatase (Pollard and Singh 1968, Pollard 1969, Jones 1969, Bailey et al. 1976), phosphoryl choline cytidyl transferase and phosphoryl choline glyceride transferase (Johnson and Kende 1971), are actually enhanced by GA_3 treatment in barley aleurone layer, but it is still not clear whether the enzymes are activated or synthesized de novo in response to GA_3.

From the histochemical study, Ashford and Jacobsen (1974) reported that acid phosphatase activity increased in cytoplasm of barley aleurone cells in response to GA_3. Bailey et al. (1976) reported promotion by GA_3 of the increase in total acid phosphatase activity and the enzyme release in barley aleurone layer. Recently, Gahan et al. (1979) have indicated cytochemically that nucleotide pyrophosphatase is localized in the cytoplasm, whereas general acid phosphatase in both the cytoplasm and cell walls of wheat aleurone cells.

Very little is known, however, about enhancement and release of acid phosphatase activity in response to GA_3 in wheat seed. This paper reports effects of GA_3 on the

enhancement and the release of several forms of acid phosphatase in wheat half-seed and some properties of these enzyme components.

Materials and Methods

Seed preparation and incubation

Wheat seeds (Triticum aestivum L. cv. Nohrin No. 61) harvested in 1977, were cut in half transversely with a razor blade. The embryoless half-seeds were sterilized in 1 % sodium hypochlorite for 20 min and rinsed more than 10 times with sterile distilled water. One hundred sterilized half-seeds were preincubated in a 200 ml-flask containing 5 ml of sterile distilled water for 24 hr at 24 °C by reciprocal shaking (120 strokes/min). After preincubation 10 or 100 half-seeds were placed aseptically into a sterile vial (3 cm in diameter, 6 cm in length) containing 1 ml of medium or 200 ml-flask containing 5 ml of medium. The medium consisted of 2 mM sodium acetate buffer (pH 5.2) and, if added, 1.25 μCi/ml [3,4,5-^{3}H]-leucine (1 Ci/mmole) and 10^{-5} M GA_3. The medium was sterilized using a Millipore filter (0.45 μm). The vials or flasks containing half-seeds were shaken for 24 hr or 48 hr at 24 °C.

Enzyme preparation

In time course experiments, the filtered medium was diluted to 5 ml with 50 mM sodium acetate buffer (pH 5.2). Incubated half-seeds were washed and then homogenized with a mortar and pestle in 5 ml per 10 half-seeds of the same acetate buffer. The homogenate and the diluted medium were

centrifuged at 20,000 x g for 20 min. The supernatants were used directly for acid phosphatase assay. For CM-cellulose column chromatography, 400 half-seeds were chopped in a Waring blender at a gentle speed (3,000 rpm) and the bulk of endosperm starch was removed by filtration through a nylon gauze. The chopped aleurone layers were further homogenized with a mortar and pestle in 50 mM sodium acetate buffer (pH 5.2). The homogenate and the medium were centrifuged at 20,000 x g for 20 min. The supernatants were brought to 75 % satulation with solid ammonium sulfate, stirred at least for 60 min and centrifuged at 20,000 x g for 20 min. The precipitate was dissolved in 10 ml of 50 mM sodium acetate buffer (pH 5.2). After dialysis against 4 l of 4 mM sodium acetate buffer (pH 5.2) for 20 hr, denatured protein was removed by centrifugation. The above procedure was carried out at 4 °C.

Ion exchange chromatography

The enzyme preparation obtained by ammonium sulfate precipitation was applied on a column (2.0 x 10 cm) of CM-cellulose previously equilibrated with 4 mM sodium acetate buffer (pH 5.2) at 4 °C. The column was washed with the same buffer and elution was carried out with a linear gradient of 0-0.4 M NaCl in 50 mM sodium acetate buffer (pH 5.2). Five ml fractions were collected. Protein content of each fraction

was measured spectrophotometrically at 280 nm.

Phosphatase assay

In determining acid phosphatase activity, p-nitrophenyl-phosphate was routinely used as a substrate. The assay mixture comprising of 0.2 ml each 12.5 mM p-nitrophenylphosphate in 0.1 M sodium acetate buffer (pH 5.2) and a suitably diluted enzyme solution was incubated for 2 to 5 min at 30 °C. The reaction was stopped by the addition of 0.5 M sidium carbonate. The amount of p-nitrophenol liberated was measured at 420 nm and converted into the equivalent amount of inorganic phosphate (Pi). Activities toward other substrates were determined by measuring Pi liberated from each substrate by the method of Allen (1940). The reaction mixture comprising of 0.2 ml of 12.5 mM substrate in 0.1 M sodium acetate buffer (pH 5.2) and 50 µl of enzyme in a total volume of 0.4 ml was incubated for 30 to 60 min at 30 °C. The reaction was stopped by the addition of ice cold 60 % perchloric acid.

Polyacrylamide gel electrophoresis

Each of the combined fractions (P-I and P-II) from CM-cellulose column chromatography of acid phosphatase in the medium was dialyzed against 4 l of 4 mM sodium acetate buffer (pH 5.2) for 20 hr at 4°C. The dialyzed solution was subjected

to electrophoresis in a gel containing 7.5 % polyacrylamide, 0.75 M Tris-HCl (pH 8.9), 0.02 % ammonium persulfate, and 0.05 % TEMED. Both electrode vessels were filled with 50 mM Tris-glycine (pH 8.4) for 2 hr at 4 °C. After electrophoresis (2.5 mA/tube), gels were stained for acid phosphatase by a modification of the Jaaska's method (1978). In brief, gels previously incubated in 0.1 M sodium acetate buffer (pH 5.2) at least for 30 min were transferred into a mixture comprising of 6.25 mM α-naphthylphosphate, 2 mM Fast Garnet GBC salt and 50 mM sodium acetate buffer (pH 5.2), followed by incubation for 15 to 30 min at 30 °C and washing with distilled water.

Measurement of radioactivity

After electrophoresis the gels were sliced into 2.5 mm-thick sections with a gel slicer. The slices were placed into separate counting vials and treated with 1 ml of 30 % hydrogen peroxide at 60 °C for 3 hr. To each vial was added 10 ml of scintillator (toluene: 700 ml, Triton x-100: 300 ml, 2,5-diphenyloxasol: 4.0 g, 1,4-bis-2-(5-phenyloxasolyl) benzene: 0.2 g). The radioactivity was counted with a Beckman liquid scintillation spectrometer (Model LS-250).

Results

Figure 1 shows a typical time course of the release of acid phosphatase from half-seed into the incubation medium in the presence or absence of 10^{-5} M GA_3. In the presence of GA_3, the enzyme release begins after a lag period of about 12 hr and continues at least up to 48 hr. On the other hand, the release in the absence of GA_3 starts after about 24 hr and with a very slow rate. The enzyme activity retained in half-seed reaches a maximum 24 hr after addition of GA_3 and then decreases rapidly, the level at 48 hr becoming two thirds of the original activity. In the water control, the activity continues to increase up to 36 hr and decreases slowly with a concomitant release of small amount of the enzyme. The maximum activity of the extract does not differ between the control and GA_3-treated half-seed.

Gibberellic acid at 10^{-6} M to 10^{-5} M was found to be sufficient for full stimulation of the release of acid phosphatase into the medium (Fig. 2).

Figure 3 shows chromatographic profiles of acid phosphatase preparations from dry and preincubated half-seeds. Two peaks of acid phosphatase activity are observed in both preparations. First peak (P-I) was eluted at 18 mM and the second (P-II) at 145 mM NaCl. No more phosphatase activity was detected even in the eluate with 1 M NaCl in 50 mM sodium acetate buffer (pH 5.2). Two peaks of phosphatase

activity eluted at 18 mM and 145 mM NaCl, respectively, were also observed in half-seeds incubated with or without 10^{-5} M GA_3 for 12 hr (Fig. 4A,B) and 24 hr (Fig. 5A,C). In the medium after 24 hr incubation of half-seeds with GA_3, three peaks of acid phosphatase were detected (Fig. 5D). The first and third peaks were eluted at the same NaCl concentration as for P-I and P-II, respectively. An additional peak was eluted at 58 mM NaCl (P-III). Only minute amounts of P-I and P-II were detected in the incubation medium without GA_3 (Fig. 5B).

Table 1 shows the relative activity toward various substrates of these phosphatase fractions. Acid phosphatase P-I of both the extract and the medium acted on p-nitrophenyl-phosphate, α-naphthylphosphate and ATP at relatively high rates among substrates tested. P-III of the medium showed the relative activity pattern similar to that of P-I. P-II showed relatively high activities toward fructose-1,6-biphosphate, inorganic pyrophosphate and ATP as well as two aryl phosphates.

P-I and P-III were most active at pH 5.7, while P-II at pH 5.5 (Fig. 7). Although the optimum pH values were very close, the overall patterns of pH dependency differed from one another.

Incorporation of [^{3}H]-leucine into these acid phosphatase fractions was studied. In all samples examined, the

radioactivity was preferentially incorporated into P-I and only slightly into P-II and P-III (Fig. 4A,B, Fig. 5A,B,C,D).

P-I and P-II from the medium, to which the bulk of the increased enzyme activity by GA_3 can be attributed, were subjected to polyacrylamide gel electrophoresis (Fig. 7). P-I gave a single phosphatase-active band (1) and P-II was resolved into two bands (1' and 2'). Band 1 and band 1' merged into each a peak of radioactivity, while band 2' showed only a low level of radioactivity.

Discussion

Acid phosphatase is one of the enzymes whose activities are enhanced by GA_3 treatment in barley aleurone layer (Pollard and Singh 1968, Jones 1969, Bailey et al, 1976). However, in wheat half-seed as in barley aleurone layer, the acid phosphatase activity was considerably high even before GA_3 treatment (Fig. 1). Ashford and Jacobsen (1974) reported that the enzyme activity was located mainly in the cell wall region but partly around aleurone grains in aleurone cells of dry barley half-seed and that a steady increase of enzyme activity was observed in these cellular sites during incubation without GA_3. Therefore, there may be some specific mechanism for activity enhancement of acid phosphatase in aleurone layer.

The increase in acid phosphatase activity in the GA_3-treated half-seed was not so apparent as in the medium. However, since the total activity (extract plus medium) reaches two-fold of the control by GA_3 treatment up to 36 hr, promotion of either synthesis or activation of acid phosphatase must occur. The fact that almost all of the increased activity is found in the medium (Fig. 1) suggests that there may be close relationships between the enhancement of acid phosphatase activity and the enzyme release into the medium by GA_3.

No difference was found in chromatographic profiles of

the extracts from dry and preincubated half-seeds, both were resolved into P-I and P-II (Fig. 3A,B). Furthermore, there was no significant difference in profiles of the control and GA_3-treated half-seeds after 12 hr incubation (Fig. 4A,B). These results indicate that GA_3 may scarsely affect the acid phosphatase components up to 12 hr incubation.

Both acid phosphatase fractions, P-I and P-II, were released from half-seed into the incubation medium by GA_3-treatment (Fig. 5D). The finding differs from the result that in barley aleurone layer only some molecular forms of acid phosphatase were selectively released into the medium in response to GA_3 (Bailey et al. 1976). However, P-I and P-II exhibited characteristic differences in pH-activity relation and relative activities toward various substrates (Table 1, Fig. 6) and so they may play each a different role in wheat seed. Acid phosphatase P-III which was found only in the medium showed close resemblance to P-I in pattern of pH dependency and substrate specificity, suggesting its possible relation to P-I in biosynthetic origin.

Incorporation of [^{3}H]-leucine into P-I fraction was much larger than that of P-II, but the gross incorporation may not necessarily dependent upon GA_3, because there was only a small difference between the GA_3-treated half-seed and the control in total incorporation (extract plus medium) (Fig. 5A,B,C,D).

Electrophoretic analysis of labelled acid phosphatase fractions from the medium showed that P-I consists of one molecular form (band 1), whereas P-II of two molecular forms (band 1' and 2') and that each of these enzyme-active bands but 2' merges into the high radioactive band (Fig. 7). These results suggest the possibility that some molecular forms of acid phosphatase released into the medium by GA_3 treatment contain enzyme proteins synthesized de novo. If band 2' enzyme also contributed to the GA_3-dependent increase of the enzyme activity, it may due to activation rather than de novo synthesis, because of its low rate of [^{3}H]-leucine incorporation.

Table 1. Relative activities toward various substrates of different fractions of wheat acid phosphatase. Fractions (P-I, P-II, P-III) were obtained by CM-cellulose column chromatography of the extract and the medium after incubation with or without GA_3 for 0 or 24 hr. The activities are indicated as the percentage of that toward p-nitrophenylphosphate.

Substrate	Relative activity (%)								
	0 hr		24 hr - GA_3		24 hr + GA_3		24 hr + GA_3		
	Extract		Extract		Extract		Medium		
	P-I	P-II	P-I	P-II	P-I	P-II	P-I	P-II	P-III
5'-Adenosine monophosphate	13	19	14	30	13	26	15	32	14
5'-Adenosine triphosphate	75	71	71	98	87	98	87	96	84
Fructose-1,6-biphosphate	20	50	16	77	27	103	36	81	25
Glucose-1-phosphate	7	33	9	36	18	39	28	47	31
Glucose-6-phosphate	12	35	11	59	15	46	19	37	16
Inorganic pyrophosphate	39	114	21	146	37	170	46	118	28
Inositol hexaphosphate	7	24	6	22	7	46	5	36	6
β-Glycerophosphate	12	32	13	48	15	28	20	29	17
α-Naphthylphosphate	89	92	95	96	83	91	87	96	84
p-Nitrophenylphosphate	100	100	100	100	100	100	100	100	100

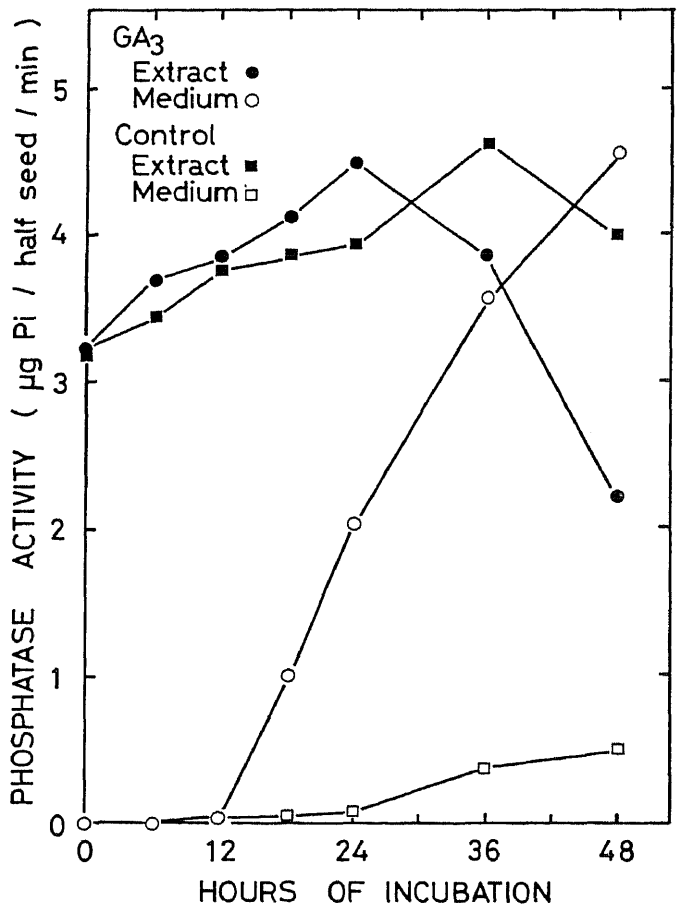

Fig. 1. Time course of the release of acid phosphatase from embryoless wheat half-seed treated with GA_3. The half-seeds preincubated for 24 hr in distilled water were incubated in the presence or absence of 10^{-5} M GA_3. The enzyme activities were measured for both the tissue extract and the medium. Each value is the mean of three samples.

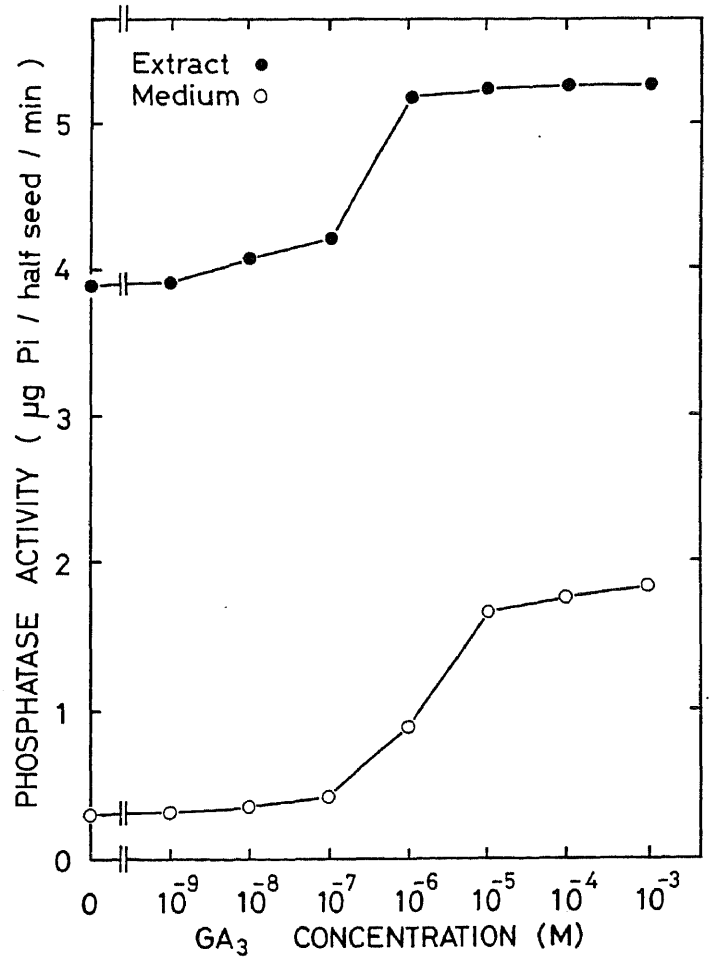

Fig. 2. Effect of GA_3 concentration on acid phosphatase release from half-seeds. The enzyme activities were measured after 24 hr incubation. Each value is the mean of three samples.

