Phytochemistry, Vol. 21, No. 3, pp. 585–590, 1982.
Printed in Great Britain.

0031-9422/82/030585-06$03.00/0
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GIBBERELLIC ACID-MEDIATED ACTIVATION OF MONOPHENOLASE IN DE-EMBRYONATED HALF-SEEDS OF WHEAT (*TRITICUM AESTIVUM*)

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(*Revised received* 15 *June* 1981)

Key Word Index—*Triticum aestivum*; Gramineae; de-embryonated half-seeds; gibberellic acid; hormonal control of monophenolase activity; enzyme stability and activation through oligomerization.

Abstract—Stimulation of monophenolase activity was observed when de-embryonated prewashed half-seeds of wheat were imbibed in a solution of gibberellic acid (GA_3, 10^{-5} M). Crude extracts, prepared from GA_3-treated half-seeds, showed *ca* a two-fold increase in monophenolase activity over the controls at pH 6.6, while a dramatic rise in enzyme activity (seven- to nine-fold) was observed at pH 9.0. The partially purified $(NH_4)_2SO_4$ fraction precipitate (30–50% saturation) also showed enhancement of enzyme activity at pH 9.0 in GA_3-treated half-seeds, while in controls, there was negligible activity at this pH. Administration of five amino acid analogues (1 mM each) to half-seeds showed no significant inhibition of GA_3-stimulated monophenolase activity, but proved very effective in decreasing (86% inhibition) the GA_3-induced amylase activity. This indicated that the hormone-regulated monophenolase activity was not dependent on *de novo* protein synthesis. Treatment of half-seeds with GA_3 modified the monophenolase and altered the electrophoretic pattern. The enzyme was relatively thermostable at 55° and the pH optimum was shifted from pH 7.0 to 9.0. In addition, the GA_3-treated half-seeds showed relatively high stability of enzyme activity in the presence of $(NH_4)_2SO_4$ ions. These alterations in the GA_3-stimulated nomophenolase suggest there is activation of preformed enzyme molecules. The appearance of slow-migrating multiple forms on acrylamide gels, in response to GA_3 treatment, is probably due to the association of fast-migrating forms. Such oligomerization could result in a conformational change leading to enzyme activation. This may be an adaptive mechanism so that the enzyme can function with varying temperature, pH and ionic strength during early stages of seed germination.

INTRODUCTION

There are a few reports in the literature where the stimulation of enzyme activity by a phytohormone is brought about by the activation of preformed enzyme proteins. In barley aluerone layers, GA_3 stimulated phosphorylcholine glyceride transferase activity through the phenomenon of activation [1]. The stimulation of β-amylase in GA_3-treated barley endosperm tissue was also brought about by the activation of zymogen molecules [2]. In mature bean leaves, the enhancement of ribulose diphosphate carboxylase by gibberellin and cytokinin was ascribed to the activation of pre-existing enzyme molecules [3]. Likewise, activation of acid phosphatase enzyme was reported in GA_3-treated barley endosperm tissue [4]. In pea stem tissue, the stimulation of β-glucan synthetase by auxin was not inhibited by actinomycin D and cycloheximide at concentrations that blocked RNA and protein synthesis respectively [5, 6]. Auxins also stimulated RNA polymerase I activity in lentil roots and soybean hypocotyls. In lentil roots, auxin seems to promote the synthesis of a protein factor which activated RNA polymerase I [7, 8], while in soybean hypocotyl tissue, auxin treatment increased the specific activity of RNA polymerase I [9, 10]. A two-fold stimulation of monophenolase activity was reported in GA_3-treated half-seeds of wheat. The hormone-triggered enzyme activity was not inhibited by actinomycin D and cycloheximide [11]. In this investigation, we have shown that the increased monophenolase activity in the GA_3-treated wheat half-seeds is brought about by the activation of preformed enzyme molecules.

