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GA_3-MODULATED MULTIPLE FORMS OF MONOPHENOLASE IN WHEAT SEED

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Key Word Index—*Triticum aestivum*; Gramineae; embryo-less wheat half-seeds; separation of monophenolase multiple forms; activation of monophenolase by GA_3 and urea.

Abstract—Multiple forms of monophenolase in wheat half-seeds were separated by molecular sieving on Sephadex G-200. A single molecular form of monophenolase was observed in control, while two multiple forms were present in GA_3-treated wheat half-seeds. A high MW (200 000 or above) multiple form (activity peak I) which eluted soon after the void volume was exclusively present in GA_3-treated half-seeds. The second activity peak (peak II) was a low MW (45 000) multiple form and its elution profile coincided in control and GA_3-treated wheat half-seeds. Both the multiple forms of monophenolase in GA_3-treated wheat half-seeds showed a pH optimum at 9.0, while the optimum enzyme activity of the control molecular form (peak II) was at pH 7.0. This indicated that the treatment of wheat half-seeds with GA_3 brought about a structural modification in monophenolase. The *in vitro* addition of trypsin enhanced the control of the molecular form of monophenolase but this treatment failed to alter the activity of multiple forms in GA_3-treated half-seeds. This differential response of monophenolase towards trypsin could be ascribed to a conformational change of the enzyme in hormone-treated half-seeds. Brief exposure of the enzyme preparation to urea (6 M) brought about an irreversible activation of monophenolase both in control and GA_3-treated wheat half-seeds.

INTRODUCTION

Activation of latent phenolases was first reported by Kenten[1, 2] in chloroplast preparations of *Vicia faba* by a short exposure to acid (pH 3.0–3.5) and alkali (pH 11.5). Similar activation of phenolases has also been reported by the *in vitro* treatment with detergents[3, 4], trypsin[5] and urea[6]. Thus, the *in vitro* treatment of phenolases with various chemicals was responsible for bringing about enzyme activation.* According to Kenten[1, 2] the increased enzyme activity was brought about by the dissociation of a protein–inhibitor complex. Subsequent studies have, however, revealed that the activation of phenolases is as a result of conformational changes in the preformed enzyme molecules[8].

The *in vivo* application of phytohormones (GA_3, auxin) and fungal infection of plant tissues was reported to stimulate the activity of phenolases. This increased activity was ascribed either to *de novo* enzyme synthesis[9–12], or to the activation of preformed enzyme molecules[13, 14]. The stimulation of phenolases by phytohormone (auxin) treatment was associated with the appearance of new multiple forms[11, 12].

A two-fold stimulation of monophenolase was reported in embryo-less wheat half-seeds by the *in vivo* application of GA_3 (10^{-5} M)[13]). The GA_3-stimulated monophenolase activity was not significantly inhibited by the administration of five amino acid analogues[14]. This indicated that *de novo* protein synthesis was not necessary for the hormone-evoked monophenolase activity. The GA_3-activated monophenolase exhibited different molecular properties, such as a shift in the pH optimum, relatively high thermostability and altered electrophoretic mobility of its multiple forms[14]. These changes in the physical properties of monophenolase reflected structural modification(s) of the preformed enzyme molecules. In the present investigation, multiple forms of monophenolase in GA_3-treated wheat half-seeds have been studied.

RESULTS AND DISCUSSION

Separation of the multiple forms of monophenolase

Previously, it was reported from this laboratory that the electrophoretic pattern of monophenolase multiple forms on polyacrylamide gels was different in control and GA_3-treated wheat half-seeds[14]. Based upon this finding, the enzyme preparations were fractionated on acrylamide gels with a view to elute and quantify different multiple forms of monophenolase. Although it was possible to confirm the differential pattern of multiple forms in the control and GA_3-treated wheat half-seeds, the slicing of gels

*A recent comprehensive review by Mayer and Harel [7] gives an excellent account of literature on the activation of phenolases in higher plants.