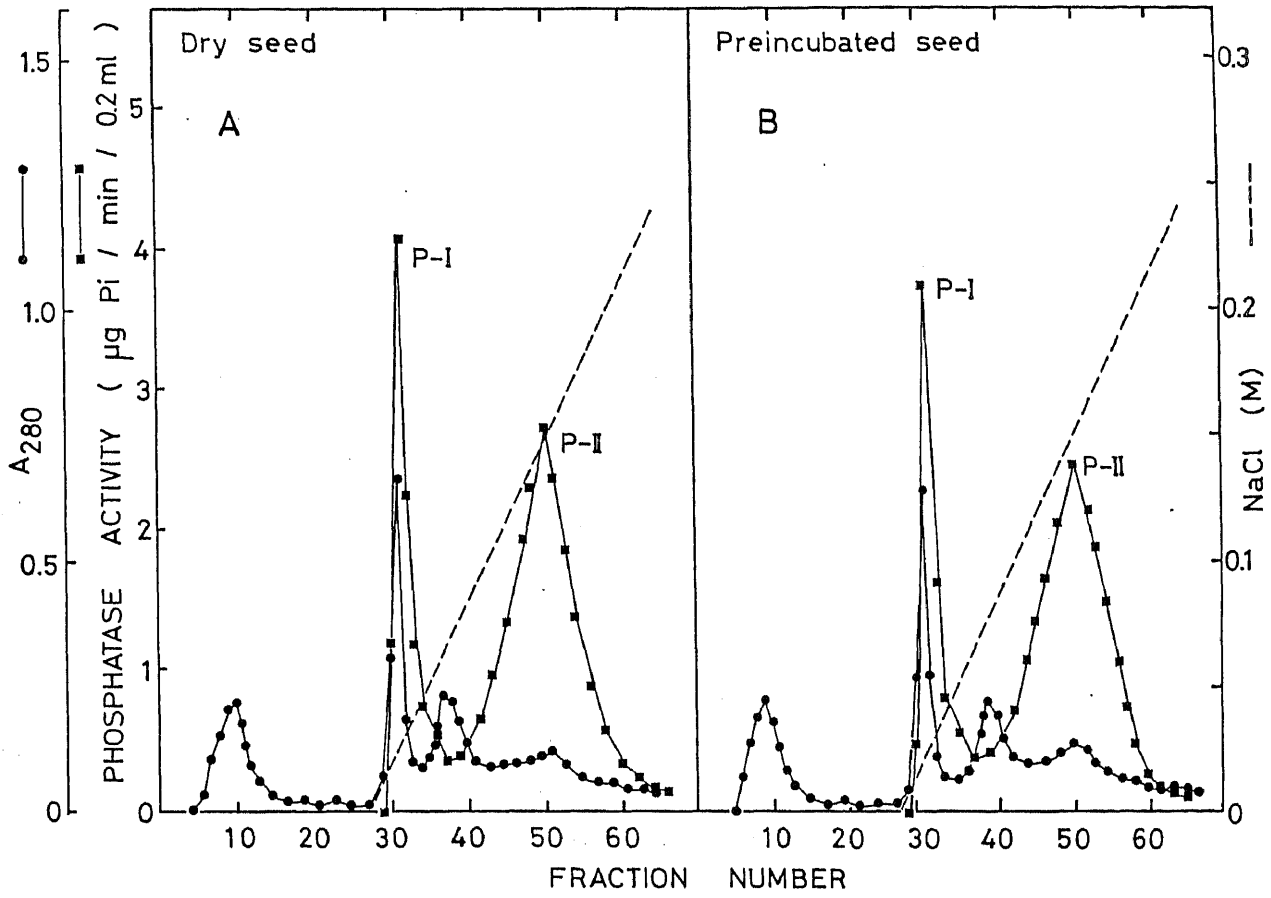

Fig. 3. Profiles of CM-cellulose column chromatography of acid phosphatase preparations from dry and preincubated half-seed. Ten ml of the enzyme preparation was applied onto the column and eluted under the conditions described in Methods.

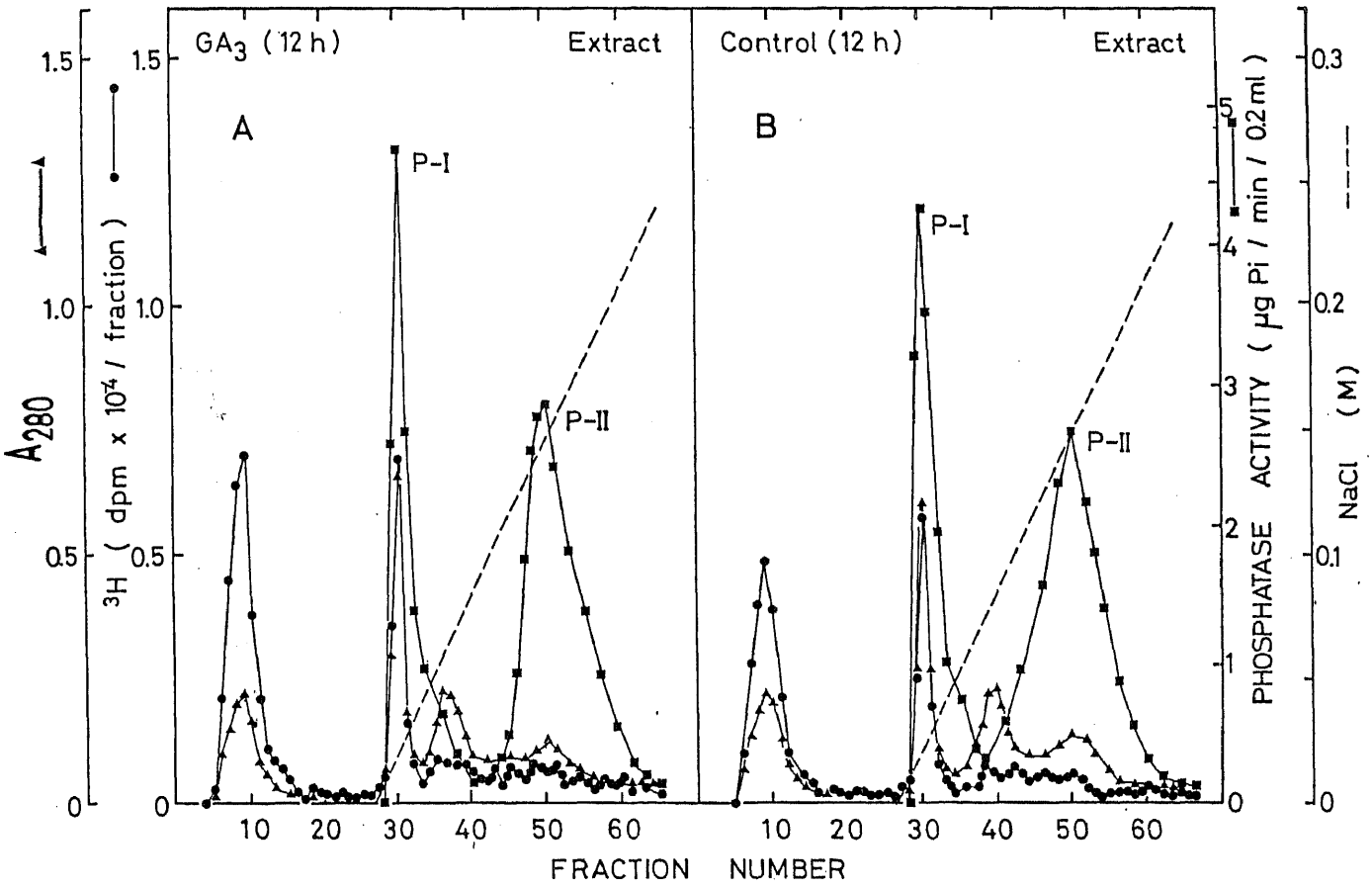

Fig. 4. Chromatographic profiles of acid phosphatase preparations from half-seed incubated for 12 hr with or without GA_3. Conditions for radioactivity labelling and CM-cellulose column chromatography are described in Methods.

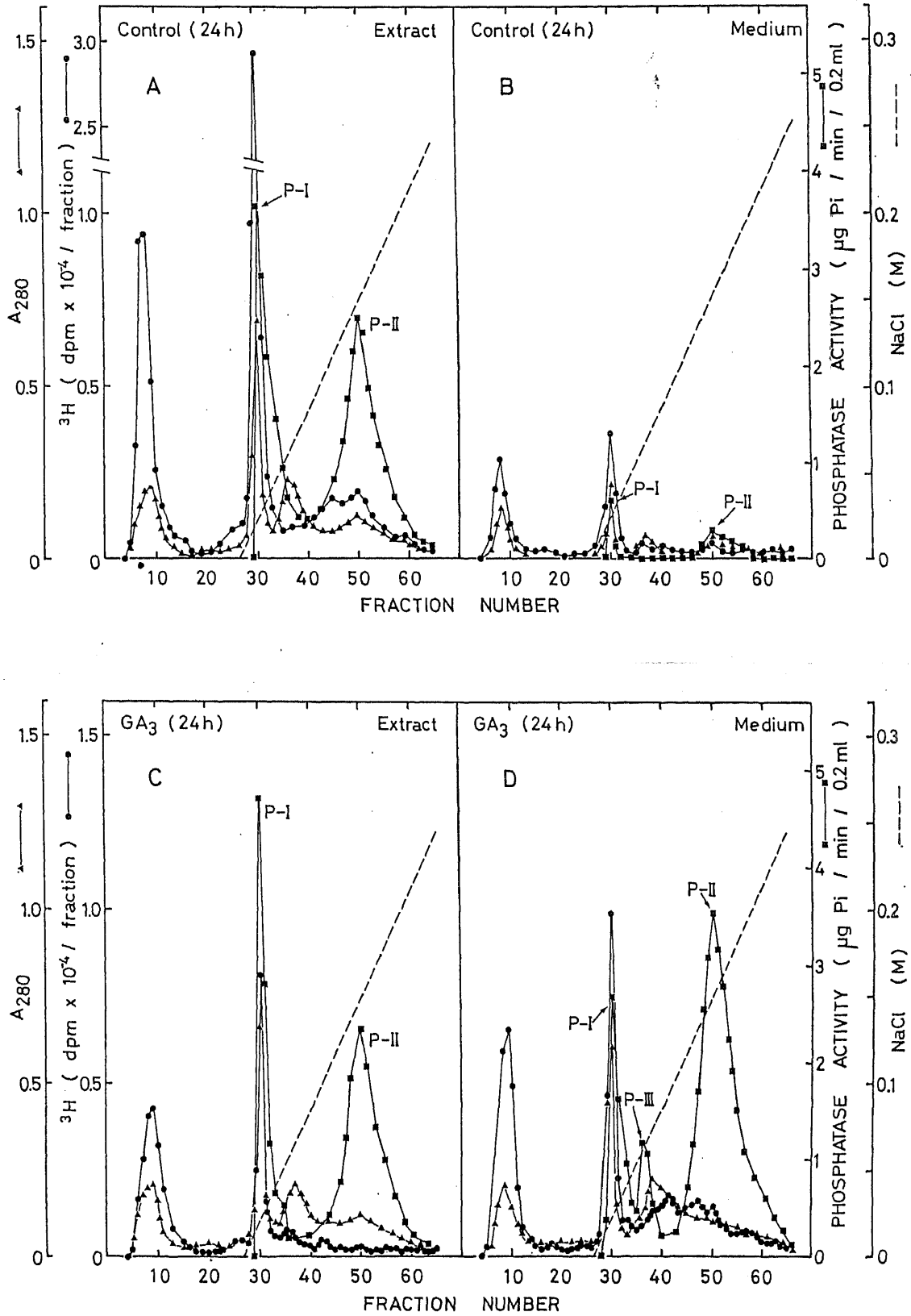

Fig. 5. Chromatographic profiles of acid phosphatase preparations from half-seed and medium after 24 hr incubation with or without GA_3. Conditions for radioactivity labelling and CM-cellulose column chromatography are described in Methods.

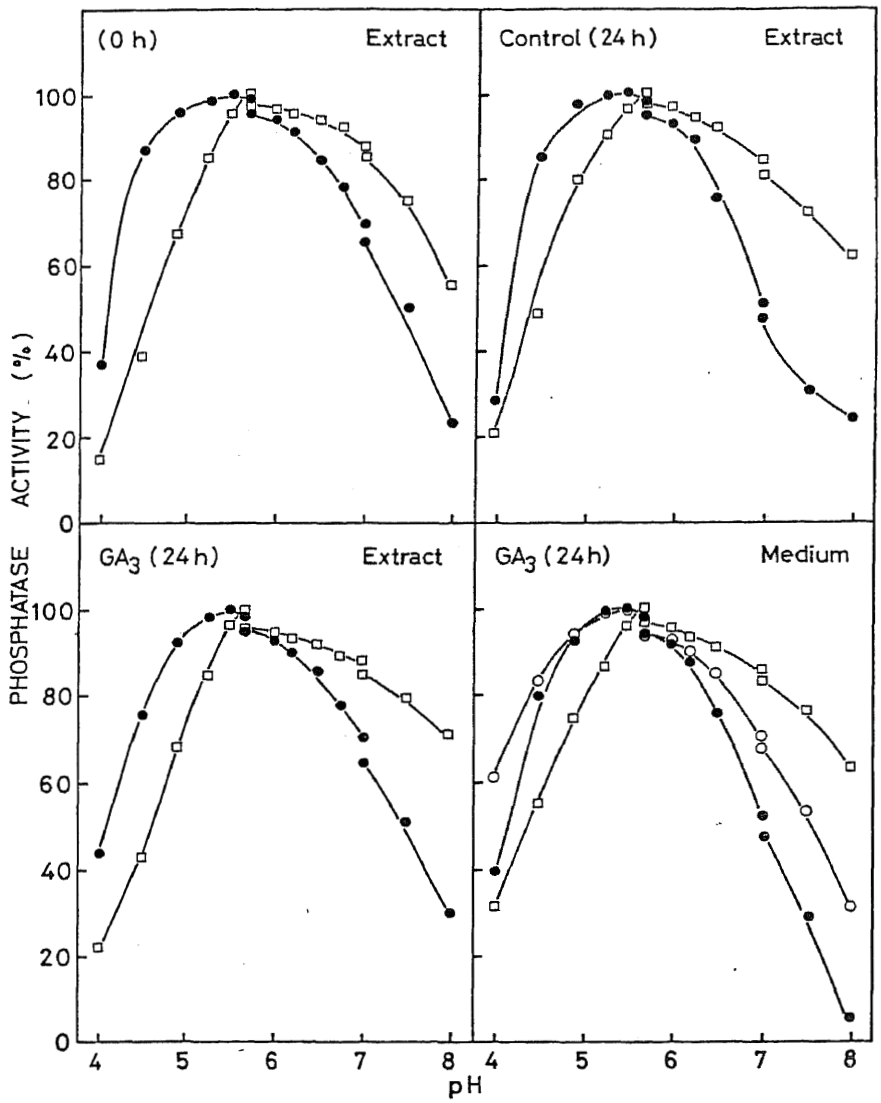

Fig. 6. Effect of pH on acid phosphatase activity of P-I, P-II and P-III fractions from half-seed and the medium. Activities for p-nitrophenylphosphate of three fractions (●, P-I; □, P-II; ○, P-III) from each sample were determined using sodium acetate (pH 4.0-5.7), MES (pH 5.7-7.0) and Tris-HCl (pH 7.0-8.0) buffers and expressed as % of the maximum. Each value is the mean of three replicates.

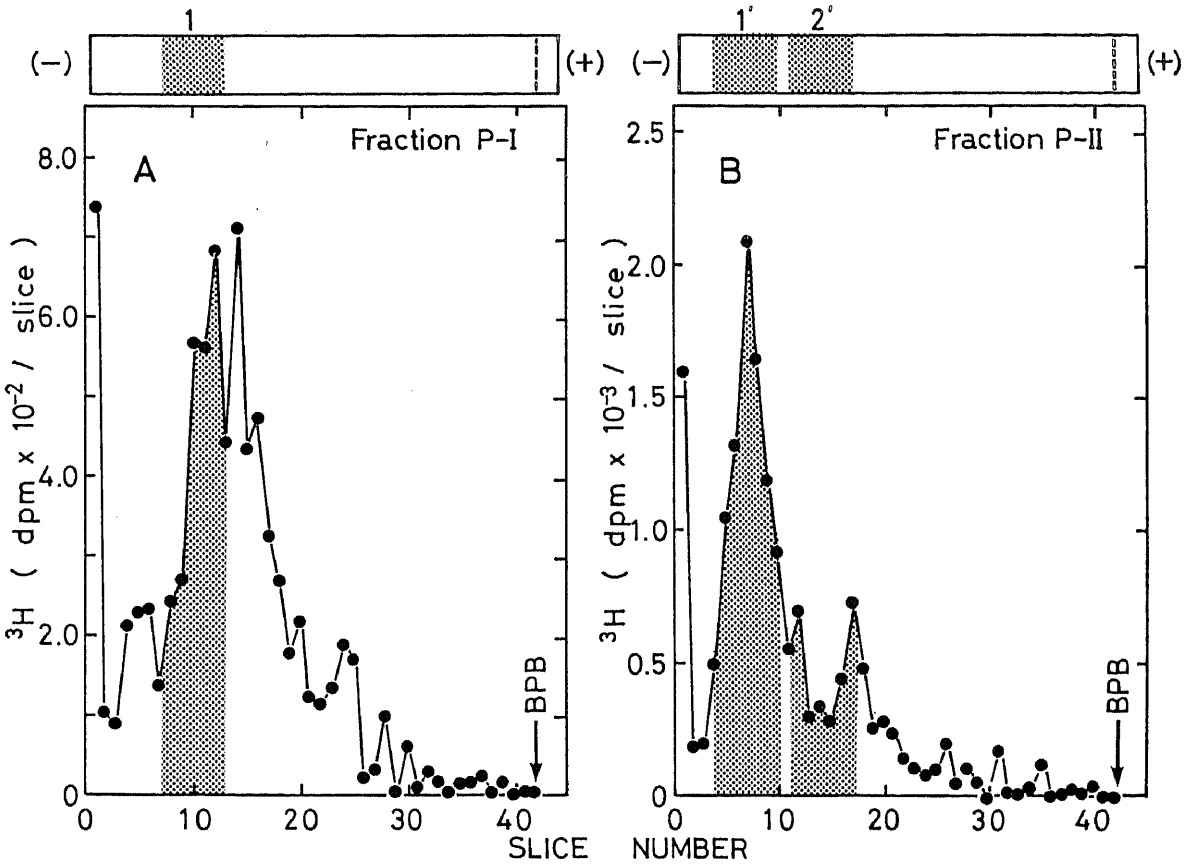

Fig. 7. Electrophoretic patterns of acid phosphatase fractions, P-I and P-II. The fractions were prepared from the medium after 24 hr incubation of the half-seed with GA_3 and [^{3}H]-leucine (Fig. 5). Conditions for gel electrophoresis are described in Methods.