RESULTS AND DISCUSSION

Effect of GA_3 *on monophenolase activity in unwashed and washed half-seeds of wheat*

Imbibition of unwashed half-seeds of wheat in soln of GA_3 (10^{-5} M) showed only 20% increase in monophenolase activity over the controls (Table 1). Half-seeds, washed exhaustively in sterile distilled water (for 6 hr), proved a more sensitive system for studying hormonal regulation of enzyme activity. A significant enhancement of enzyme activity (about 80–100%) was achieved by treating washed half-seeds with GA_3 (10^{-5} M) (Table 1, Fig. 1). Prewashing of half-seeds substantially decreased (about two-fold) the basal level of monophenolase activity. The stimulatory effect of GA_3 became more pronounced when the basal enzyme activity was lowered in controls as a result of washing treatment (Table 1). The half-seeds probably contain endogenous GA_3 which if leached by washing, resulted in the deactivation of the monophenolase. Exogenous addition of GA_3 to the washed half-seeds presumably activated the

Table 1. Effect of gibberellic acid (GA_3) on monophenolase activity in unwashed and washed half-seeds of wheat

	Monophenolase activity			
	Unwashed half-seeds		Washed half-seeds	
Additions	Absorbance units at 420 nm/g dry wt of half-seeds	% control	Absorbance units at 420 nm/g dry wt of half-seeds	% control
Control	1.20	100	0.62	100
GA_3, 10^{-5} M	1.44	120	1.22	196

The half-seeds were imbibed in sterile water at 25° in the dark for 48 hr. GA_3 was present throughout the period of imbibition. The half-seeds were washed in sterile water at 20° for a period of 6 hr and then kept for imbibition for 48 hr. The enzyme activity was assayed in crude extracts at pH 6.6.

monophenolase. Subsequent experiments were, therefore, performed with washed half-seeds.

Insensitivity of GA_3-stimulated monophenolase activity to amino acid analogues

The effect of amino acid analogues was examined in GA_3-treated half-seeds as a probe to understand the mode of enzyme regulation. Addition of five amino acid analogues (1 mM each) to control and GA_3-treated half-seeds did not effectively inhibit the monophenolase activity; there was only a 20% decrease in enzyme activity in control and GA_3-treated half-seeds (Table 2).

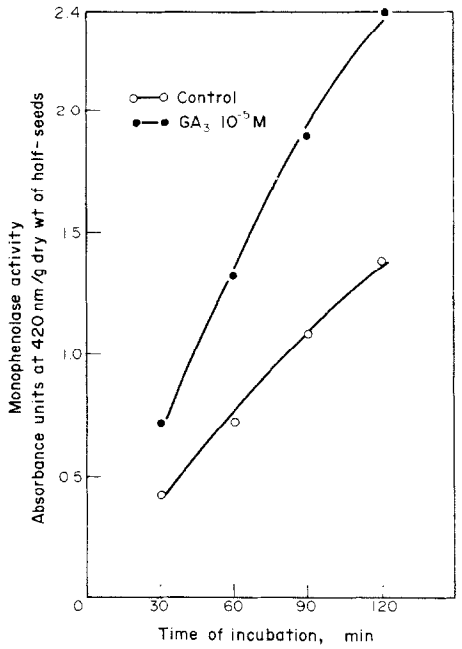

Fig. 1. Kinetics of monophenolase activity in the control and GA_3-treated half-seeds of wheat. The prewashed half-seeds were imbibed in sterile water in the dark at 25° in the presence and absence of GA_3 (10^{-5} M) for 48 hr. The enzyme activity was assayed in crude extracts at pH 6.6.