followed by elution in buffer did not give satisfactory results for the quantification of the activity bands. Since phenolases are known to exist in multimeric forms in many plants[12, 15, 16], we resorted to the gel filtration technique for the separation of the monophenolase multiple forms of wheat half-seeds. For this purpose, the crude extracts were initially subjected to the ammonium sulphate fractionation method. The bulk of the monophenolase activity was present in the ammonium sulphate fraction precipitate (30–50% saturation). This enzyme preparation was fractionated on Sephadex G-200. The control enzyme fraction yielded a single activity peak (peak II), while the GA_3-fraction showed two distinct separable activity peaks on Sephadex G-200. The activity peak (peak I), which eluted soon after the void volume ($V_0 = 60.0$ ml), corresponded to the slow migrating activity band on acrylamide gels. This multiple form was exclusively present in GA_3-treated wheat half-seeds and its MW is 200 000 or above. The second activity peak (peak II) represented a low MW multiple form and its MW is *ca* 45 000 as determined by the gel filtration technique. The elution profile of peak II coincided in control and GA_3-treated half-seeds (Fig. 1). Thus, it seems logical to consider that the additional high MW multiple form (peak I) of monophenolase is produced in response to hormone treatment. It is further postulated that the GA_3-induced multiple form could have been formed by the association of preformed low MW multiple form following some structural modification(s). Such a contention is supported by the observation that GA_3-mediated activation of monophenolase does not require *de novo* protein synthesis[14].

Effect of pH on the multiple forms of monophenolase

A significant shift in the pH optimum of monophenolase activity was reported in GA_3-treated wheat half-seeds. Whereas the optimum activity of the control enzyme was at pH 7.0, the pH optimum of monophenolase activity in hormone-treated half-seeds was at 9.0[14]. However, it was not possible to ascertain from the previous study whether one or both the multiple forms of monophenolase in GA_3-treated half-seeds underwent structural modification(s) resulting in the shift of pH optimum. The separation of monophenolase multiple forms achieved in the present study permitted us to determine the pH optimum of the individual multiple forms. It was observed that both the multiple forms of monophenolase in GA_3-treated wheat half-seeds possessed an optimum enzyme activity at pH 9.0. In contrast, the molecular form in control half-seeds showed an optimum enzyme activity at pH 7.0 (Fig. 2). This marked shift in the pH optimum of the enzyme activity caused by GA_3 reflected a conformational change in the multiple forms. Thus it seems that GA_3 conferred a structural modification in both the multiple forms (peak I, peak II) irrespective of their molecular size.

Effect of trypsin on monophenolase activity

Phenolases in chloroplast preparations of different plant tissues are known to be activated by the *in vitro* addition of trypsin[5, 17, 18]. In the present study, the *in vitro* addition of trypsin (150 μg/incubation) to the multiple forms (peak I, peak II) isolated from GA_3-treated half-seeds revealed no significant enzyme activation (data not presented). In contrast, the *in vitro* addition of trypsin (150 μg/incubation) to the molecular form (peak II) isolated from control half-seeds resulted in *ca* 30% increase in monophenolase activity at pH 7. At pH 9 the monophenolase activity in controls was virtually abolished and this was restored by the *in vitro* addition of trypsin (Table 1). However, the trypsin-mediated stabilization of the

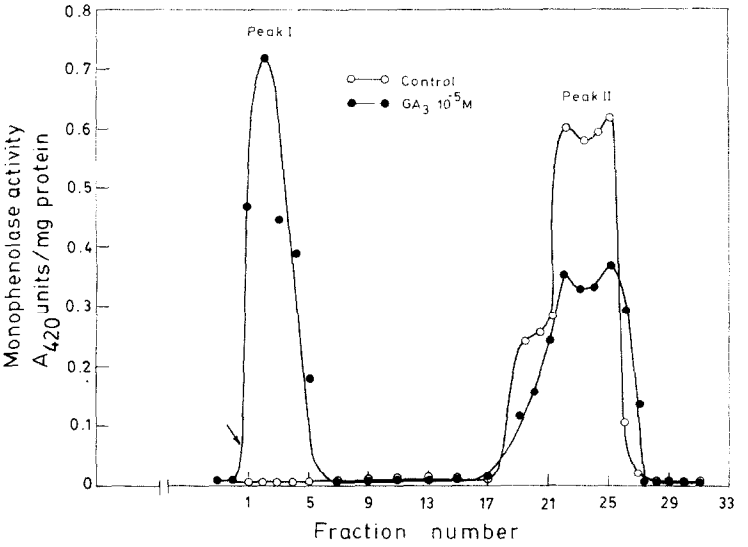

Fig. 1. Separation of monophenolase multiple forms in control and GA_3-treated wheat half-seeds by molecular sieving on Sephadex G-200. The notch in peak II indicated that there may be two forms with a slight difference in their MW. The arrow indicates the void volume ($V_0 = 60$ ml) of the column.