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CHAPTER II

INHIBITORY EFFECTS OF ABSCISIC ACID ON GIBBERELLIC ACID-INDUCED ACID PHOSPHATASE ACTIVITY IN WHEAT HALF-SEEDS

Chapter II

Inhibitory effects of abscisic acid on gibberellic acid-induced acid phosphatase activity in wheat half-seeds

Abstract

Abscisic acid (ABA) at 10^{-4} M completely inhibited gibberellic acid (GA_3)-stimulated release of acid phosphatase from wheat half-seeds into an incubation medium. The same treatment with ABA, however, did not affect GA_3-induced acid phosphatase in the half-seeds. The mode of inhibition of ABA on acid phosphatase release into the medium was apparently similar to that of cordycepin. These results are discussed in relation to the effect of ABA on the synthesis and release of α-amylase.

Introduction

Recently, we reported that GA_3 enhanced acid phosphatase activity in wheat half-seeds and the release of the enzyme into an incubation medium (Akiyama and Suzuki 1980). It is a well documented fact that ABA inhibits GA_3-induced α-amylase synthesis in barley aleurone layer (Chrispeels and Varner 1966, Chrispeels and Varner 1967, Jacobsen 1973, Ho and Varner 1976). Inhibition of GA_3-induced release of acid phosphatase by ABA has been also observed in barley seeds (Pollard and Nelson 1971).

We have carried out an analysis of the effects of ABA on acid phosphatase activity in wheat half-seeds and its release into the incubation medium. Furthermore, several other metabolic inhibitors were employed to see any relevant mode of action to ABA. The outcome of experiments will be described in this paper.

Materials and Methods

Seeds of Triticum aestivum L. cv. Nohrin No. 61 were transversely cut into halves with a razor blade. The halves without embryo (hereafter denoted half-seeds) were sterilized in 1 % sodium hypochlorite solution for 20 min and rinsed more than ten times with sterile distilled water.

One hundred half-seeds were preincubated in 5 ml of distilled water in a 200-ml flask on a reciprocal shaker at 120 strokes per min at 24 °C for 24 hr. After preincubation 10 half-seeds were transferred to each vial (3 cm in diameter, 6 cm in length) containing 1 ml of the test medium. GA_3, ABA, CHI and cordycepin were added to the basal medium (2 mM sodium acetate buffer, pH 5.2) as specified in each experiment. The vials were incubated by shaking as above. All media were sterilized using a Millipore filter, pore size 0.45 μm, and glassware had been sterilized. Half-seeds were handled in a sterile laminar flow chamber.

At the end of incubation half-seeds were collected on a glass filter, washed with deionized-distilled water and then homogenized with a mortar and pestle in 5 ml of 50 mM sodium acetate buffer (pH 5.2). The filtered medium was diluted with the same buffer to make a final volume of 5 ml. The homogenates and the diluted media were separately centrifuged at 20,000 x g for 20 min. The supernatants were used for the measurement of acid phosphatase activity.

The assay mixture comprising 0.1 ml of each 12.5 mM p-nitrophenylphosphate in 0.1 M sodium acetate buffer (pH 5.2) and a suitably diluted enzyme solution was incubated at 30 °C for 10 min, followed by the addition of 0.1 ml of 0.5 M sodium carbonate to terminate the enzyme reaction. The amount of p-nitrophenol liberated was measured at 420 nm. In one experiment, α-amylase was assayed by the blue value method (Obata and Suzuki 1976) and a unit was defined as the activity causing a decrease of 1.0 in absorbancy at 700 nm per min.

All the experimental values are the means ± standard error of triplicated experiments.

Results

Influence of ABA on the GA_3-induced activity of acid phosphatase and α-amylase

The activity of acid phosphatase in incubated half-seeds was not reduced but rather increased by the addition of ABA ranging from 10^{-8} M to 10^{-4} M, especially in the presence of GA_3 (Fig. 1A). On the other hand, increasing concentrations of ABA inhibited GA_3-induced release of acid phosphatase into the incubation medium (Fig. 1B). ABA at 10^{-4} M completely supressed the effect of GA_3 on the enzyme release. As a comparative experiment the influence of ABA on GA_3-induced activation of α-amylase was measured. Figure 2 shows that ABA not only inhibited the release of α-amylase into the medium, but also the enhancement of the enzyme activity in the half-seeds. Again, 10^{-4} M ABA completely repressed the GA_3-induced release of the enzyme.

Influence of ABA on GA_3-induced change in acid phosphatase activity

The time course of changes in acid phosphatase activity was investigated in the presence or absence of GA_3 and/or ABA. In half-seeds incubated without ABA, a gradual increase of acid phosphatase activity was seen up to about 24 hr and thereafter decreased regardless of GA_3 treatment (Fig. 3A). The addition of GA_3 stimulated the enzyme release into the

medium (Fig. 3B). In the presence of 10^{-5} M ABA, the enzyme activity increased similarly by about 24 hr, but the decrease in the following hours did not occur (Fig. 3C). As expected, ABA dramatically inhibited GA_3-stimulated release of acid phosphatase into the medium (Fig. 3D). This inhibition became visible after 16 hr-incubation period which may correspond to the onset of GA_3-induced enzyme release into the medium.

Influence of ABA, cordycepin, and CHI on acid phosphatase activity

Preliminary experiments indicated that the release of acid phosphatase into the incubation medium containing GA_3 was not initiated before 12 hr-incubation of the half-seeds. Therefore, 12 hr incubation cycles were selected to investigate the effect of the inhibitors. Table 1 summarizes the outcome of the experiments. Inclusion of ABA in the first 12 hr incubation period resulted in a 85 % reduction of acid phosphatase activity in the medium, whereas the enzyme activity in half-seeds was almost not affected. Similar results were observed with cordycepin. However, the presence of CHI in the first incubation period reduced the enzyme activity in half-seeds to half of the control. When ABA, cordycepin, and CHI were supplied in the second 12 hr period, acid phosphatase in the medium was reduced to 42, 45, and 21 % of the control, respectively.

Discussion

As in a previous paper (Akiyama and Suzuki 1980), a gradual increase in the level of acid phosphatase activity in wheat half-seeds was seen regardless of GA_3 treatment up to 24 hr incubation. The rate in the presence of GA_3, however, was greater than in the absence of it. Further incubation of the half-seeds resulted in a decrease of the level of retained enzyme activity. GA_3 remarkably stimulated the rate of enzyme release into the incubation medium. This effect was detected at 16 hr incubation and thereafter became more prominant. Concerning α-amylase of wheat half-seeds, both the synthesis and the release were dependent on GA_3 as in barley half-seeds.

ABA is known to depress the GA_3-enhanced synthesis of α-amylase in barley aleurone layer (Chrispeels and Varner 1966, Chrispeels and Varner 1967, Jacobsen 1973, Ho and Varner 1976). Previously Pollard and Nelson (1971) have reported that ABA inhibits GA_3-induced acid phosphatase release from barley half-seeds into the incubation medium. However, they did not mention about influence of ABA on the enzyme level in the half-seeds. Our present results with wheat half-seeds demonstrated that ABA scarcely inhibited the increase in acid phosphatase activity of the half-seeds, but supressed the enzyme release into the medium. Moreover, ABA inhibited the decrease in acid phosphatase activity

in the half-seeds that had been in the active phase of enzyme release at about 24 hr after incubation. This fact is probably accounted to the suppression of the enzyme release.

On the other hand, ABA not only reduced α-amylase in the half-seeds but also its release into the medium. Thus, the mechanism of action of ABA in the GA_3-promoted synthesis (or activation) and release of particular enzymes in the half-seeds may not be the same.

Ho and Varner (1976) suggested that the inhibition by ABA of α-amylase synthesis in barley aleurone layer may be affected through ABA-induced synthesis or activation of certain RNA, which prevents translation of α-amylase mRNA. To see any possibilities of the inhibition of transcription or translation by ABA in the present case, comparative experiments using cordycepin and CHI were carried out. ABA and cordycepin were apparently similar in their mode of action, both inhibited only the release of acid phosphatase into the medium. Furthermore, the inhibition degree was evidently high when the treatment with ABA and cordycepin was applied during the first half period of 24 hr incubation. Thus, ABA seems to interfere with RNA synthesis relating to early development of a membraneous machinery for the enzyme secretion. However, our study by centrifugal fractionation of the wheat aleurone cell homogenate indicated that only a very small portion of the acid phosphatase

can be recovered in particulate fractions with which more than 21 % of the total α-amylase is associated (Akiyama and Suzuki 1980). This suggests a difference in the way of release between acid phosphatase, at least the enzyme pre-existing in the aleurone cells, and the newly synthesized α-amylase.

CHI inhibited both enhancement of acid phosphatase in half-seeds and its release into the medium by GA_3. Thus, GA_3 seems to act at translational level to increase the acid phosphatase activity in wheat half-seeds. The GA_3-stimulated synthesis of acid phosphatase will be dealt in a forthcoming paper.

Table 1. Influence of several inhibitors on the GA_3-induced activity in wheat half-seeds and the incubation medium. ABA (10^{-5} M), codycepin (100 µg/ml) or CHI (10 µg/ml) was contained in the incubation medium for either the first or the following 12 hr. All media contained 10^{-5} M GA_3.

Treatment with inhibitor		Acid phosphatase activity µmoles p-nitrophenol/half-seeds/min	
First 12 hr	Following 12 hr	half-seed	Medium
None	None	685 ± 23(100)*	364 ± 69(100)
ABA	None	685 ± 54(100)	55 ± 11(15)
None	ABA	669 ± 52(98)	152 ± 30(42)
Cordycepin	None	575 ± 30(84)	47 ± 8(13)
None	Cordycepin	685 ± 47(100)	165 ± 26(45)
CHI	None	336 ± 29(53)	43 ± 8(12)
None	CHI	619 ± 82(90)	75 ± 8(21)

* Values in parentheses express percentage to the control treated with GA_3 alone.

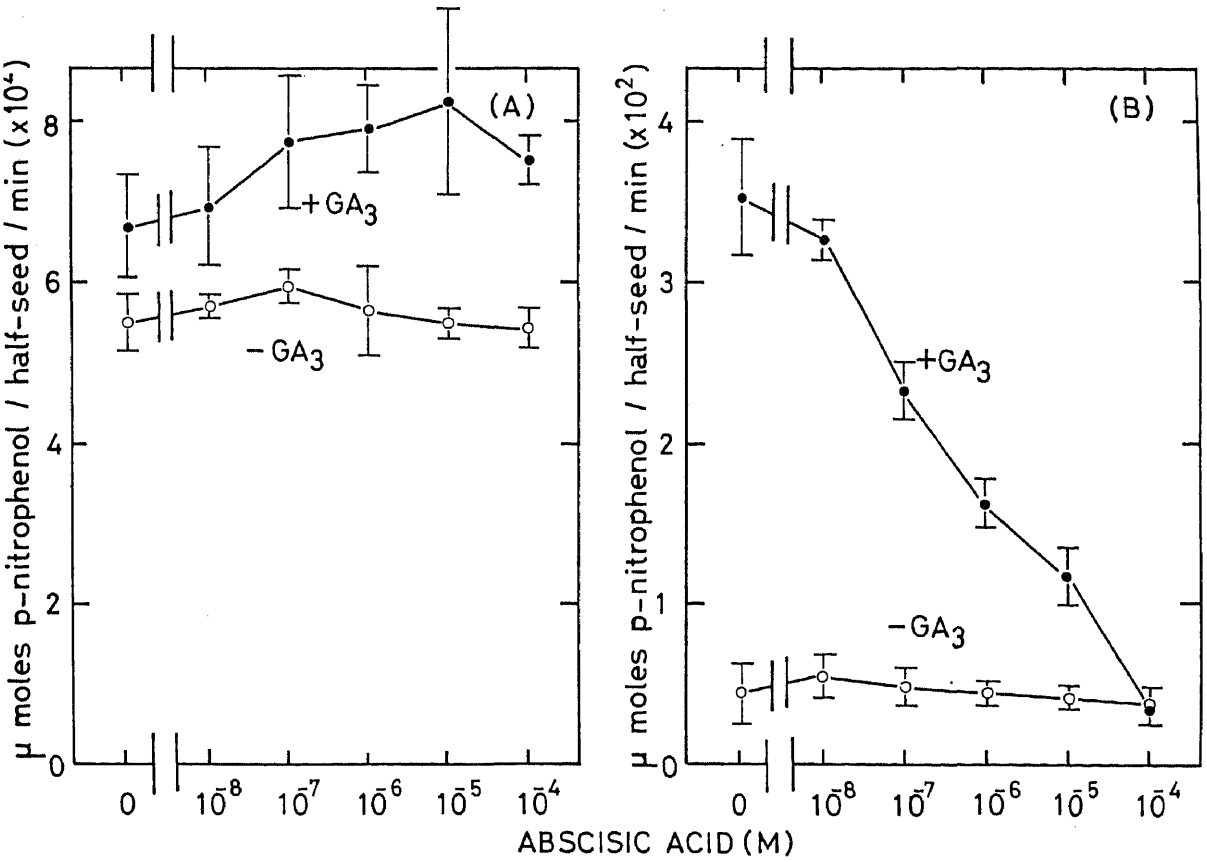

Fig. 1. Effect of ABA on acid phosphatase activity in wheat half-seeds (A) and in the incubation medium (B). Half-seeds were treated with various concentrations of ABA in the presence or absence of 10^{-5} M GA_3 for 24 hr.

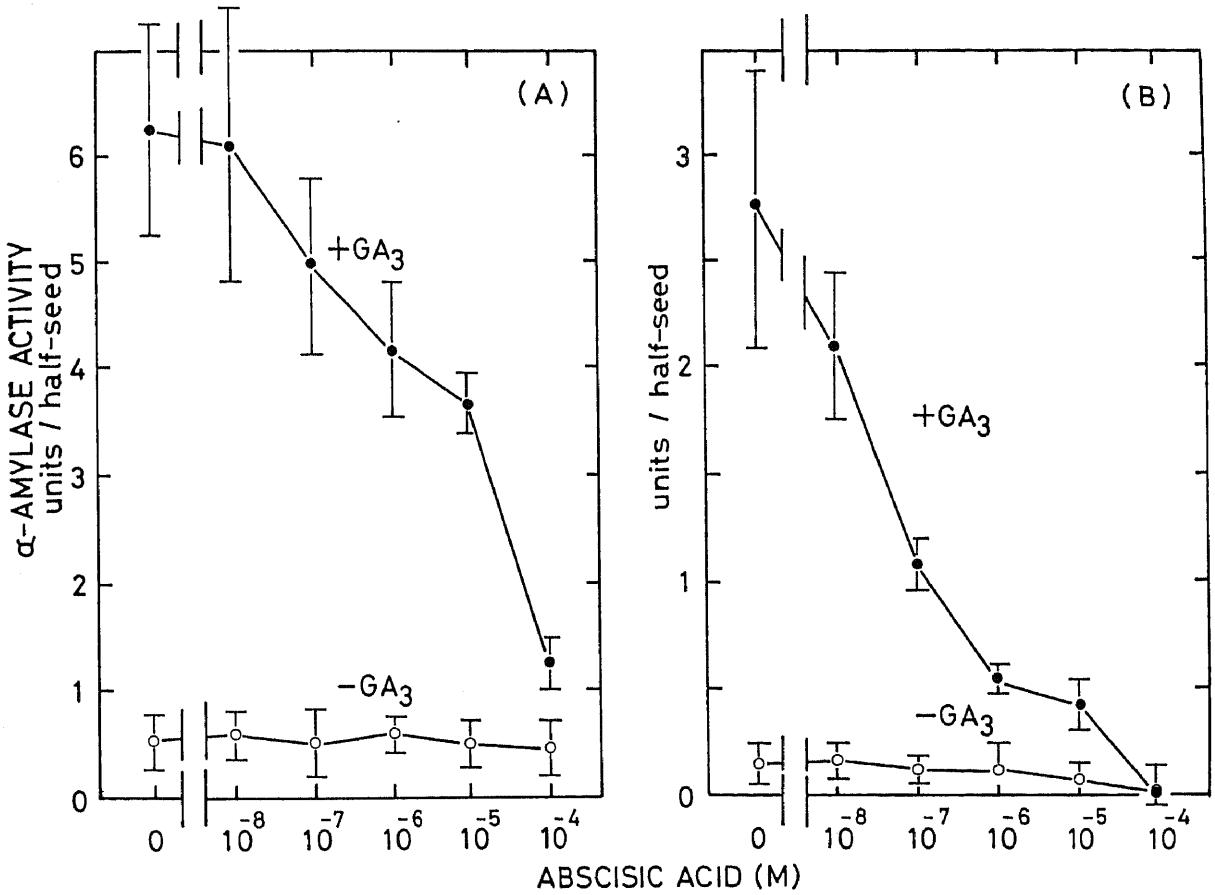

Fig. 2. Effects of ABA on α-amylase activity in wheat half-seeds (A) and in the incubation medium (B). Half-seeds were treated with various concentrations of ABA in the presence or absence of 10^{-5} M GA_3 for 24 hr.