Inhibition of GA_3-stimulated amylase activity by amino acid analogues

Since amino acid analogues did not substantially inhibit GA_3-stimulated monophenolase activity, it was considered necessary to ascertain their effective penetration into half-seeds. For this purpose, their effect was tested on GA_3-induced amylase activity in wheat half-seeds. A six-fold stimulation of amylase activity was observed. Amino acid analogues completely blocked the GA_3-stimulated amylase activity, thus proving the entry of the analogues in half-seeds in effective concentrations. Competition of analogues (1 mM each) with high concentration of amino acids (2 mM each) substantially reversed the inhibition of

Table 2. Effect of amino acid analogues on monophenolase activity in the control and GA_3-treated half-seeds of wheat

	Monophenolase activity	
Additions	Absorbance units at 420 nm/g dry wt of half-seeds	% activity
Control	0.67	100
Five amino acid analogues (1 mM each)	0.54	80.6
GA_3, 10^{-5} M	1.50	100
GA_3, 10^{-5} M + five amino acid analogues (1 mM each)	1.20	80.0

The washed half-seeds of wheat were imbibed in sterile water for 48 hr at 25° in the dark. The half-seeds were washed for 6 hr in sterile water at 20° and imbibed in the continuous presence of five amino acid analogues (1 mM each of L-canavanine, DL-ethionine, DL-7-azatryptophan, DL-*o*-fluorophenylalanine, L-thioproline) with and without GA_3 (10^{-5} M). The enzyme activity was assayed in crude extracts at pH 6.6. The values given are an average of two experiments.

GA_3-stimulated amylase activity (Table 3). Thus, these amino acid analogues are effective inhibitors, provided the stimulation of enzyme is dependent on *de novo* protein synthesis. The failure of analogues to block GA_3-mediated stimulation of monophenolase activity to any substantial level thus supports the hypothesis that the enzyme is regulated by activation.

Increased thermostability of monophenolase activity in GA_3-treated half-seeds

If the stimulation of monophenolase activity by GA_3 is brought about by the activation of preformed enzymes, distinct differences should be observable in the properties of monophenolase in control and GA_3-treated half-seeds. This approach seemed promising since heat-coagulated proteins in crude extracts of control and GA_3-treated half-seeds showed a differential sedimentation behaviour. This preliminary observation prompted us to compare the thermolability of monophenolase activity in control and GA_3-treated half-seeds. Crude extracts were maintained at 55° in a water-bath for different intervals of time (1–9 min). After heat-treatment, the samples were instantly chilled in ice and the precipitated proteins removed by centrifugation (30000 g, 20 min). The enzyme activity was assayed in a clear supernatant fraction at 37°. Crude extracts of controls showed a substantial loss of monophenolase activity (about 70% decrease) following heat treatment, while no significant decrease in the enzyme activity (about 10% decrease) was observed in crude extracts of GA_3-treated half-seeds (Fig. 2). Heat inactivation experiments were also performed with the $(NH_4)_2SO_4$ fraction precipitate (30–50% saturation; desalted on Sephadex G-25) prepared from GA_3-treated half-seeds. The partially purified fraction also exhibited thermostability of monophenolase activity at 55°, as was observed in crude preparations. This excluded the role of any cofactor in conferring enzyme stability in our crude extracts. It is envisaged that GA_3-induced heat stability of monophenolase activity reflects a conformational change in the preformed enzyme.

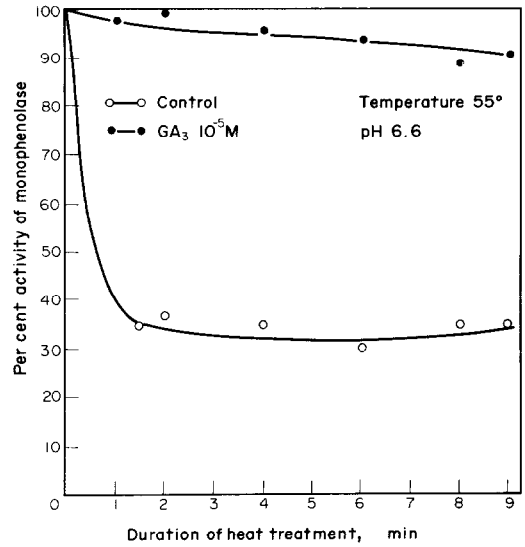

Fig. 2. Heat inactivation of monophenolase enzyme in the control and GA_3-treated half-seeds. The prewashed half-seeds of wheat were imbibed in sterile water at 25° in the dark for 48 hr. The crude extracts were maintained at 55° for different intervals of time and were chilled immediately after heat treatment. The precipitated protein was removed by centrifugation and the enzyme activity was assayed in the clear supernatant fraction.