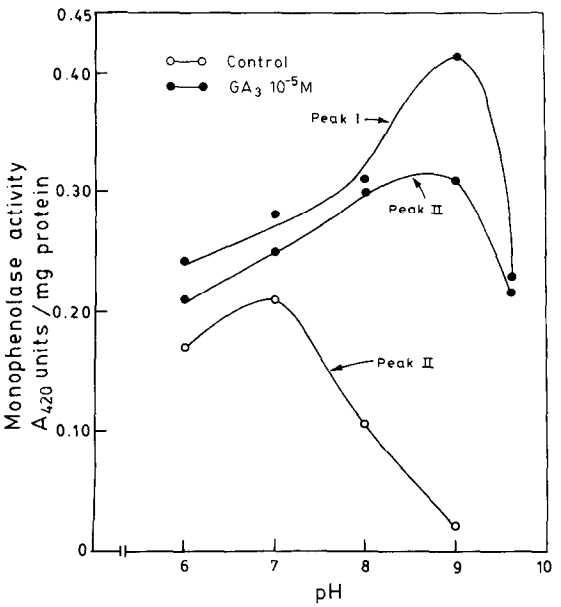

Fig. 2. The pH activity profiles of monophenolase multiple forms depicting a shift in pH optimum towards alkalinity in GA_3-treated wheat half-seeds. The enzyme activities of each multiple form (peaks I and II), separated by gel filtration on Sephadex G-200, were assayed at different pH values (pH 6.0–9.6).

control monophenolase could not be ascribed to the proteolytic action of trypsin. This became evident when it was observed that trypsin, inactivated by trichloroacetic acid (5%), was equally effective in stabilizing the activity of the control monophenolase multiple form at pH 9. A similar stabilization of monophenolase activity was also observed by the *in vitro* addition of bovine serum albumin and casein (150 μg/incubation). However, this stabilization response was observed only after a short lag phase of 30 min at 37° (data not presented). Apparently, the stabilization of monophenolase is brought about by the non-specific protein–protein interaction. Earlier, Tolbert[5] had observed that the increased activity of phenolases by trypsin was not due to proteolytic action of trypsin since it occurred even with denatured trypsin preparation. This was in contrast to the tryptic activation of polyphenol oxidase in sugar beet chloroplasts. In this case, limited proteolysis resulted in the solubilization and activation of membrane-bound phenolases[17, 18].

Effect of urea on monophenolase activity

Many workers have reported the activation and shift in the pH optimum of phenolases by urea treatment[6, 19, 20]. We also tested the effect of urea on wheat monophenolase. The ammonium sulphate fraction precipitates (30–50% saturation), prepared from control and GA_3-treated half-seeds, were treated with urea (6 M) for 30 min at 4° and subsequently dialysed. This exposure of enzyme fractions to urea brought about an irreversible activation of monophenolase both in control and GA_3-treated half-seeds at pH 7 and 9 (Table 2). However, the sp. act. of monophenolase in the enzyme preparations from GA_3-treated half-seeds remained higher than that of the control, even after urea treatment.

Since the enhancement of monophenolase activity in GA_3-treated half-seeds is not dependent on protein synthesis[14], the formation of a high MW multiple form (peak I) in response to hormone treatment could have been brought about by the association of a low MW multiple form. This hypothesis was tested by treating the enzyme fraction (isolated from GA_3-treated half-seeds) with 6 M urea (30 min at 4°), followed by its fractionation on Sephadex G-200. It was observed that the denaturing agent brought about a partial inclusion of the high MW multiple form (peak I) and also resulted in its activation (Fig. 3). The appearance of an additional activity peak in the urea-treated sample could be due to partial dissociation of the original activity peak. Lack of complete dissociation of the high MW multiple form (peak I) to a low MW multiple form (peak II) by urea treatment could be ascribed various reasons, e.g. the formation of disulphide covalent bonds, or the rapid reassociation of smaller subunits during their elution from the Sephadex column. There was no shift in the position of the low MW multiple form (peak II),

Table 1. Stabilization of monophenolase activity in control wheat half-seeds by the *in vitro* addition of trypsin

	Monophenolase activity of isolated multiple form (peak II)			
	pH 7		pH 9	
Additions	A_{420} units/mg protein	Relative activity	A_{420} units/mg protein	Relative activity
Untreated	0.14	1.00	0.03	1.00
Active trypsin (150 μg)	0.18	1.30	0.20	6.67
Inactive trypsin (150 μg)	0.17	1.21	0.19	6.33

The $(NH_4)_2SO_4$ fraction precipitate (30–50% saturation), prepared from control half-seeds, was fractionated by Sephadex G-200. The activity of the isolated multiple form was assayed at pH 7 and 9 after the *in vitro* addition of trypsin to the incubation mixture. The values given represent an average of two experiments.