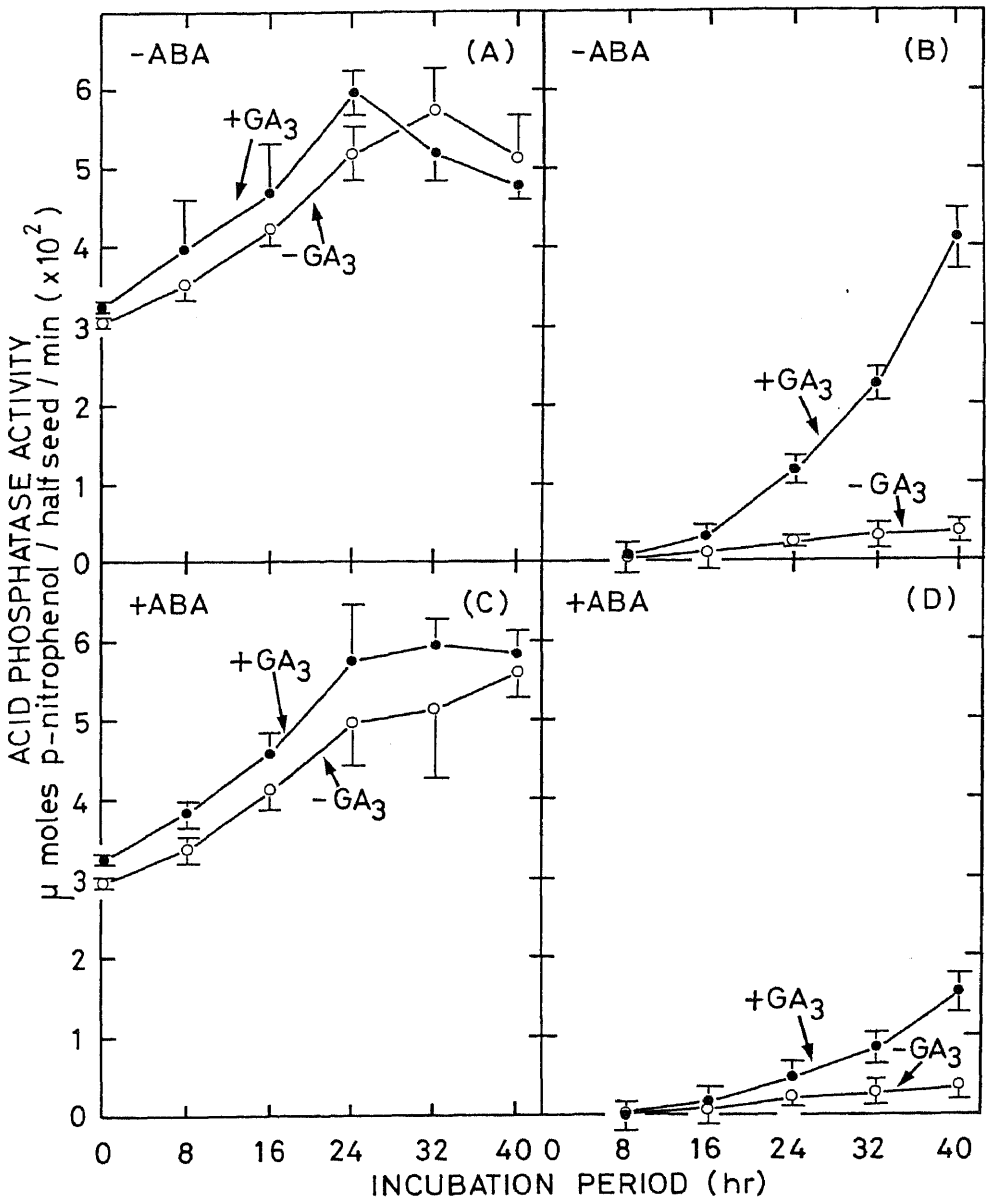

Fig. 3. Time course of changes in the acid phosphatase in wheat half-seeds (A,C) and in the incubation medium (B,D) during incubation in the presence or absence of 10^{-5} M GA_3 and/or 10^{-5} M ABA.

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CHAPTER III

LOCALIZATION OF ACID PHOSPHATASE IN ALEURONE LAYERS OF WHEAT SEED

Chapter III

Localization of acid phosphatase in aleurone layers of wheat seed

Abstract

Histochemical observations of acid phosphatase in aleurone layers of wheat seeds using α-naphthylphosphate as a substrate indicated that most of this enzyme was located in cytoplasm, but not in cell wall. Subcellular fractionation experiments of aleurone cells by sucrose density gradient centrifugation showed that predominant amount of acid phosphatase activity was detected in the soluble fraction regardless of the presence or absence of gibberellic acid (GA_3) in the incubation medium. In the presence of GA_3, however, a small but definite portion of acid phosphatase activity could be found in the particulate fractions showing α-amylase activity.

Introduction

Acid phosphatase is an enzyme which hydrolyzes a variety of orthophosphate esters. Catalytic function of this enzyme in germinating seeds is so important in terms of hydrolyzing phosphate compounds and eventually providing free phosphate pool for various metabolic pathways. Previously we have reported the presence of a measurable amount of acid phosphatase in dry wheat seeds (Akiyama ans Suzuki 1980). We have also demonstrated that gibberellic acid (GA_3) considerably increased the activity of acid phosphatase in embryoless wheat half-seeds and its release into the incubation medium. Histochemical investigation by Gahan et al. (1979) on the localization of acid phosphatase indicated that when naphthol AS-BI phosphate was used as a substrate, acid phosphatase was detected mainly in the cytoplasm of aleurone cells of dry wheat seeds.

We have carried out similar investigation as Gahan et al. (1979) and confirmed their observations. Furthermore, using subcellular fractionation techniques attempts have been made to see whether or not this enzyme is associated with particular intracellular structures. The results of experiments will be presented here.

Materials and Methods

Preparation of half-seeds

Wheat seeds (Triticum aestivum L. cv. Nohrin No. 61) were cut into half transversely with a razor blade and embryoless halves (half-seeds) were provided for experimentations. For the purpose of sterilization, half-seeds were immersed in a solution containing 1 % sodium hypochlorite for 20 min, and rinsed more than ten times with sterile distilled water.

One hundred half-seeds were preincubated with 5 ml of sterile distilled water in a 200 ml-flask at 24 °C. After preincubation for 24 hr, they were transferred into a 200 ml-flask containing 5 ml of 2 mM sodium acetate buffer (pH 5.2) with or without 10^{-5} M GA_3. The GA_3 solution was Millipore-sterilized before use. The flask containing half-seeds was further incubated at 24 °C for 24 hr in a reciprocal shaker at 120 strokes per min. Every containers for the incubation of half-seeds were sterilized previously and seeds were handled in a sterile laminar flow chamber.

Fixation of dry seeds for microscopic observation of acid phosphatase

Dry wheat seeds were fixed in a 3 % (w/w) glutaraldehyde solution in 50 mM cacodylate buffer (pH 7.4) for 20 hr at 0 °C.

Fixed and frozen sections (7 μm thick) were subjected to acid phosphatase localization test according to the modified method of Grogg and Pearse (1952). As a control, sections were incubated in the reaction mixture without the substrate. The reaction was stopped by adding 7.5 % acetic acid. Observations were made using an Olympus BH phase microscope.

Subcellular fractionation of aleurone layer of wheat half-seeds using sucrose density gradient

Two hundreds half-seeds were chopped with a Waring blender at about 3,000 rpm in a medium containing 0.35 M sucrose, 25 mM Tris-HCl (pH 7.3), 10 mM KCl, 1 mM $MgCl_2.6H_2O$ and 1 mM EDTA. The bulk of endosperm starch was removed by passing the chopped material thruogh a nylon gauze and by washing twice with the same medium. The aleurone layers retained on the nylon gauze were then homogenized with a mortar and pestle containing 10 ml of the same medium. The homogenate was centrifuged at 900 x g for 10 min. After centrifugation the supernatant was saved, 6 ml of which was applied onto sucrose step gradients consisting of 4 ml of 50, 35, 25, 20 % and 10 ml of 17 % sucrose solution which contained 25 mM Tris-HCl (pH 7.3), 10 mM KCl, 1 mM $MgCl_2.6H_2O$, and 1 mM EDTA. The gradients were centrifuged at 25,000 rpm for 2 hr in a Beckman SW 27 rotor. Forty five drops (1.3 ml/fraction) were collected from the bottom of the gradient. Absorbance

at 280 nm was determined using Shimadzu UV-200 spectrophotometer. All operations of the above procedure were carried out at 4 °C.

Measurement of enzyme activity

Acid phosphatase activity was measured in the same way as described elsewhere (Akiyama and Suzuki 1980) using p-nitrophenylphosphate as a substrate. NADH-dependent cytochrome c reductase was assayed according to the method of Bowles and Kauss (1976). α-Amylase activity was assayed by the method of Obata and Suzuki with slight modification (1976).

Results and Discussion

Figure 1B shows deep staining in the cytoplasmic portion of aleurone cells of a dry wheat seed. If the substrate for acid phosphatase is not present, staining in the cytoplasm is dim (Fig. 1A). In both cases, localization of acid phosphatase in cell wall regions is not apparent. Similar results were obtained when half-seeds were incubated in the presence or absence of GA_3 (data not shown). Though traces of acid phosphatase were detected in the cell wall, the cytoplasmic activity was predominant. Our observations coincide with those of Gahan et al. (1979).

Regarding subcellular localization of seed enzymes, a well studied example can be seen with α-amylase of gramineous plants. Several workers have reported that GA_3-induced α-amylase is associated with endoplasmic reticulum (Firn 1975, Gibson and Paleg 1972, Gibson and Paleg 1976 Locy and Kende 1978). Figure 2A shows subcellular fractionation profiles of a discontinuous density gradient centrifugation of the homogenate of aleurone layer incubated in the presence of GA_3. As a control, data are presented with aleurone layer not treated with GA_3 (Fig. 2B). As apparently indicated here, NADH-dependent cytochrome c reductase, a marker enzyme for endoplasmic reticulum, is stimulated by GA_3 treatment and is detected in the interface zones of a gradient. Moreover α-amylase activity in the interface

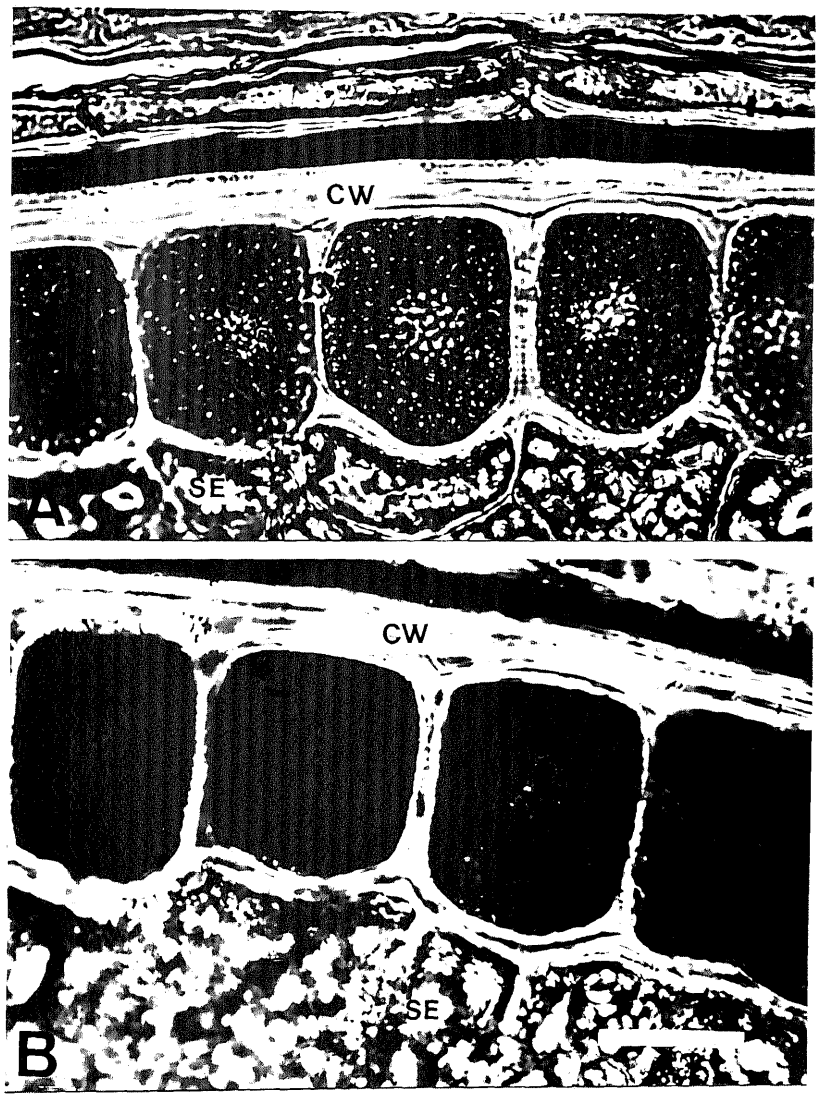
zones is much higher in GA_3-treated aleurone layer (21 % of total α-amylase activity in a gradient) than in the control.

In contrast to α-amylase, only a trace amount of acid phosphatase was detected in the interface zones. The bulk of the enzyme activity was present in the supernatant fraction. Gibson and Paleg (1972) reported that much of RNase activity of barley aleurone cells was soluble in earlier periods of GA_3 treatment but may be associated with particulates in later periods. Since acid phosphatase activity is detected in subcellular compartments such as aleurone grain in barley (Paux 1965), it may be likely that acid phosphatase previously contained in subcellular compartments is released into soluble fraction during homogenization of aleurone cells.

Pyliotis et al. (1979) reported that although the greater part of acid phosphatase activity in GA_3-treated barley aleurone layers occurred in the periplasm and the digested wall region, some part was associated with the endoplasmic reticulum. Bailey et al. (1976) and we (1980) suggested de novo synthesis of a certain form of acid phosphatase in barley and wheat seeds, respectively. Furthermore, our unpublished data have demonstrated at least one component of acid phosphatase in wheat half-seeds is synthesized de novo in response to GA_3. Acid phosphatase found in the particular fraction, possibly associated with endoplasmic reticulum, appears to correspond to such an enzyme synthesized

de novo. Much remains to be studied before any definite statement about the localization of acid phosphatase in the aleurone layer.

Fig. 1. Frozen sections of aleurone layer of dry wheat seeds. (A) control; a section was incubated in the reaction mixture without substrate, (B) a section stained for acid phosphatase using α-naphthylphosphate as a substrate. Although black and white picture does not represent precise colorations, presence of acid phosphatase in (B) was seen as a deep red color which contrasts to light brown in the aleurone cells of (A). (CW) cell wall, (SE) starcy endosperm. Bar represents 20 μm.


CW
SE
CW
SE
B

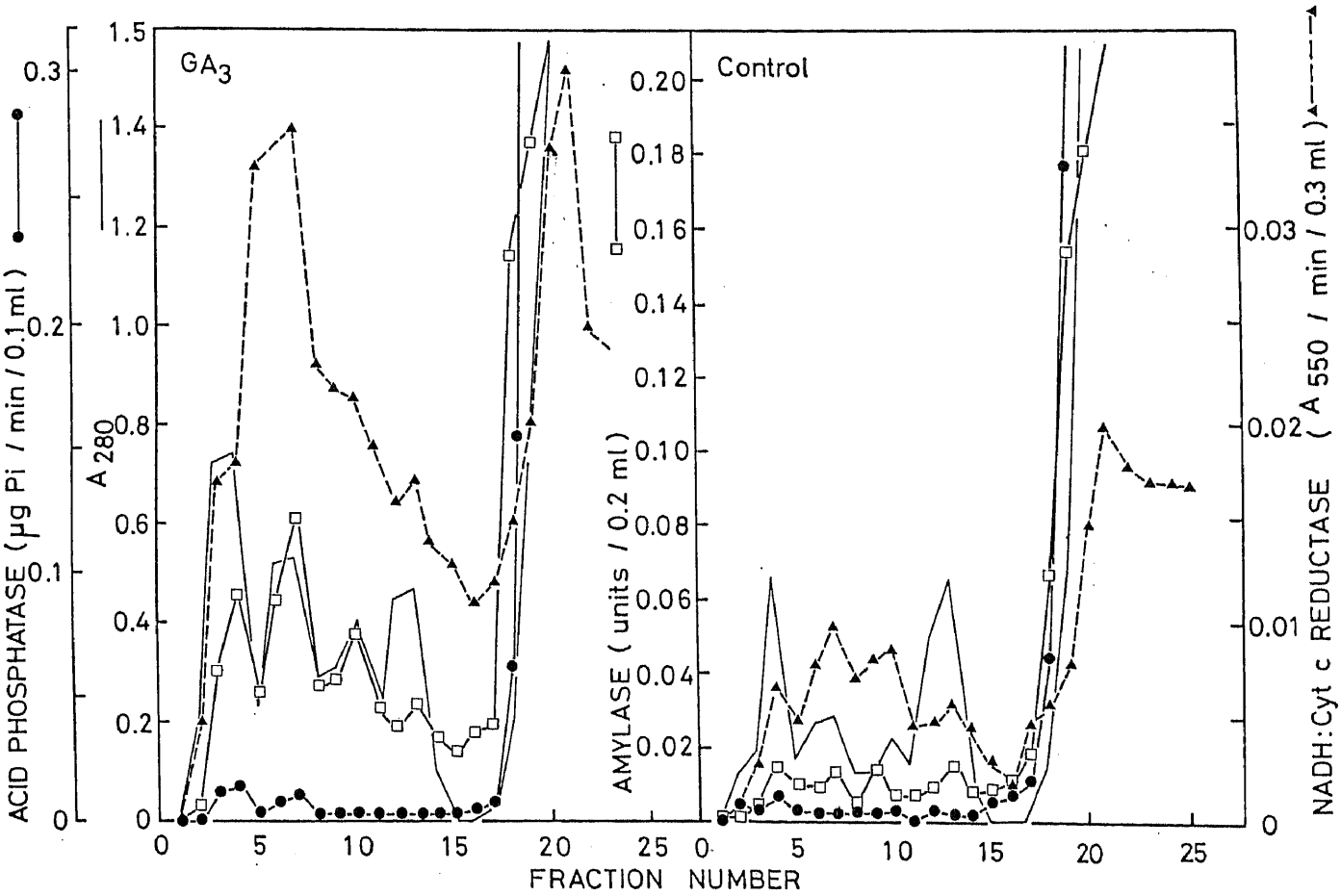


Fig. 2. Sucrose density gradient centrifugation of homogenates of wheat aleurone layers treated with or without GA_3. Crude homogenates were centrifuged at 700 x g for 10 min and a portion of the supernatant was directly applied onto the sucrose step gradients. (A) 10^{-5} M GA_3, (B) control ($-GA_3$). ——— A_{280}, ▲----▲ NADH:Cyt. c reductase, ●———● acid phosphatase, □———□ α-amylase.

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CHAPTER IV

GIBBERELLIC ACID-INDUCED INCREASE IN ACTIVITY OF A PARTICULAR ISOZYME OF ACID PHOSPHATASE IN WHEAT SEED

Chapter IV

Gibberellic acid-induced increase in activity of a particular isozyme of acid phosphatase in wheat seed

Abstract

Embryoless half-seeds of Triticum aestivum L. contain at least 9 acid phosphatase isozymes possessing isoelectric pH ranging from 4.0 to 7.2. Treatment with GA_3 resulted in activation of a particular isozyme of pI 4.0. Three major isozymes of pI 4.0, 4.9, and 6.2, respectively, differed in their relative specificities. Similar increase of pI 4 isozyme was also observed in endosperm of germinating wheat seeds.

Introduction

Occurence of multiple forms of acid phosphatase (EC.3.1.3.2) has been reported in various plant materials including wheat germ (Joyce and Glisolia 1960), rice ear (Ikawa et al. 1964), maize carnel (Efron 1970), bean hypocotyl (Mizuta and Suda 1980), barley seeds (Bailey et al. 1976) and also tissue cultured cells of rice (Igaue et al. 1976) as well as tobacco (Ueki and Sato 1977). Multiplicity of this enzyme has been characterized by means of column chromatography and gel electrophoresis. Use of isoelectric focusing in analysis of phosphatase isozyme has provided valuable information about phyletic lines of wheat (Nakai 1973) as well as rice plants (Pai et al. 1975). We reported that gibberellic acid (GA_3) enhanced acid phosphatase activity in wheat half-seeds by giving a predominant effect on the acid enzyme fraction resolved by CM-cellulose column chromatography and that it also stimulated the enzyme release into the incubation medium (Akiyama and Suzuki 1980). In this study, it have been found that acid phosphatase of wheat half-seeds can be separated into deveral distinctive forms by gel isoelectric focusing and that among these enzymes possessing pI values between 4.0 and 7.2, a particular one of very acidic pI was dramatically activated by GA_3. Moreover, similar change of the isozyme pattern has been observed to occur in the endosperm during germination of wheat.