Table 3. Inhibition of GA_3-stimulated amylase activity by five amino acid analogues in embryoless half-seeds of wheat

Additions	Amylase activity Enzyme units/g dry wt of half-seeds	Relative activity
Control	86	1.00
GA_3, 10^{-5} M	527	6.12
GA_3, 10^{-5} M + five amino acid analogues (1 mM each)	73	0.84
GA_3, 10^{-5} M + five amino acid analogues (1 mM each) + five amino acids (2 mM each)	392	4.55

The half-seeds were imbibed for 48 hr at 25° in the dark. Gibberellic acid (10^{-5} M), five amino acid analogues (1 mM each of L-canavanine, DL-ethionine, DL-7-azatryptophan, DL-*o*-fluorophenylalanine, L-thioproline) and five amino acids (2 mM each of L-arginine, DL-methionine, DL-tryptophan, DL-phenylalanine, L-proline) were present throughout the course of imbibition. Amylase activity was assayed in crude extracts at pH 6.0.

GA_3-mediated shift in the pH optimum of monophenolase activity

We next compared the pH optimum of monophenolase activity in control and GA_3-treated half-seeds, enzyme activity being plotted as a function of pH. Monophenolase activity was assayed using buffers (0.05 M) of different pH values (pH 5.0–10.0). In crude extracts of controls, the maximum enzyme activity was observed at pH 7.0, while in GA_3-treated half-seeds, a peak activity was obtained at pH 9.0 (Fig. 3). This marked shift in the pH optimum, in response to GA_3 provided additional support in favour of activation of monophenolase. In another set of experiments, monophenolase activity was compared in crude extracts of controls and GA_3-treated half-seeds at pHs 7 and 9. The hormone evoked *ca* 1.8-fold increase in enzyme activity at pH 7.0, while a seven- to nine-fold stimulation of enzyme activity was witnessed at pH 9.0 (Table 4). Similar results were obtained when the partially purified $(NH_4)_2SO_4$ fraction precipitate (30–50% saturation) was prepared from the control and GA_3-treated half-seeds. After desalting on Sephadex G-25, the enzyme activity was tested at pH 7.0 and 9.0. The results were identical to those observed in crude extracts (Table 5). Thus, the difference in the pH optimum cannot be attributed to

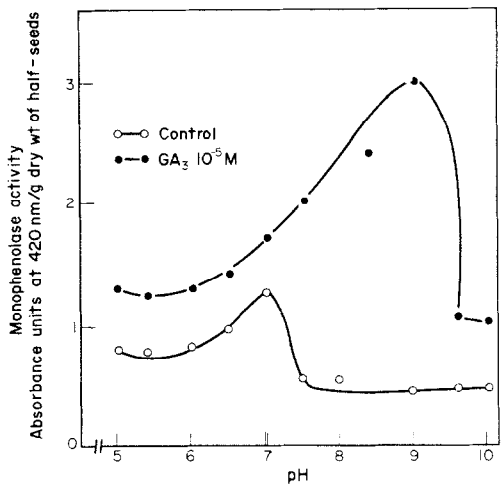

Fig. 3. The pH curves of monophenolase in the control and GA_3-treated half-seeds. The prewashed half-seeds of wheat were imbibed in sterile water at 25° in the dark for 48 hr. The crude extracts, prepared from the control and GA_3-treated half-seeds, were assayed for monophenolase activity at a wide range of pH values (pH 5.0–10.0).

the presence of a cofactor in crude extracts. Since the pH optimum differs, it was considered necessary to perform heat inactivation experiments at the respective pH optima. After heat-treatment, the pH of the incubation medium was adjusted to pH 7.0 and pH 9.0 using appropriate buffers (0.05 M). Heat inactivation curves followed a pattern (see Fig. 2) similar to that observed earlier at pH 6.6. This indicated that the thermostability of monophenolase activity in GA_3-treated half-seeds is maintained over a wide range of pH.