Materials and Methods

Preparation of half-seeds

Seeds of Triticum aestivum L. cv. Nohrin No. 61 were transversely cut into halves with a razor blade. The halves without embryos (hereafter denote half-seeds) were immersed in 1 % sodium hypochlorite solution for 20 min and rinsed more than 10 times with sterile distilled water.

Incubation of half-seeds

Fifty half-seeds were preincubated with 2.5 ml sterile distilled water in a 100 ml-flask at 24 °C for 24 hr. Incubation was carried out by reciprocal shaking at 120 strokes per min. Preincubated half-seeds were transferred into a 100 ml-flask containing 2.5 ml of 2 mM sodium acetate buffer (pH 5.2). GA_3 and cycloheximide (CHI) (Sigma Chemical Company) were used at 10^{-5} M and 10 μg/ml, respectively. All glassware had been sterilized and seeds were handled in a sterile laminar flow chamber. At the end of 24 hr incubation half-seeds were collected on a sintered glass filter, washed with deionized-distilled water and then homogenized with a mortar and pestle in 6 ml of 50 mM sodium acetate buffer (pH 5.2). The homogenate and the filtered medium were separately centrifuged at 20,000 x g for 20 min at 4 °C. The supernatants were used as the samples for gel electrophoreis.

Germination of seeds

Dry wheat seeds were sterilized in the same way as for half-seeds and placed on a wet filter paper to germinate at 24 °C. After 24, 48, and 72 hr of imbibition periods, germinating seeds were dissected into appropriate portions such as endosperm, embryo, shoot and root, from which extracts were obtained as described above.

Polyacrylamide gel isoelectric focusing

One hundred μl of crude protein sample was applied on a gel containing 7.5 % polyacrylamide, 1 % Ampholine (pH 3.5-10, LKB), 20 % glycerol, 0.02 % ammonium persulfate, and 0.05 % TEMED. Anode and cathode vessels were filled with 20 mM HCl and 20 mM ethylenediamine, respectively. Electrophoresis was performed at a constant voltage of 200 V for 17 hr at 4 °C. After electrophoresis, the gel was subjected to staining of acid phosphatase using α-naphthylphosphate as a substrate and Fast Garnet GBC salt by the method described in previous paper (Akiyama and Suzuki 1980). The stained isozyme bands were scanned at 520 nm with a Shimadzu dual wavelength scanner CS-900.

Extraction of isozymes from gel slices and assay for acid phosphatase activity

For location of acid phosphatase isozymes, the gels were faintly stained and each of the bands was cut off

by a razor blade. Slices corresponding to the respective band from 10 gel columns were combined and homogenized in a glass-glass homogenizer with 3 ml of 0.1 M sodium acetate buffer (pH 5.2) at 4 °C. The homogenate was centrifuged at 20,000 x g for 20 min and the supernatant was dialyzed against 50 mM sodium acetate buffer (pH 5.2) for 20 hr at 4 °C. The dialyzed solution was used for the determination of substrate specificity. Acid phosphatase activities were assayed by measuring inorganic phosphate liberated from each substrate according to the method of Allen (1940).

Results

Acid phosphatase isozymes in half-seeds incubated under various conditions

As shown in Fig. 1, acid phosphatase in the sample from wheat half-seeds preincubated with water was resolved by gel isoelectric focusing at least 9 isozymes possessing different pIs in the range of 4.0 to 7.2. The sample from half-seeds incubated in the absence of GA_3 for 24 hr showed almost the same pattern as for preincubated half-seeds, except a slight increase of pI 6.2 (band 7) isozyme (Fig. 2A). However, when half-seeds were incubated with 10^{-5} M GA_3 for 24 hr, a remarkable increase in the activity of pI 4.0 (band 1) isozyme was observed, while no such stimulation of other isozymes was apparent (Fig. 2B). As reported previously, GA_3 stimulates not only acid phosphatase activity, but also release of the enzyme into the incubation medium (Akiyama and Suzuki 1980). Profile of acid phosphatase isozymes released into the medium was essentially similar to that of the enzyme in half-seeds (Fig. 2C). The GA_3-induced stimulation and release of acid phosphatase is inhibited by CHI (Chapter II). Figure 2D shows that the sample from half-seeds treated with GA_3 and CHI gives a pattern similar to that of the zero time control (Fig. 1). For comparison of changes in the relative amount of these isozymes in different samples, three major bands (pI 4.0, 4.9, and 6.2)

were selected. Table 1 indicates a dramatic increase in pI 4 isozyme activity by GA_3 and complete inhibition of it by CHI.

Isozyme patterns in germinating seeds

To see whether such stimulation of a particular isozyme of acid phosphatase as observed in GA_3-treated half-seeds occurs also in the endosperm during germination of wheat, several portions of germinating seeds were analyzed for respective isozyme patterns. Figure 3 shows change of the isozyme pattern in the endosperm portion during 3 days of germination. Activity of an acid phosphatase isozyme of pI 4 increased progressively with time. Changes in its relative amount during germination was most prominant among three major isozymes (Table 2). In the embryo including scutellum portion of ungerminated seeds, pI 4 isozyme was also contained and increased conspicuously as compared with others after 24 hr incubation (Fig. 4B). The presence of pI 4 isozyme was found in the shoot and root portions from 48 hr-germinated seeds (Fig. 4C,D).

Substrate specificities of some isozyme

Three major acid phosphatase isozymes in the samples from half-seeds incubated with or without GA_3 were compared in their substrate specificity. Table 3 shows that the

specificity patterns of these three isozymes are more or less different from one another, but no appreciable difference is observed between the corresponding isozymes from two samples.

Discussion

The aim of this study was to explore possible effect of GA_3 on the pattern of acid phosphatase isozyme and to provide more information about GA_3-induced increase in acid phosphatase activity in wheat half-seeds and release of the enzyme into the incubation medium.

Acid phosphatase in embryoless halves of ungerminated wheat seeds was found to consist of at least 9 isozymes by isoelectric focusing. Of particular interest was the finding that activity of only a particular isozyme of pI 4.0 was stimulated dramatically by GA_3 treatment. GA_3 also stimulated the release of this isozyme, together with others, into the medium, though there could not be clearly found any selective release among isozymes.

Three major isozymes (pI 4.0, 4.9, and 6.2) showed their own specificity toward various phosphomonoesters and pyrophosphates. However, there was no appreciable difference between the corresponding isozymes from half-seeds incubated with or without GA_3. This indicates that GA_3 may not induce qualitative change in the isozyme pattern.

Marked increase of pI 4 isozyme was also found in the endosperm of germinating wheat seeds. Similar change observed in the embryo seems to be due to the accompanying scutellum, for cells of scutellum and aleurone tissue resemble each other functionally as well as ultrastructurally (Katayama and

Suzuki 1980, Tanaka et al. 1976).

Bailey et al. (1976) reported that a single molecular form of acid phosphatase was present in dry half-seeds of barley, whereas on incubation two further form were synthesized de novo and that GA_3 stimulated activation, but not de novo synthesis, of all three forms of the enzyme. CHI was found here to inhibit the rise of pI 4 isozyme by GA_3 completely. We have also found that GA_3-promoted rise of acid phosphatase activity in wheat half-seeds was repressed by CHI but not by cordycepin (unpublished data). These results suggest that GA_3 may stimulate de novo synthesis of pI 4 isozyme and/or a certain activating enzyme. Problem in this respect is presently under study.

Table 1. Relative amounts of acid phosphatase isozyme in wheat half-seeds incubated under various conditions. Relative amounts of three major isozymes in the half-seeds were estimated by measuring the area under each peak in the densitograms shown in Fig. 1 and 2.

Incubation (hr)	Addition	Isozyme		
		Band-1 (pI 4.0)	Band-6 (pI 4.9)	Band-7 (pI 6.2)
0		100%	100%	100%
24	—	78	71	114
24	GA_3	264(408)[a]	84(110)	111(151)
24	GA_3+CHI	86	115	100

[a] Total amount (half-seeds + medium)

Table 2. Relative amounts of acid phosphatase isozymes in the endosperm portion of germinating wheat seeds. Relative amounts of three major isozymes were estimated by measuring the area under each peak in the densitograms shown in Fig. 3.

Isozyme	Germination period (hr)			
	0	24	48	72
Band-1 (pI 4.0)	100[a]	900	1335	1910
Band-6 (pI 4.9)	100	96	83	78
Band-7 (pI 6.2)	100	101	115	196

Table 3. Relative activities of acid phosphatase isozymes to various substrates. Samples from half-seeds incubated with or without 10^{-5} M GA_3 for 24 hr were separately subjected to gel electrofocusing and three major isozymes extracted from the gels were assayed for each substrate. Relative activity is expressed in percentage to the activity for inorganic pyrophosphate.

Substrate (6.25 mM)	- GA_3			+ GA_3		
	Band-1	Band-6	Band-7	Band-1	Band-6	Band-7
5'- Adenosine monophosphate	43	72	11	13	5	3
5'-adenosine triphosphate	132	52	90	142	31	110
Fructose-1,6-biphosphate	72	33	69	52	11	80
Glucose-1-phosphate	68	36	52	63	15	23
Glucose-6-phosphate	41	4	13	16	5	12
β-Glycerophosphate	42	9	11	20	3	4
Inorganic pyrophosphate	100	100	100	100	100	100
Inositol hexaphosphate	8	10	17	7	5	2
α-Naphthylphosphate	82	20	40	44	3	38
p-Nitrophenylphosphate	161	71	82	184	15	132

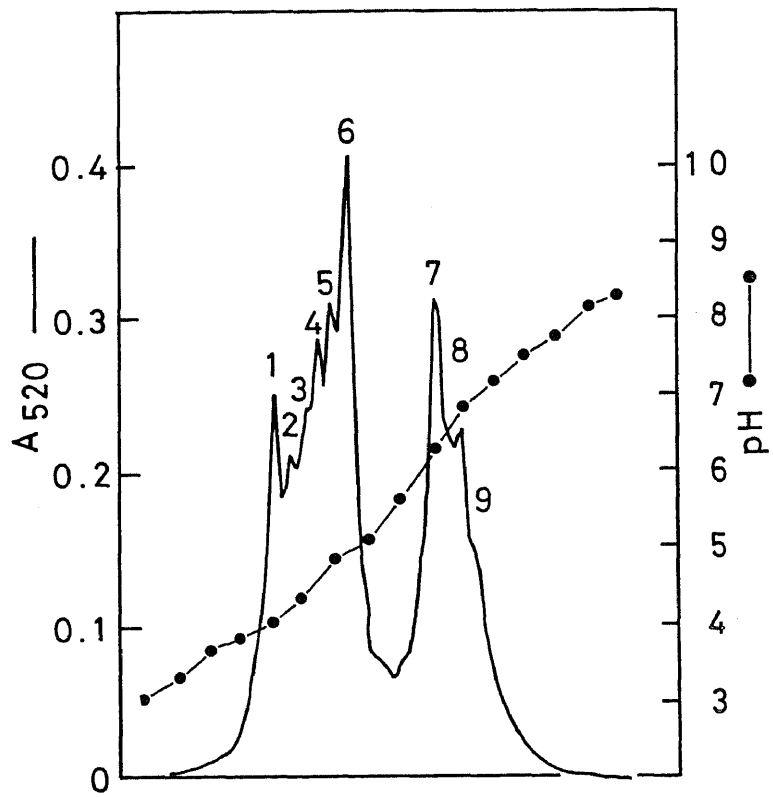

Fig. 1. Densitometric tracing of the pattern of acid phosphatase isozymes in wheat half-seeds preincubated with water for 24 hr. pH gradient was established using Ampholine (pH 3.5-10). One hundred µl of the crude protein sample equivalent to one half-seed was applied to the gel. Acid phosphatase activity was detected by the diazo-dye method.

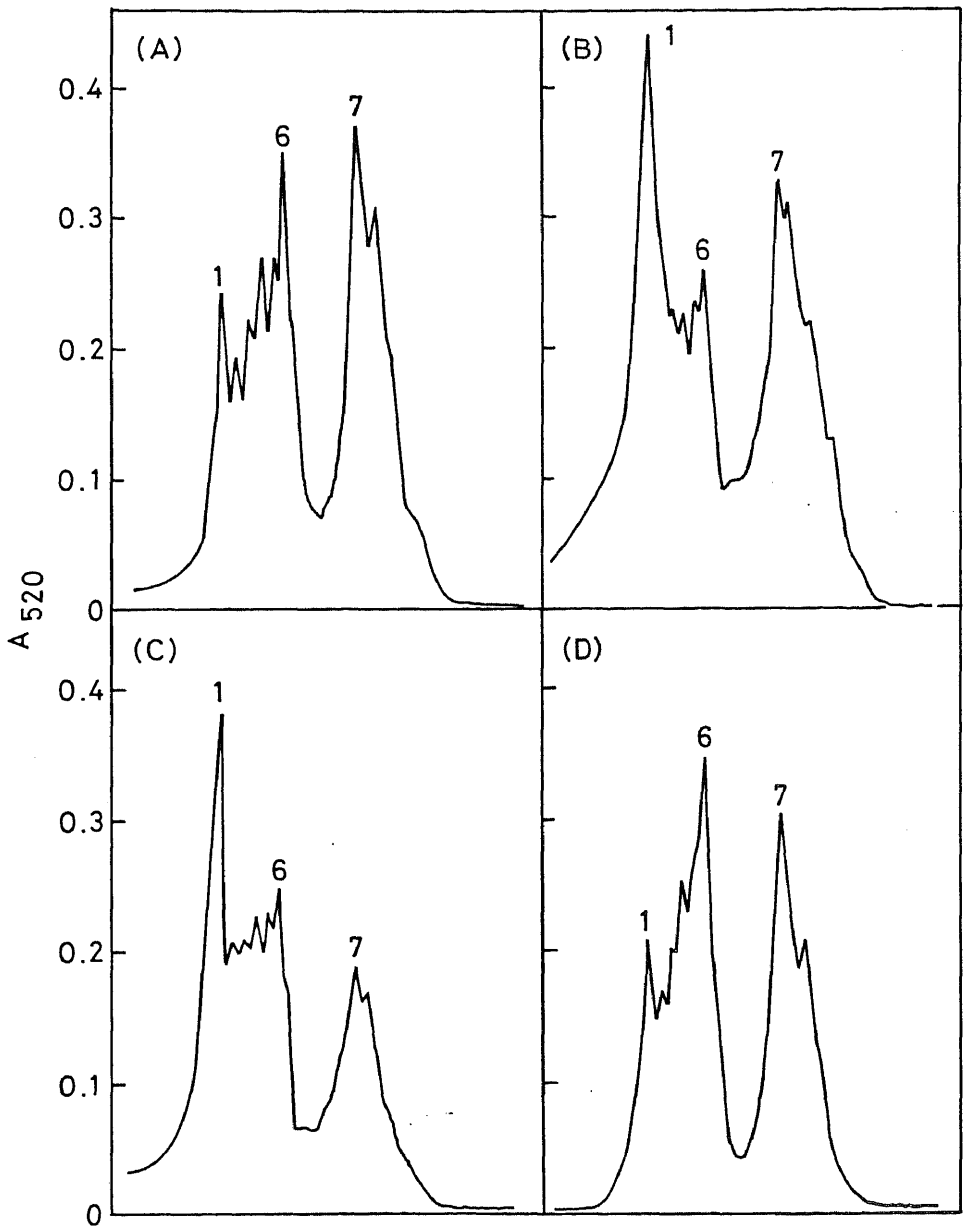

Fig. 2. Patterns of acid phosphatase isozymes in wheat half-seeds incubated under different conditions. Samples prepared from (A) half-seeds incubated without GA_3 for 24 hr, (B) half-seeds incubated with 10^{-5} M GA_3 for 24 hr, (C) medium after 24 hr incubation with GA_3, (D) half-seeds incubated with GA_3 + 10 µg/ml CHI for 24 hr were examined under the same conditions as in Fig. 1.

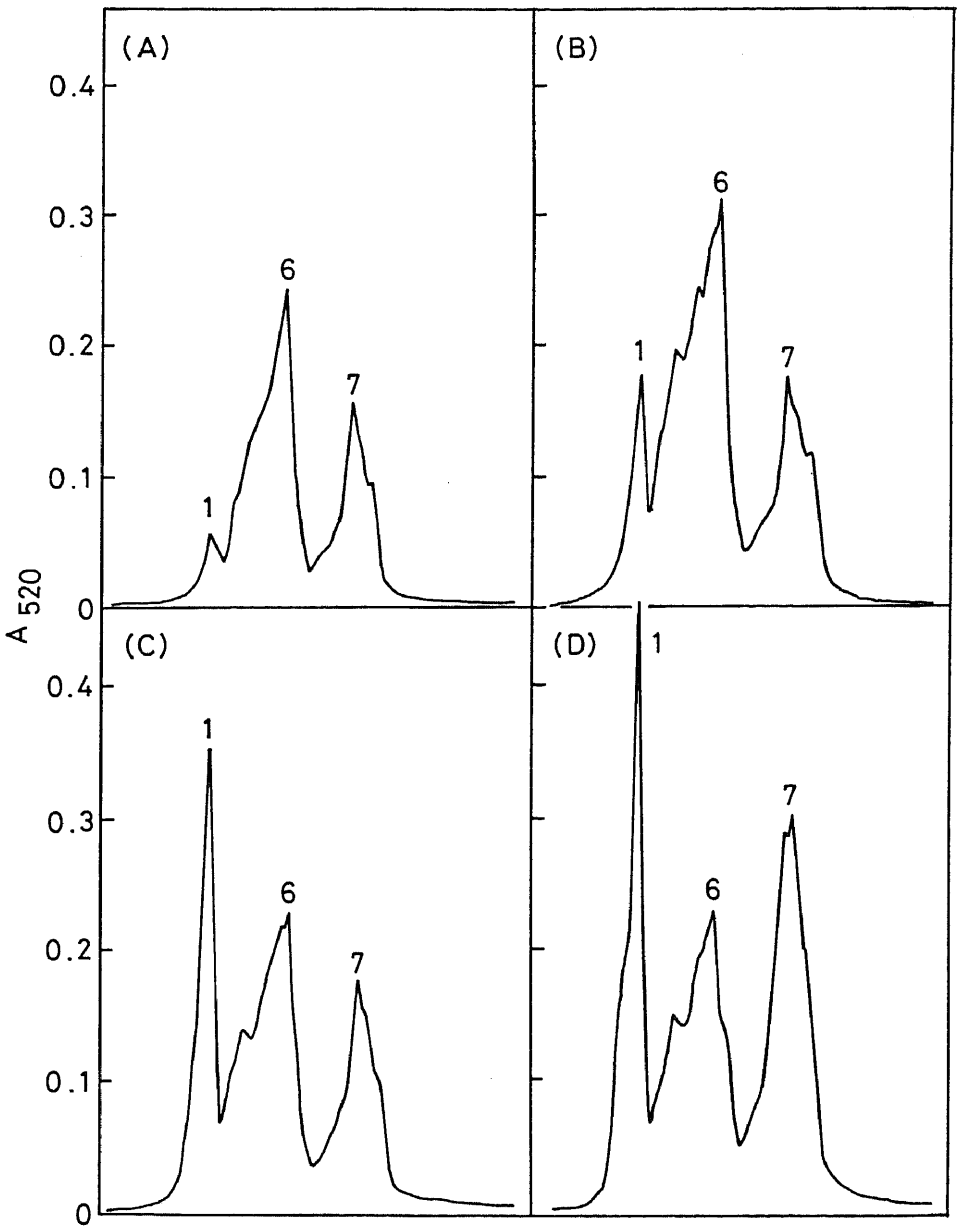

Fig. 3. Patterns of acid phosphatase isozymes in the endosperm portions of germinating wheat seeds. Samples were obtained by removing the embryo region from (A) ungerminated dry seeds and (B-D) germinating seeds at 24 hr, 48 hr and 72 hr incubation, respectively. See the legend of Fig. 1 for other conditions.

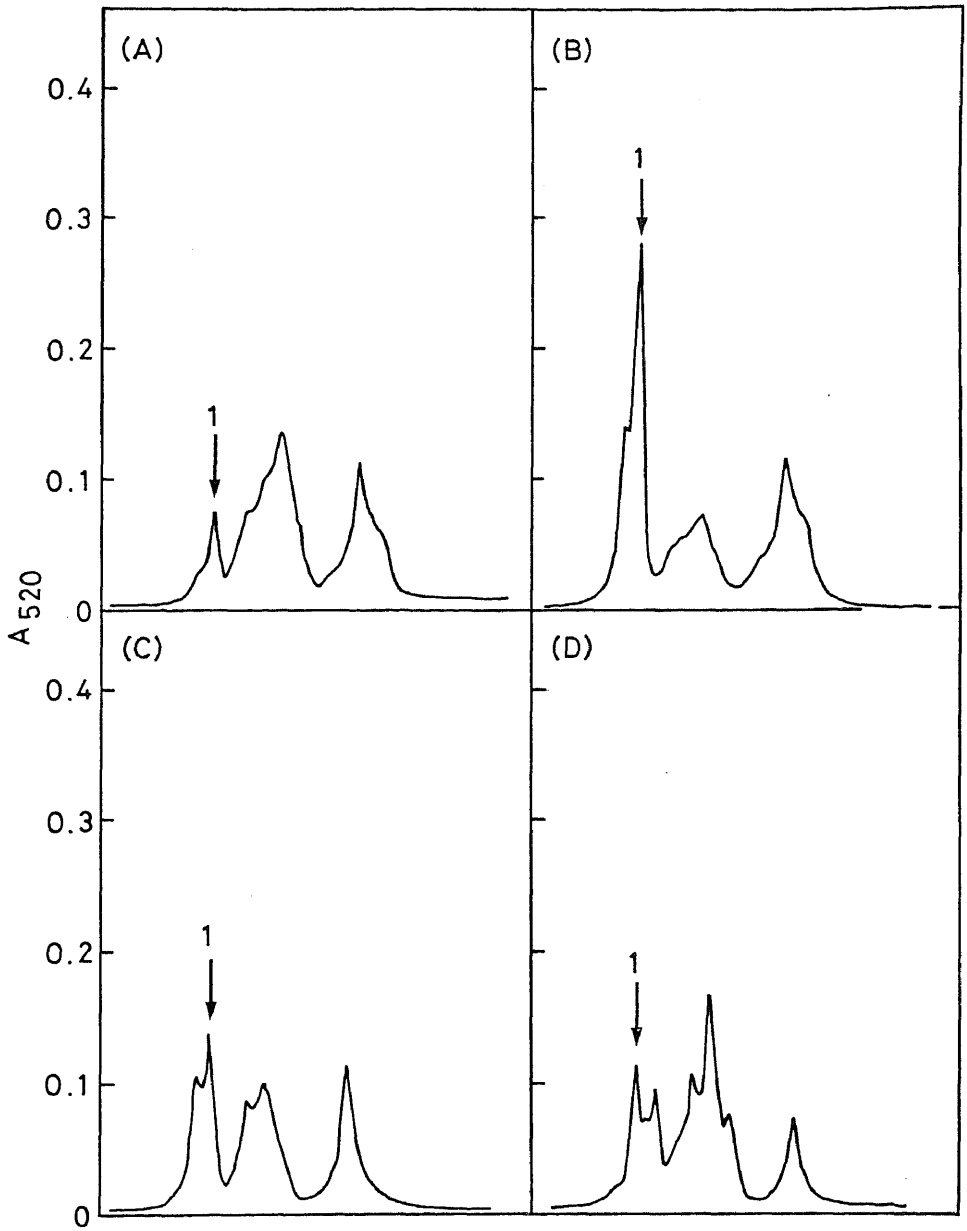

Fig. 4. Patterns of acid phosphatase isozymes in several parts of germinating wheat seeds. Samples were prepared from embryo (+ scutellum) portions of (A) dry and (B) 24 hr-imbibed seeds, (C) shoot and (D) root portions of 48 hr-germinated seeds. See legend of Fig. 1 for other conditions.

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CHAPTER V

PURIFICATION OF A GIBBERELLIC ACID-STIMULATED ISOZYME OF ACID PHOSPHATASE IN WHEAT ENDOSPERM

Chapter V

Purification of a gibberellic acid-stimulated isozyme of acid phosphatase in wheat endosperm

Abstract

A simplified procedure for purification of an acid phosphatase isozyme from tissue extracts was described. It consists of only three steps: concentration of the cell free extract by ammonium sulfate precipitation, fractional concentration of phosphatases by tricalcium phosphate adsorption and fractionation of the isozyme by gel isoelectric focusing. A combination of SDS-gel electrophoresis with the procedure seems to be useful in following the isozyme synthesis by radio-isotope labelling.

Introduction

Previously we reported the embryoless half-seeds of wheat (Triticum aestivum L.) contained at least 9 acid phosphatase isozymes differing in isoelectric pH and that GA_3 dramatically increased the activity of a particular acid phosphatase isozyme possessing isoelectric pH at 4.0 (pH 4 isozyme) (cf. Chapter IV). The finding raised the question whether pI 4 isozyme is synthesized de novo or activated under the influence of GA_3. To persue the synthesis of a particular protein, it was needed to establish as simple a purification procedure of pI 4 isozyme as possible. This paper describes the results of studies on this line.

Materials and Methods

Preparation and incubation of half-seeds

Embryoless half-seeds of Triticum aestivum L. cv. Nohrin No. 61 were prepared as described previously (Akiyama and Suzuki 1980). One hundred half-seeds were incubated in 5 ml of sterile distilled water in a 200 ml-flask at 24 °C for 24 hr by shaking (120 strokes/min). After preincubation half-seeds were transferred to a 200 ml-flask containing 5 ml of 10^{-5} M GA_3 and 2 mM sodium acetate buffer (pH 5.2) and incubated for further 24 hr.

Purification of pI 4 isozyme

All procedure described below were carried out at 4 °C.

Preparation of crude extract: Half-seeds were chopped in Warring blender at 3,000 rpm with 50 ml of 50 mM sodium acetate buffer, pH 5.2 (buffer A). Aleurone layers were collected by passing the chopped material through a nylon gauze and washed twice with buffer A, followed by homogenization in a mortar and pestle with 20 ml of buffer A. The homogenate was centrifuged at 20,000 x g for 20 min. The pellet was once more subjected to the homogenization and centrifugation. The supernatants were combined and used as a starting enzyme preparation.

Ammonium sulfate precipitation: Solid ammonium sulfate was added to the crude enzyme extract up to 75 % saturation.

The resulting precipitate was collected by centrifugation at 20,000 x g for 20 min and dissolved in 10 ml of buffer A. The solution was dialyzed overnight against 4 l of 4 mM sodium acetate buffer, pH 5.2 (buffer B) and centrifuged at 20,000 x g for 20 min.

Ion exchange chromatography: The enzyme solution (8 ml) obtained by ammonium sulfate precipitation was applied on a CM-cellulose column (2.0 x 10 cm) previously equilibrated with buffer B. The column was washed with the same buffer and elution was carried out with a linear gradient of 0-0.4 M NaCl in buffer A. The eluate was collected in 5 ml fractions.

Gel filtration: Appropriate fractions from CM-cellulose column chromatography were combined, concentrated to ca. 2 ml using collodion bag (SM 13200; Sartorius Co.,Ltd.) and applied on a Sephadex G-100 column (2.0 x 90 cm) previously equilibrated with buffer A. Elution was carried out with buffer A. In order to estimate molecular weight of acid phosphatase isozyme, the column was calibrated using marker proteins such as bovine serum alubumin (66,000), bovine blood hemoglobin (64,500), egg albumin (45,000), and cytochrome c (13,000).

Tricalcium phosphate adsorption: According to the method of Turner and Turner (1960), 7 g of tricalcium phosphate was added to 10 ml of the crude enzyme solution

obtained by ammonium sulfate precipitation. The resulting slurry was centrifuged at 20,000 x g for 10 min. From the precipitate washed twice with buffer B, acid phosphatase was desorbed by suspending in 10 ml of 0.2 M sodium phosphate buffer (pH 7.4) and centrifuged at 20,000 x g for 10 min. The supernatant was dialyzed overnight against 4 l of buffer B.

Acid phosphatase assay

The reaction mixture comprising 0.2 ml of each 12.5 mM p-nitrophenylphosphate in buffer A and a suitably diluted enzyme solution was incubated at 30 °C for 2 to 5 min. The enzyme reaction was stopped by the addition of 0.2 ml of 0.5 M sodium carbonate. The amount of p-nitrophenol liberated was measured at 420 nm.

Polyacrylamide gel isoelectric focusing

The enzyme solution was previously concentrated to an appropriate volume using the collodion bag. One hundred µl of the sample was applied on a gel cylinder containing 7.5 % polyacrylamide, 1 % Ampholine (pH 3.5-10; LKB), 20 % glycerol, 0.02 % ammonium persulfate, and 0.05 % TEMED. Anode and cathode vessels were filled with 20 mM HCl and 20 mM ethylenediamine, respectively. Electrophoresis was performed under a constant voltage at 200 V for 20 hr at 4 °C.

After electrophoresis, the gel was subjected to staining acid phosphatase using α-naphthylphosphate and Fast Garnet GBC salt as described in previous paper (Akiyama and Suzuki 1980). The stained isozyme bands were scanned at 520 nm using a Shimadzu dual wave length CS-900 scanner.

SDS-polyacrylamide gel electrophoresis

Stained band of pI 4 isozyme was cut off by a razor blade and incubated with 0.2 ml of 0.1 M sodium phosphate buffer (pH 7.2) containing 2 % SDS and 2 % 2-mercaptoethanol at 37 °C for 20 hr. The gel slice was then transferred to the top of a gel column containing 10 % polyacrylamide, 0.5 M Tris-HCl (pH 8.8), 0.1 % SDS, 0.02 % ammonium persulfate and 0.05 % TEMED. Both electrode vessels were filled with 25 mM Tris-glycine buffer (pH 8.4) containing 0.1 % SDS. After electrophoresis (6 mA/tube) at 24 °C for 4 hr, the gel was stained with 0.25 % Coomassie brilliant blue, followed by destaining with 25 % ethanol - 8 % acetic acid solution, and was scanned at 600 nm. Calibration proteins used for molecular weight determination were bovine serum albumin, egg albumin, pepsin (34,000), lactoalbumin (18,400) and lysozyme (14,300).

Results and Discussion

Figure 1 shows the isoelectric focusing profile of acid phosphatase isozyme in the enzyme solution obtained by ammonium sulfate precipitation of the crude extract from GA_3-treated wheat half-seeds. At least 9 isozyme peaks can be seen in the profile, which is essentially the same as reported previously. The increase of pI 4 isozyme by GA_3 is also discernible.

CM-cellulose column chromatography of the preparation of ammonium sulfate precipitation step gave two peaks of acid phosphatase (Fig. 2). More acidic peak was denoted peak I (P-I), whereas another one peak II (P-II) as in the previous paper (Akiyama and Suzuki 1980). Analysis by isoelectric focusing showed that P-I was composed mostly of pI 4 isozyme (Fig. 3A).

P-I was then subjected to gel filtration on a calibrated Shephadex G-100 column. A single peak was detected at the position corresponding to a molecular weight of ca. 55,000 daltons (Fig. 4). Isoelectric focusing of the peak fraction indicated that a certain degree of improvement in the purity of pI 4 isozyme was attained in this gel filtration step (Fig. 5).

Tricalcium phosphate adsorption is reported to be an effective way to fractionate phosphatase from crude enzyme preparations (Turner and Turner 1960). A preparation obtained

by the tricalcium phosphate treatment of ammonium sulfate-precipitated enzymes was analyzed by isoelectric focusing (Fig. 6). The resulting profile was practically the same as for the preparation in ammonium sulfate step (Fig. 1), indicating that all the acid phosphatase isozymes could be obtained without missing during the treatment.

Each of the pI 4 bands of various acid phosphatase preparations was subjected to SDS-polyacrylamide gel electrophoresis to see about its purity as protein (Fig. 7). A major protein peak moving moderately was detected in every pI 4 isozyme preparation tested. Its molecular weight was determined to be 62,000 daltons (Fig. 8). Acid phosphatase P-I, composed mostly of pI 4 isozyme, showed a molecular weight of 55,000 daltons (Fig. 4). However, the gel filtration analysis using Sephadex is known to give an underestimated molecular weight for glycoprotein like acid phosphatase molecule and hence, the above result seem to indicate that pI 4 isozyme of acid phosphatase is a monomaric protein of molecular weight ca. 62,000 daltons.

As seen from Fig. 7, contaminating proteins could be removed to some extent by a consecutive chromatographic fractionation on CM-cellulose and Sephadex G-100. Worth mentioning in this connection is that tricalcium phosphate treatment was strikingly effective in removing such protein contaminants. Thus, a simple procedure consisting of direct

treatment of the ammonium sulfate-precipitated sample with tricalcium phosphate and the following isoelectric focusing was found to be enough to obtain pI 4 isozyme in a purer form than that prepared through purification steps attempted here. The problem whether GA_3 stimulates *de novo* synthesis of pI 4 isozyme of acid phosphatase is under study using the improved procedure.

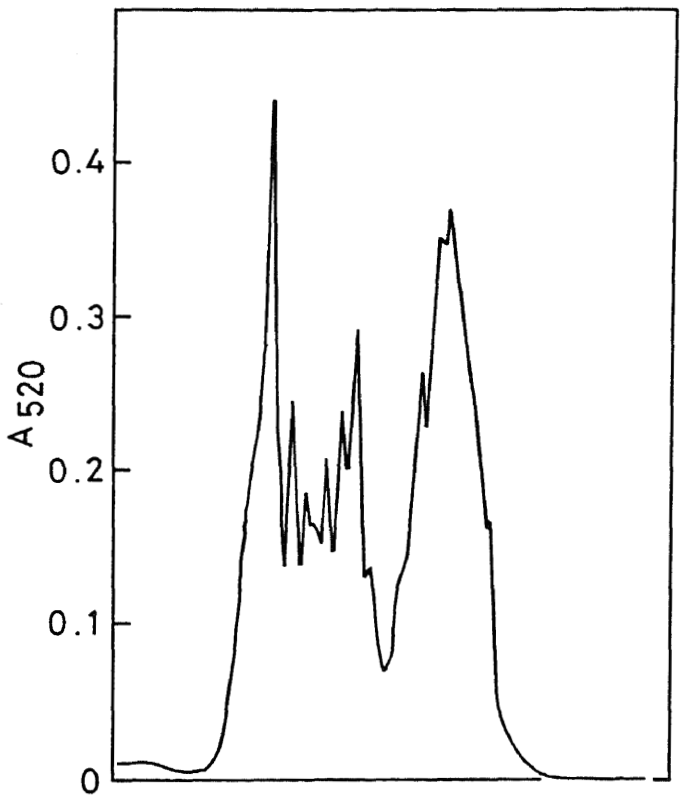

Fig. 1. Isoelectric focusing of wheat endosperm acid phosphatase preparation of ammonium sulfate precipitation step.

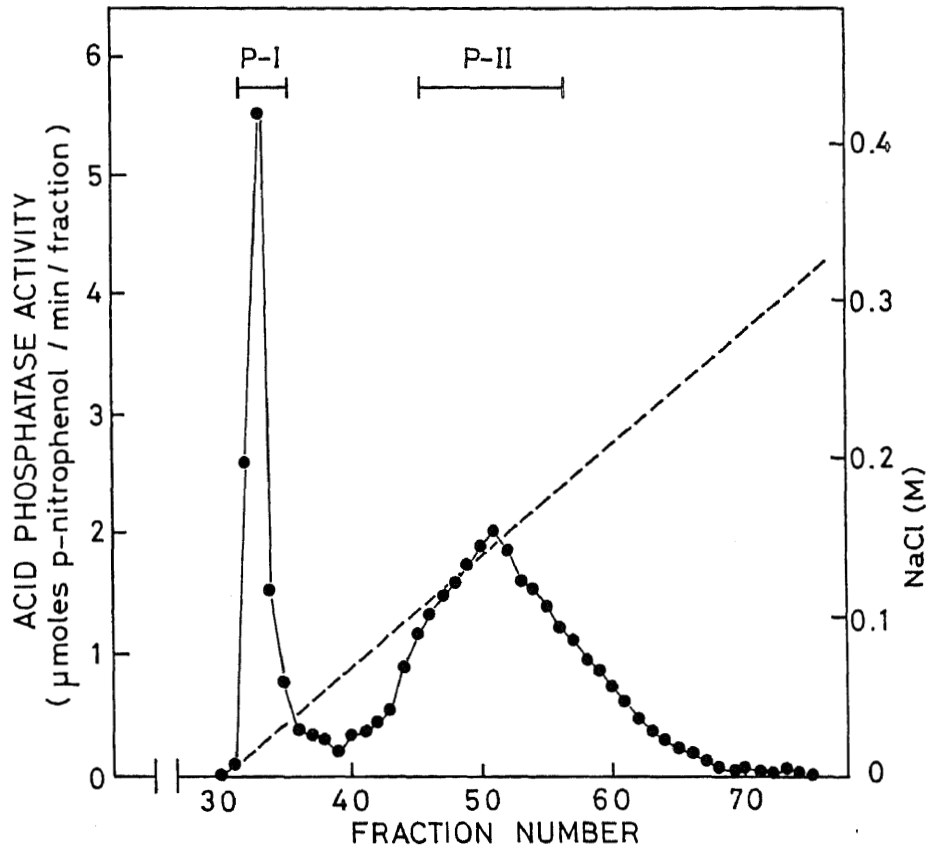

Fig. 2. CM-cellulose column chromatography of acid phosphatase preparation of ammonium sulfate precipitation step.