Effect of ionic strength on GA_3-stimulated monophenolase activity

A differential response of monophenolase activity was witnessed when crude extracts of control and GA_3-treated half-seeds were assayed in the presence of $(NH_4)_2SO_4$ (5–50 mM). In controls, the ionic strength of the salt brought a strong inhibition (about 80% decrease) of enzyme activity, whereas in crude extracts of GA_3-treated half-seeds, the enzyme was relatively stable (*ca* 25% decrease). This provided additional proof in support of altered molecular properties of this enzyme in GA_3-treated half-seeds. The

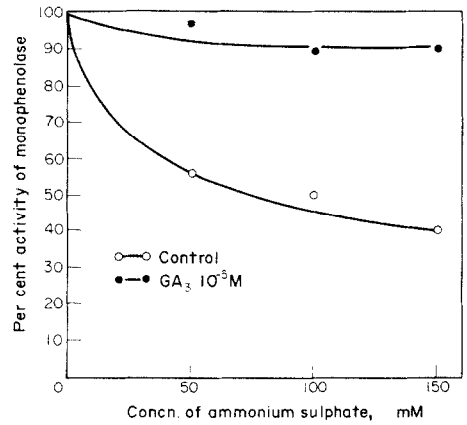

Fig. 4. Effect of $(NH_4)_2SO_4$ ions on the monophenolase activity in partially purified fractions of the control and GA_3-treated wheat half-seeds. The crude homogenates, prepared from the control and GA_3-treated half-seeds (imbibed for 48 hr), were fractionated with solid $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ fraction precipitate (30–50% saturation) was desalted on Sephadex G-25 and the monophenolase activity was assayed in presence of $(NH_4)_2SO_4$ ions (50–150 mM).

Table 4. Effect of pH on monophenolase activity in crude extracts of the control and GA_3-treated half-seeds of wheat

	Monophenolase activity at pH 7.0		Monophenolase activity at pH 9.0	
Additions	Absorbance units at 420 nm/g dry wt of half-seeds	Relative activity	Absorbance units at 420 nm/g dry wt of half-seeds	Relative activity
Experiment 1				
Control	1.00	1.00	0.43	1.00
GA_3, 10^{-5} M	1.92	1.92	3.12	7.43
Experiment 2				
Control	0.96	1.00	0.36	1.00
GA_3, 10^{-5} M	1.72	1.79	3.30	9.17

Crude extracts were prepared from control and GA_3-treated half-seeds which were imbibed for 48 hr in dark at 25°. Monophenolase activity was assayed in both fractions at pH 7.0 and 9.0.

Table 5. Effect of pH on monophenolase activity in the $(NH_4)_2SO_4$ fraction precipitate of the control and GA_3-treated half-seeds of wheat

Additions	Monophenolase activity at pH 7.0		Monophenolase activity at pH 9.0	
	Absorbance units at 420 nm	Relative activity	Absorbance units at 420 nm	Relative activity
Experiment 1				
Control	0.16	1.00	0.05	1.00
GA_3, 10^{-5} M	0.28	1.75	0.40	8.00
Experiment 2				
Control	0.15	1.00	0.05	1.00
GA_3, 10^{-5} M	0.27	1.80	0.39	7.80

The $(NH_4)_2SO_4$ fraction precipitate (30–50% saturation) was prepared from the control and GA_3-treated half-seeds. This partially purified fraction was desalted on Sephadex G-25 and monophenolase activity was assayed at pH 7.0 and 9.0. Each assay mixture contained 1.5 mg of $(NH_4)_2SO_4$ fraction precipitate.

differential response of enzyme activity towards ionic strength could not be ascribed to the presence of a cofactor in crude extracts. This became evident from our subsequent experiments with partially purified fractions. The $(NH_4)_2SO_4$ fraction precipitate (30–50% saturation) was prepared from crude extracts of the control and GA_3-treated half-seeds and desalted on Sephadex G-25. In this case too, the assay of enzyme in the presence of $(NH_4)_2SO_4$ ions (50–150 mM) revealed a relatively high stability of GA_3-stimulated monophenolase activity over that of controls (Fig. 4).

GA_3-*mediated altered electrophoretic mobility of monophenolase multiple forms*

The possibility that GA_3 treatment of half-seeds may have altered the electrophoretic mobility of monophenolase was tested by fractionating crude extracts on acrylamide gels. Electrophoresis showed two juxtaposed fast-moving bands of monophenolase following staining with tyramine soln. The crude extracts of GA_3-treated half-seeds also showed two fast-moving bands at positions similar to those in controls. In addition, there was a prominent slow-migrating band. Such a slow band could represent the association of smaller enzyme protein subunits into a larger polymeric form.* A similar difference in the pattern of multiple forms was observed in the $(NH_4)_2SO_4$ fraction precipitate (0–50% saturation) of control and GA_3-treated half-seeds. The altered electrophoretic pattern of multiple forms could be responsible for the activation of monophenolase in GA_3-treated half-seeds of wheat.

*Gel filtration of the $(NH_4)_2SO_4$ fraction precipitate (30–50% saturation) on Sephadex G-200 revealed a single activity peak of monophenolase in controls, whereas two activity peaks were observed in GA_3-treated half-seeds. The first activity peak (peak I) which eluted soon after the void volume is a high-MW polymeric form of monophenolase and was exclusively present in GA_3-treated half-seeds. The position of the second activity peak (peak II) was identical in control and GA_3-treated half-seeds (Saluja and Sachar, unpublished results).

EXPERIMENTAL

Half-seed imbibition. Wheat grains (*Triticum aestivum* var. HD 2009) were manually cut transversely into two halves and the de-embryonated portion of the seed was used for experimentation. Prior to imbibition, the half-seeds were washed in sterile distilled H_2O (1 g half-seed/100 ml H_2O) for 6 hr at 20° by constant stirring. The washed half-seeds were surface sterilized with 0.02% $HgCl_2$ for 10 min and rinsed several times with sterile distilled H_2O. The half-seeds were imbibed under aseptic conditions in chloramphenicol soln (50 μg/ml) on sterilized coarse white sand. The Petri plates containing half-seeds were maintained in the dark at 25° in a BOD incubator throughout the period of germination.

Treatment to embryoless half-seeds. The effect of gibberellic acid (GA_3, 10^{-5} M) and five different amino acid analogues (1 mM each of L-canavanine, DL-ethionine, DL-7-azatryptophan, DL-*o*-fluorophenylalanine, L-thioproline) was tested on monophenolase and amylase activities in imbibed half-seeds of wheat. The effect of amino acid analogues on amylase activity was counteracted by the simultaneous addition of five amino acids (2 mM each of L-arginine, DL-methionine, DL-ethionine, DL-tryptophan, DL-phenylalanine and L-proline).

Preparation of crude extracts. H_2O-imbibed (48 hr) half-seeds (2 g) were homogenized in 6.0 ml Pi buffer (0.05 M, pH 6.6) and spun at 20000 g for 15 min at 4°. The supernatant (crude extract) was used for the assay of monophenolase and amylase activities.

Preparation of partially purified fraction. Control and GA_3-treated half-seeds (15 g), imbibed for 48 hr, were homogenized in 100 ml Pi buffer (0.05 M, pH 6.6), containing PVP (2% w/v) and acid-washed sand. The homogenate was

passed through two layers of muslin cloth and was centrifuged at 18000 g for 15 min. The clear supernatant was fractionated with solid $(NH_4)_2SO_4$ (0–30% satn) at 4°. The pellet fraction was discarded, while the supernatant fraction was further fractionated with $(NH_4)_2SO_4$ (30–50% satn). The pptd protein was centrifuged (18000 g for 20 min.) and the pellet was suspended in 3 ml of Tris–HCl buffer (0.05 M, pH 7.0). The $(NH_4)_2SO_4$ fraction ppt. (30–50% satn) was desalted on a Sephadex G-25 column (1.8 cm × 16 cm). The desalted fractions were pooled and used for the assay of enzyme activity at pH 7.0 and 9.0. This fraction was also employed for studying the effect of $(NH_4)_2SO_4$ ions and for heat inactivation studies.