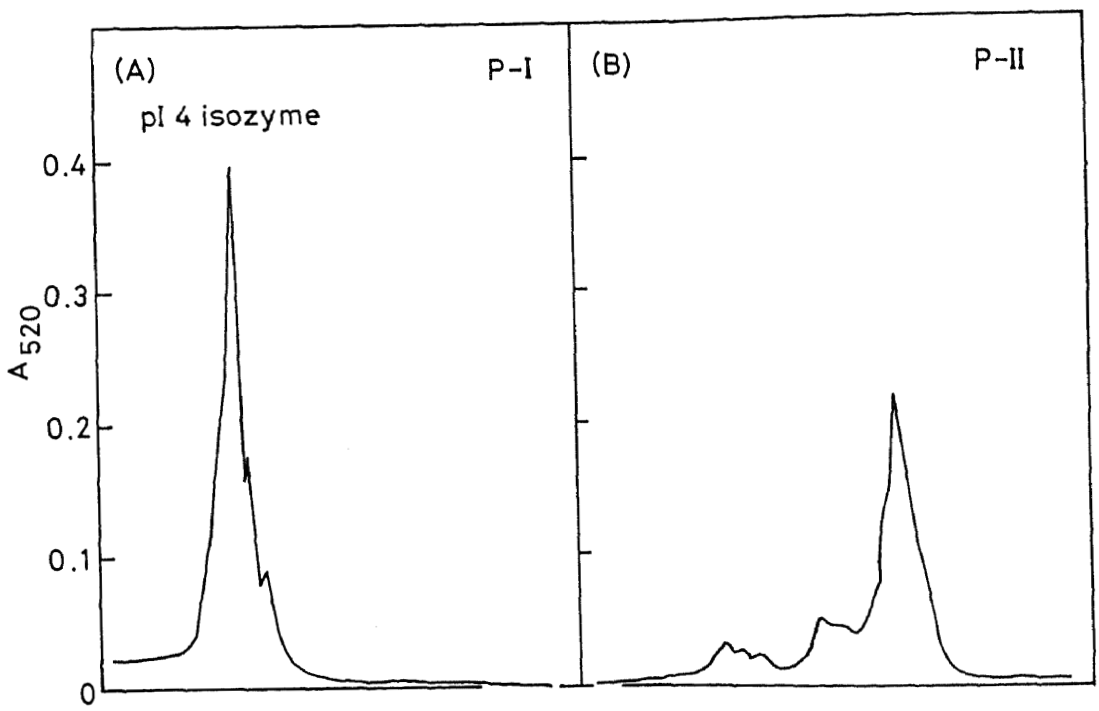

Fig. 3. Isoelectric focusing of acid phosphatase preparations (P-I and P-II) of CM-cellulose column chromatography step.

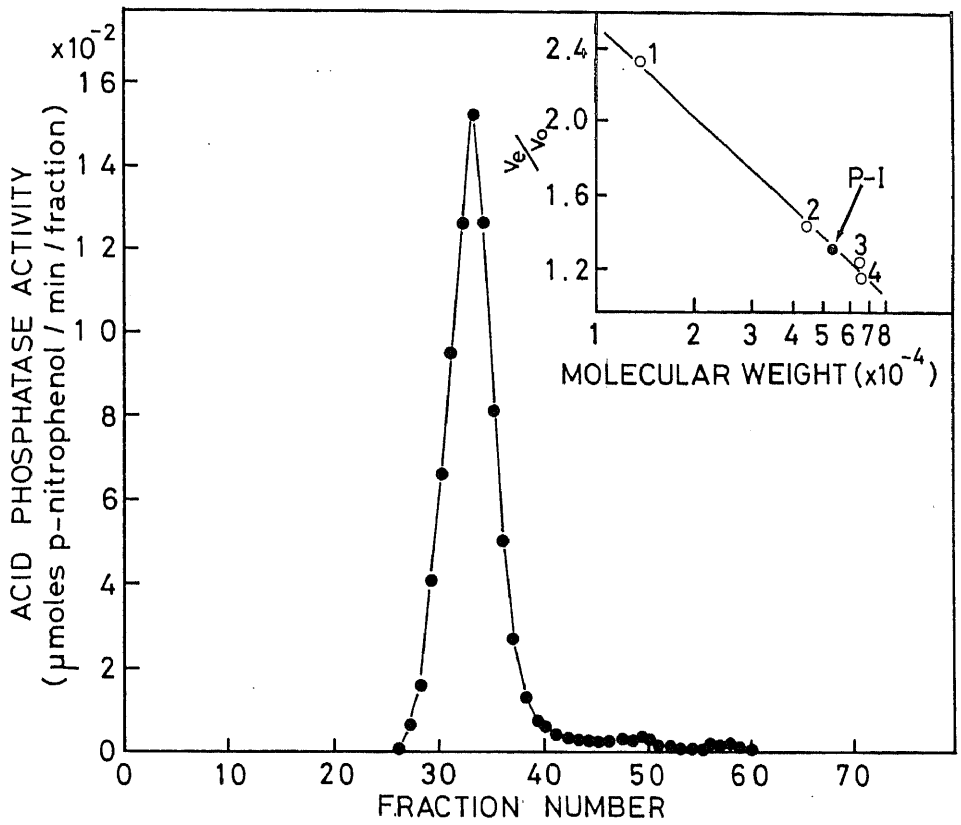

Fig. 4. Gel filtration of acid phosphatase preparation P-I (Fig. 3) on a calibrated column of Sephadex G-100. Standard proteins used for calibration are 1, bovine serum albumin (66,000); 2, bovine blood hemoglobin (64,500); 3, egg albumin (45,000); and 4, cytochrome c (13,000).

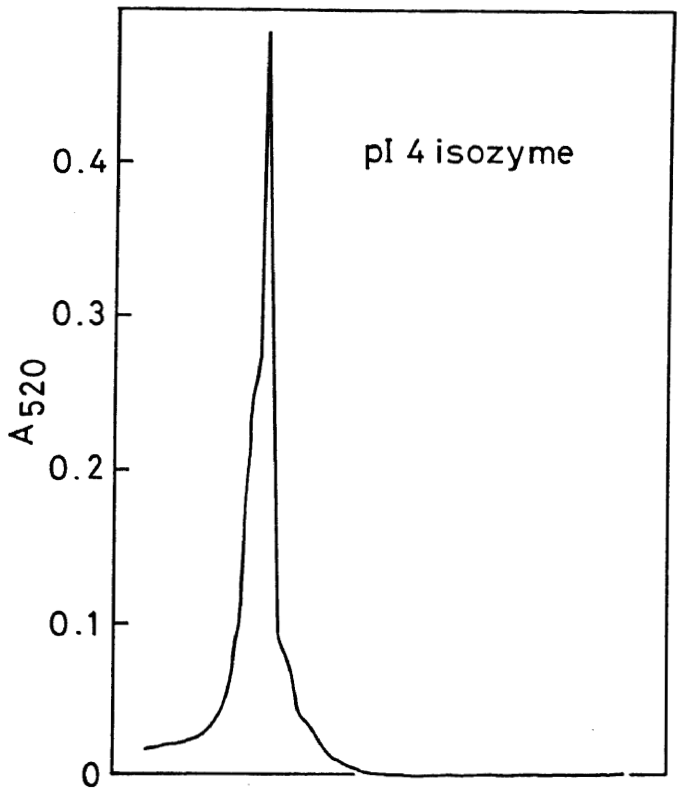

Fig. 5. Isoelectric focusing of acid phosphatase preparation P-I after Sephadex G-100 gel filtration.

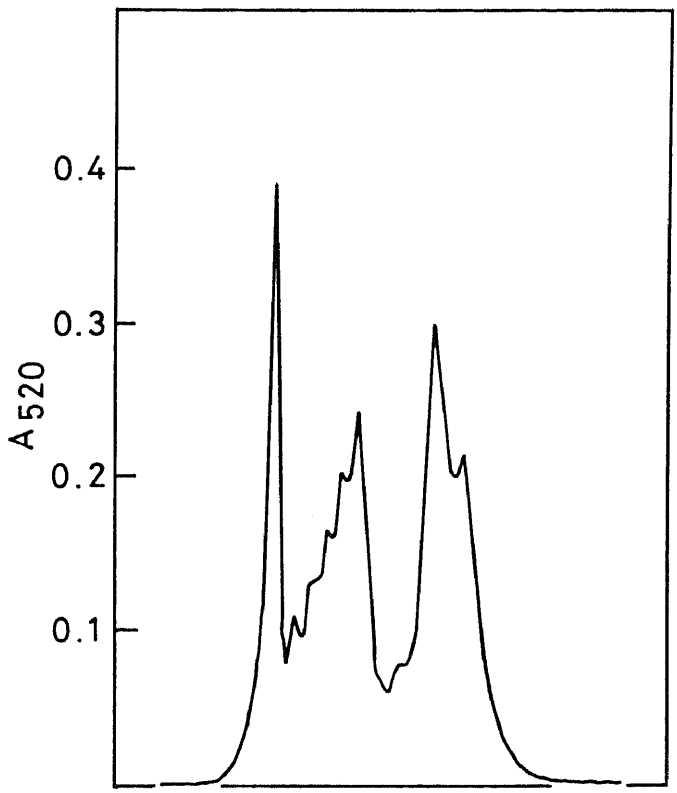

Fig. 6. Isoelectric focusing of acid phosphatase isozyme preparation obtained by tricalcium phosphate adsorption following ammonium sulfate step.

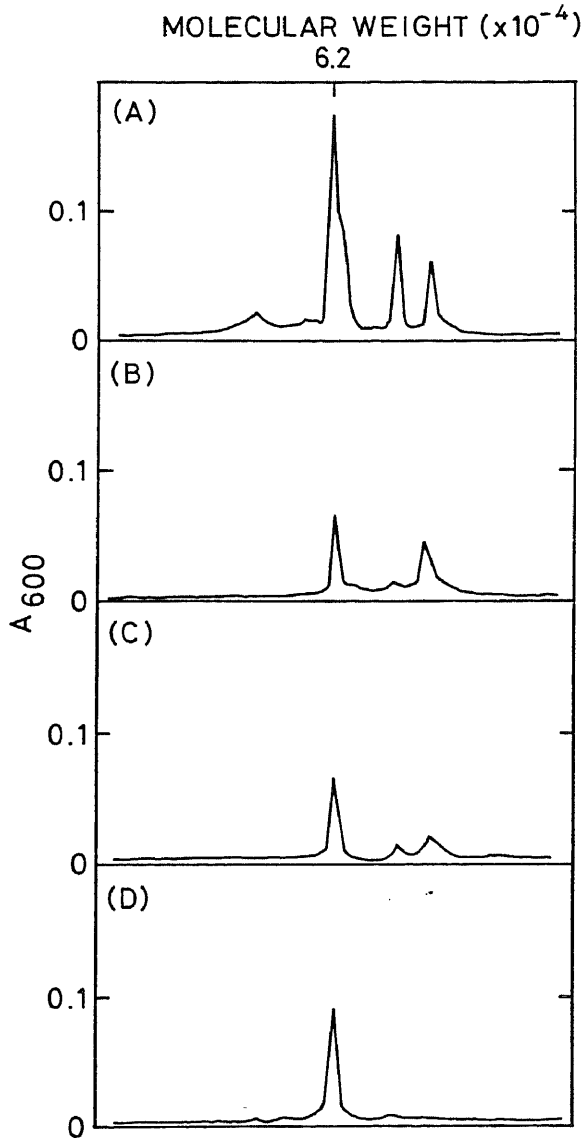

Fig. 7. SDS-polyacrylamide gel electrophoresis of pI 4 isozyme preparations. Each of the pI 4 isozyme bands from the acid phosphatase preparations of (A) ammonium sulfate step, (B) P-I of CM-cellulose chromatography step, (C) P-I of gel filtration step and (D) tricalcium phosphate adsorption step (Fig. 6) was subjected to electrophoresis on SDS-containing gel.

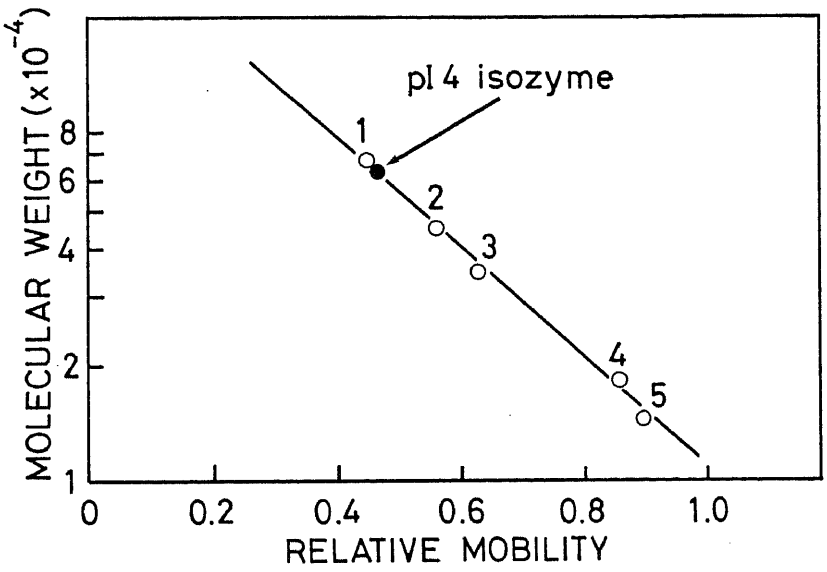

Fig. 8. Estimation of molecular weight of pI 4 acid phosphatase isozyme by SDS-polyacrylamide gel electrophoresis. pI 4 isozyme band from enzyme preparation treated with tri-calcium phosphate was used (cf. Fig. 7D). Calibration proteins used are 1, bovine serum albumin (66,000); 2, egg albumin (45,000); 3, pepsin (34,700); 4, lactoalbumin (18,400) and 5, lysozyme (14,300).

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CHAPTER VI

GIBBERELLIC ACID-STIMULATED *DE NOVO* SYNTHESIS OF A PARTICULAR ISOZYME OF ACID PHOSPHATASE IN WHEAT HALF-SEEDS

Chapter VI

Gibberellic acid-stimulated de novo synthesis of a particular isozyme of acid phosphatase in wheat half-seeds

Abstract

Gibberellic acid (GA_3) stimulated incorporation of both [^{35}S]-methionine and [^{3}H]-uridine into acid-insoluble materials in the cell free extract of wheat half-seeds. The incorporation of [^{35}S]-methionine into an acid phosphatase isozyme possessing isoelectric pH at 4.0 (pI 4 isozyme) was also promoted by GA_3. GA_3-stimulated de novo synthesis of pI 4 isozyme was almost completely inhibited by cycloheximide, but not by cordycepin. The results suggest the presence of preformed mRNA coding for pI 4 isozyme of acid phosphatase in wheat dry seeds and the translational stimulation of the enzyme synthesis by GA_3.

Introduction

We previously demonstrated that the increase in acid phosphatase activity observed in wheat half-seeds treated with GA_3 is due to largely to an isozyme of pI 4.0 in aleurone layers of the endosperm and that the increase of this particular isozyme can be found also in the endosperm of germinating wheat seeds (Akiyama, Uchimiya and Suzuki).

We also developed an efficient procedure for analysis of acid phosphatase isozyme pattern and fractionation of pI 4 isozyme using a dual gel electrophoretic runs, polyacrylamide gel isoelectric focusing and SDS-polyacrylamide gel electrophoresis (Chapter V). Using this procedure we have carried out the experiments to study whether or not this enzyme is synthesized de novo in GA_3-treated wheat half-seeds and the results are reported here.

Materials ans Methods

Preparation of half-seeds

Embryoless half-seeds Triticum aestivum L. cv. Nohrin No. 61 were prepared as reported previously (Akiyma and Suzuki 1980).

Incubation of half-seeds

One hundred half-seeds were incubated in 5 ml of sterile distilled water contained in a 200 ml-flask at 24 °C for 24 hr by shaking (120 strokes/min). After preincubation half-seeds were transferred to a 200 ml-flask containing 5 ml of 2 mM sodium acetate buffer (pH 5.2) and incubated further for 24 hr. GA_3, CHI, and cordycepin were added as specified in each experiment.

Labelling of protein and RNA

To study protein and RNA synthesis, half-seeds were incubated in the presence of [^{35}S]-methionine (6.7 μCi/ml) and [^{3}H]-uridine (4.5 μCi/ml). After 24 hr labelling, 10 half-seeds were homogenized in a mortar and pestle with 3 ml of 0.1 M Tris-HCl buffer (pH 7.4) containing 20 mM NaCl at 4 °C. The homogenate was centrifuged at 20,000 x g for 20 min. To 0.1 ml aliquot of the supernatant, an equal volume of 10 % TCA was added. The precipitate was collected on Whatman GF/C glass microfiber filter by filtration and

washed twice successively with 10 ml of 5 % TCA, distilled water and 70 % ethanol. After drying, the filter was transferred into a counting vial and the radioactivity was counted with a Beckman liquid scintillation spectrometer (Model LS-250) in 5 ml of a scintillator (toluene: 1000 ml, 2,5,-diphenyloxasol: 4.0 g, 1,4-Bis-2-(5-phenyloxasolyl)benzene: 0.1 g).

Preparation of protein sample

The extract of half-seeds was prepared as previously described (Akiyama and Suzuki 1980) and proteins precipitable at 75 % satulation with ammonium sulfate were collected and subjected to tricalcium phosphate treatment as described in Chapter V.

Isoelectric and SDS-polyacrylamide gel electrophoresis

Acid phosphatase isozymes in the protein sample were fractionated by polyacrylamide gel isoelectric focusing, stained by the diazo-dye method and scanned at 520 nm. The band of pI 4 isozyme was then subjected to SDS-polyacrylamide gel electrophoresis as described in Chapter V. In both cases the gel was sliced to 2 mm-thick pieces and dispensed to vials. After treatment with 0.5 ml of 30 % hydrogen peroxide overnight at 60 °C, 5 ml of a scintillator (toluene: 700 ml, Triton x-100: 300 ml, 1,4-Bis-2-(5-phenyloxasolyl)benzene: 0.2 g, 2,5-diphenyloxasol: 4.0 g) was added to each vial and counted for radioactivity as above.

Results

The influence of GA_3, cordycepin and CHI on RNA and protein synthesis

The effect of GA_3, cordycepin and CHI on the incorporation of [^{3}H]-uridine into acid-insoluble materials in the half-seed extract was tested (Fig. 1). GA_3 stimulated incorporation, especially for the second 12 hr. Cordycepin and CHI inhibited the GA_3-stimulated incorporation by about 50-70 % irrespective of observation time. The effect on the incorporation of [^{35}S]-methionine was similarly tested (Fig. 2). GA_3 stimulated [^{35}S]-methionine incorporation into acid-insoluble materials at a constant rate during 24 hr. The effect of GA_3 for the second 12 hr but almost not for the first 12 hr was supressed by CHI and cordycepin up to 90 %.

The influence of GA_3, cordycepin and CHI on acid phosphatase activity and incorporation of [^{35}S]-methionine

As reported previously, at least 9 isozymes were found in all of the isoelectric focusing profiles of the enzyme preparations from half-seeds incubated under various conditions (Fig. 3). [^{35}S]-methionine incorporation was found to be generally associated with more acidic proteins. GA_3 caused an appreciable enhancement of both the enzyme activity and the radioactivity in the region of acidic proteins including pI 4 isozyme of acid phosphatase (Fig. 3B). In the

presence of cordycepin, this effect of GA_3 was reduced only slightly (Fig. 3C). On the contrary, CHI inhibited the GA_3 effect almost completely (Fig. 3D).

To analyze [^{35}S]-methionine incorporation into pI 4 isozyme and the influence thereon of GA_3, cordycepin and CHI, the respective pI 4 bands were extracted and subjected to SDS-polyacrylamide gel electrophoresis. Determination of radioactivity in gel slices revealed the existence of a single labeled protein of 62,000 daltons in each sample (Fig. 4). Furthermore, the changing pattern of the rate of ^{35}S-incorporation in response to treatment with GA_3, cordycepin and CHI was quite similar to that observed in Fig. 2: stimulation by GA_3, cancellation by CHI and indifference to cordycepin of GA_3 effect.

Discussion

The aim of this study was to approach to the question whether GA_3-induced increase in pI 4 isozyme activity is caused by de novo synthesis of this enzyme or by its activation.

Comparison of the effects of GA_3 and inhibitors of RNA and cytoplasmic protein synthesis on uridine and methionine incorporations into acid-insoluble polymers seem to suggest that GA_3 may not only stimulate some translation-dependent transcription and/or post-transcriptional process occurring in earlier periods of its action but also some early protein synthesis depending on preformed RNA and being less sensitive to CHI in gross. At least, GA_3 stimulation of methionine incorporation into protein of half-seeds could be confirmed.

According to the fractionation procedure developed in Chapter V, incorporation of [^{35}S]-methionine into pI 4 acid phosphatase was studied. The results showed that pI 4 isozyme of half-seeds was synthesized de novo at a low basal rate even without the addition of GA_3, whereas GA_3 stimulated the rate dramatically, and that this stimulation was completely inhibited by CHI but not by cordycepin. These findings strongly suggest the occurence of preformed mRNA for pI 4 isozyme of acid phosphatase in wheat half-seeds. Preliminary experiments using the antibody for pI 4 isozyme gave the results supporting the above suggestion.

The existence of stored mRNA has been reported for various plant seeds (Maherchandani and Naylor 1966, Payne 1976, Peumans and Carlier 1977). However, little is known about hormonal activation of mRNA during germination. Efforts are being made firstly to obtain direct evidence for the presence of mRNA in wheat dry seeds.

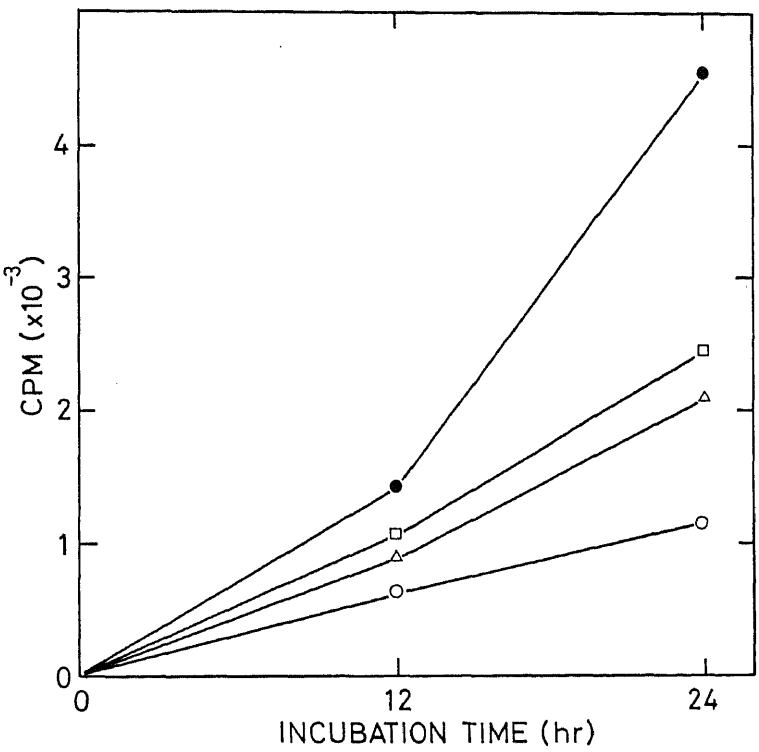

Fig. 1. [^{3}H]-Uridine incorporation into TCA-insoluble fraction of extracts from half-seeds treated with GA_3 or GA_3 and inhibitors. ●, GA_3 (10^{-5} M); □, GA_3 + cordycepin (100 µg/ml); △, GA_3 + CHI (10 µg/ml); ○, control (-GA_3).

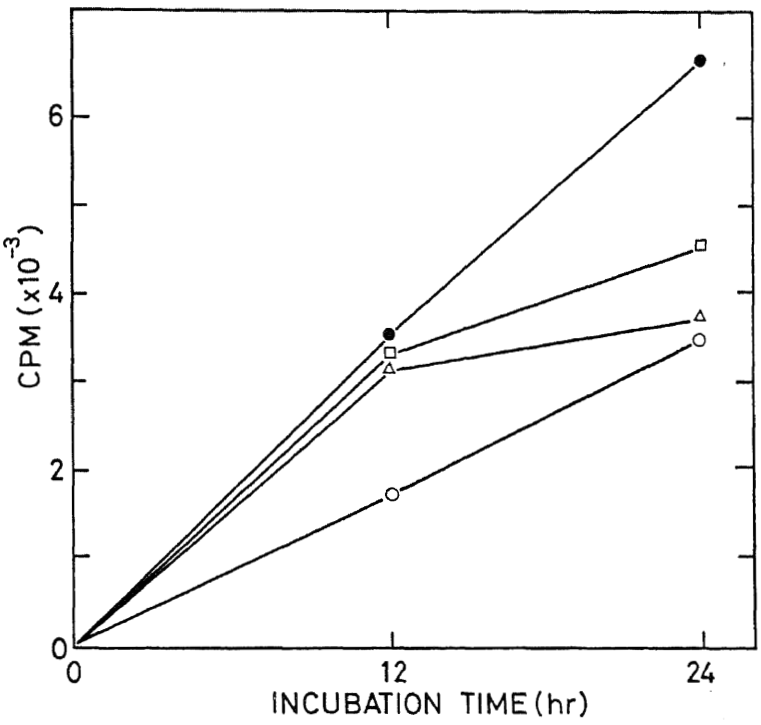

Fig. 2. [^{35}S]-Methionine incorporation into TCA-insoluble fraction of extracts from half-seeds treated with GA_3 or GA_3 and inhibitors. ●, GA_3 (10^{-5} M); □, GA_3 + cordycepin (100 µg/ml); △, GA_3 + CHI (10 µg/ml); ○, control (-GA_3).

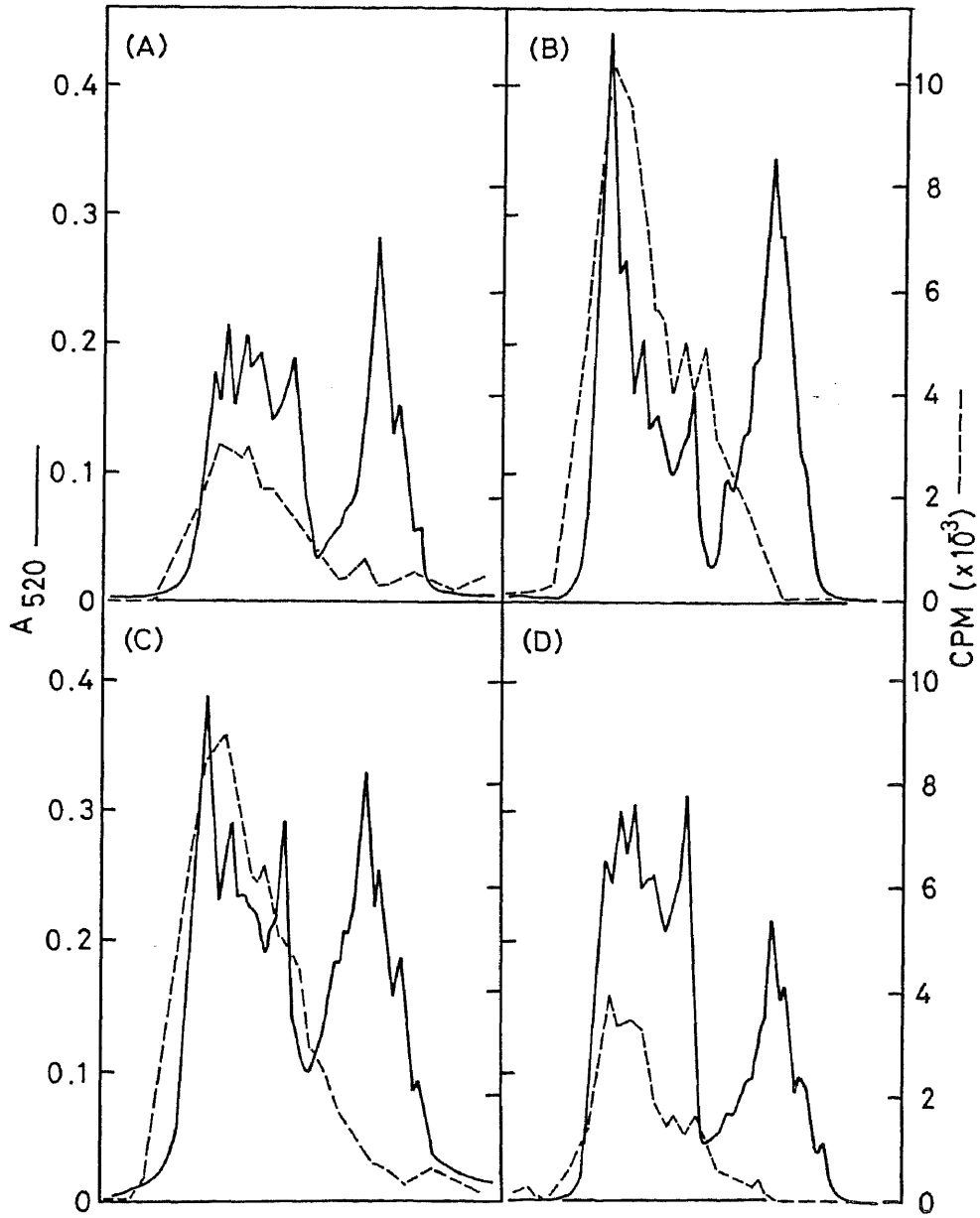

Fig. 3. Isoelectric focusing patterns of acid phosphatase isozymes and labelling with [^{35}S]-methionine in protein sample from half-seeds treated with GA_3 or GA_3 and inhibitors for 24 hr. (A) -GA_3, (B) 10^{-5} M GA_3, (C) 10^{-5} M GA_3 + 100 µg/ml cordycepin, (D) 10^{-5} M GA_3 + 10 µg/ml CHI.

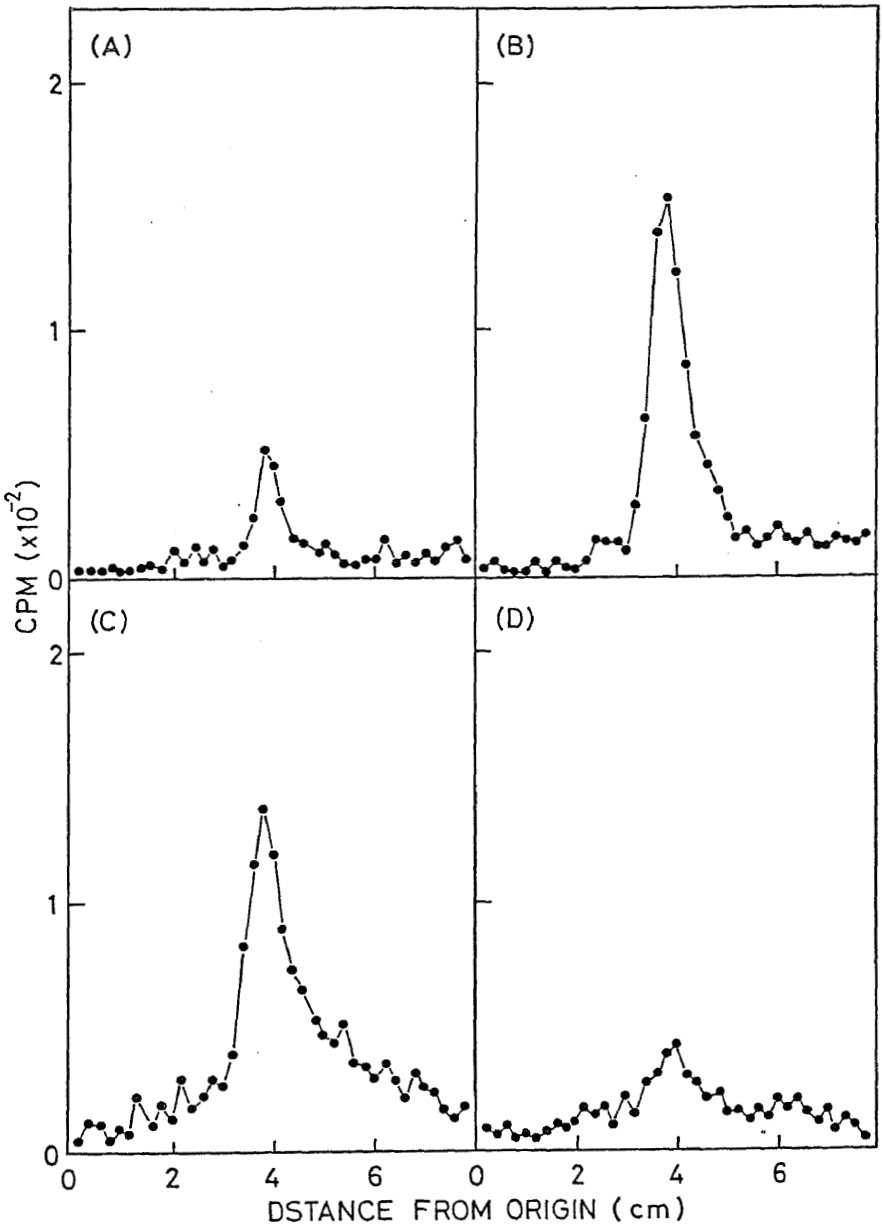

Fig. 4. SDS-gel electrophoretic patterns of [^{35}S]-methionine labelling in fractions of pI 4 acid phosphatase isozyme (Fig. 3). (A) -GA_3, (B) 10^{-5} M GA_3 + 100 µg/ml cordycepin, (C) 10^{-5} M GA_3 + 10 µg/ml CHI.

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Conclusions

Though a measurable amount of acid phosphatase was present in dry wheat half-seeds, GA_3 considerably enhanced the activity in embryoless half-seeds and its release into the incubation medium. Acid phosphatase preparations from the half-seeds and the medium was separated into two major fractions by CM-cellulose column chromatography. However, chromatographic profiles of acid phosphatase were almost the same between GA_3-treated and the control half-seeds. These two enzyme fractions differed from each other in substrate specificity and pH dependency of the activity.

Both the increase of acid phosphatase activity in half-seeds and its release into the medium were inhibited by CHI. Cordycepin inhibited the release of the enzyme into the medium, whereas it hardly affected the increase of the activity in half-seeds. This suggests that GA_3 may increase the acid phosphatase activity in half-seeds through its effect on translational process.

Cytoplasmic localization of acid phosphatase in wheat aleurone cell was confirmed by histochemical observations. Subcellular fractionation experiments showed that bulk of the enzyme activity was in the soluble fraction regardless of GA_3 treatment and only a low level of the activity was associated with particulate fractions from the GA_3-treated tissue. It may be likely, however, that acid phosphatase

previously contained in subcellular compartments was released readily into the soluble fraction during homogenization of aleurone layers.

Using polyacrylamide gel isoelectric focusing, acid phosphatase of wheat half-seeds was found to be composed of at least 9 different isozymes with each isoelectric pH ranging from 4.0 to 7.2. Among these isozymes, only a particular isozyme possessing isoelectric pH of 4.0 (pI 4 isozyme) was enhanced significantly by GA_3. The isozyme pattern of the enzyme release into the medium by GA_3 was similar to that of the retained enzyme and therefore there seems to be no selective release among the isozyme.

A similar increase of pI 4 isozyme of acid phosphatase was found to occur also in the endosperm portion of germinating seeds. The finding supports the observations with the half-seeds treated with GA_3 reflect the actual phenomenon which may be induced by gibberellin originated from the embryo in germinating seeds.

A simple procedure for isolation of pI 4 isozyme using isoelectric focusing and SDS-gel electrophoresis was established and by this procedure, the incorporation of [^{35}S]-methionine into pI 4 isozyme of acid phosphatase was compared among half-seeds incubated in the presence or absence of GA_3, CHI and cordycepin. The results were: (1) GA_3 stimulates *de novo* synthesis of pI 4 isozyme, (2) CHI completely inhibits this

stimulation by GA_3 and (3) cordycepin does not produce any significant effect on the GA_3 stimulation. These facts strongly suggest that the preformed mRNA for pI 4 isozyme of acid phosphatase may exist in wheat half-seeds, even though there remains a possibility of GA_3-dependent activation of mRNA.

There are many instances suggesting the presence of long-lived mRNA in various plant seeds. However, little is known about such mRNA for a definite enzyme. Efforts are being made to prepare mRNA fraction from wheat half-seeds and to demonstrate the polypeptide homologous to pI 4 isozyme of acid phosphatase *in vitro*.

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