Assay of monophenolase activity. Different substrates were tested for the assay of monophenolase activity. These were *m*-cresol, phenol, tyrosine and tyramine (4 mM each). Of these, tyramine proved superior, and was therefore routinely employed for the assay of monophenolase activity. The incubation mixture comprised 2.0 ml of tyramine substrate (8 mM), 1.5 ml Pi buffer (0.05 M, pH 6.6) and 0.5 ml crude extract. The assay mixture (4.0 ml) was incubated for 1 hr at 37° and the absorbance measured at 420 nm. Addition of boiled crude extracts to the incubation mixture served as control for the enzyme-catalysed reaction. The enzyme activity was expressed as absorbance units at 420 nm/g dry wt of half-seeds. A similar assay procedure was adopted for the assay of the $(NH_4)_2SO_4$ fraction ppt. (30–50% satn).

Assay of amylase activity. Slightly modified procedure of ref. [12] was adopted for the assay of amylase activity in imbibed half-seeds of wheat. The incubation mixture contained 1 ml starch soln (0.15%), and 1 ml dil. crude extract (pH 6.0) and was incubated at 37° for 3 min. The reaction was terminated by the addition of 1 ml iodine reagent and finally diluted with 5 ml H_2O. The absorbance was measured at 610 nm. Boiled crude extracts served as controls. A unit of amylase activity is defined as that amount of enzyme which hydrolysed 10 μg of starch in 3 min at 37° in an incubation mixture of 2 ml.

Heat inactivation studies on monophenolase. The thermostability of monophenolase activity was determined in control and GA_3-treated half-seeds by maintaining crude extracts at 55° in temp.-controlled water-bath. After heat treatment, 2 ml aliquots of extracts were taken at different intervals of time (1–9 min) and chilled instantaneously in ice. The turbidity in the samples, produced by heat-treatment, was removed by centrifugation (30000 g for 20 min). The clear supernatant fraction was employed for the assay of monophenolase activity at different pH values (pH 6.6, 7.0 and 9.0). Heat inactivation curves were plotted as per cent monophenolase enzyme activity as a function of time. The stability of monophenolase was also tested in the $(NH_4)_2SO_4$ fraction ppt. (30–50% satn) by following the above procedure.

Effect of pH on monophenolase activity. The pH optimum of monophenolase activity in control and GA_3-treated half-seeds was estimated by assaying the enzyme at different pH values (pH 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.4, 9.0, 9.6, 10.0). A wide range of buffer solns (0.05 M) were prepared as described in ref. [13]. The enzyme activity was assayed as described earlier.

Effect of ionic strength on monophenolase activity. Different concns of $(NH_4)_2SO_4$ (5–150 mM) were added in the incubation mixture to study the effect of ionic strength on the monophenolase activity in crude extracts and $(NH_4)_2SO_4$ fraction ppt. (30–50% satn) of control and GA_3-treated half-seeds.

Fractionation of monophenolase multiple forms by polyacrylamide gel electrophoresis. Crude extracts and the $(NH_4)_2SO_4$ fraction ppt. (0–50% satn, desalted on Sephadex G-25) were fractionated on polyacrylamide gels (15%) using Tris–glycine buffer (pH 8.3). The procedures of refs. [14] and [15] were adopted for the acrylamide gel electrophoresis. The samples (0.25 ml containing 1 mg protein) were concd on spacer gels. After electrophoresis, the gel columns were incubated in a tyramine soln (1 mg/ml; at 37° for 1 hr) for developing the activity bands of monophenolase.

Protein estimation. Protein was estimated following the procedure of ref. [16].

Acknowledgement—The financial assistance from the University Grants Commission is gratefully acknowledged.

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