Table 2. The *in vitro* activation of monophenolase enzyme by urea in control and GA_3-treated wheat half-seeds

		Monophenolase activity			
		pH 7		pH 9	
Treatments	Additions	A_{420} units/mg protein	Relative activity	A_{420} units/mg protein	Relative activity
Control	(a) Untreated	0.10	1.00	0.04	1.00
	(b) Urea (6 M)	0.24	2.40	0.23	5.75
GA_3 (10^{-5} M)	(a) Untreated	0.15	1.00	0.22	1.00
	(b) Urea (6 M)	0.42	2.80	0.60	2.73

The $(NH_4)_2SO_4$ fraction precipitate (30–50% saturation) was prepared from control and GA_3-treated half-seeds. Both enzyme fractions were treated with 6 M urea for 30 min at 4° and dialysed overnight against Tris–HCl buffer (20 mM, pH 7). Monophenolase activity was assayed in urea-treated and untreated fractions at pH 7 and 9.

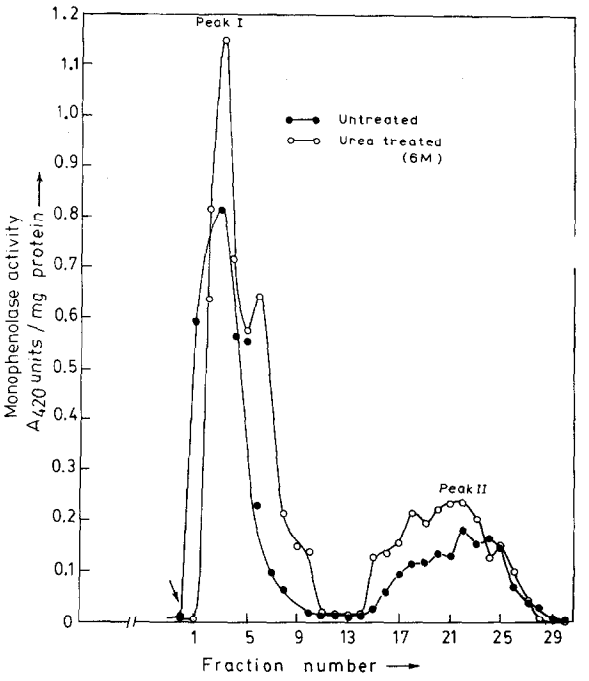

Fig. 3. Stimulatory effect of urea on the monophenolase multiple forms, isolated from GA_3-treated wheat half-seeds. The $(NH_4)_2SO_4$ fraction precipitate (30–50% saturation), prepared from GA_3-treated half-seeds, was incubated with urea (6 M) for 30 min at 4°. An aliquot (2 ml; containing 32.4 mg protein) of urea-treated and untreated was fractionated on Sephadex G-200 for the separation of monophenolase multiple forms. The arrow indicates the void volume (V_0 = 60 ml) of the column.

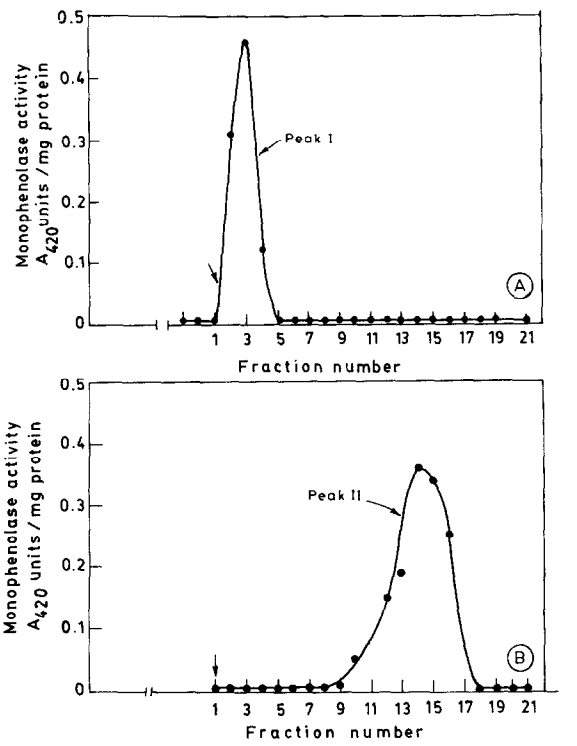

Fig. 4. Enrichment of individual multiple forms and their refractionation on Sephadex G-200. The $(NH_4)_2SO_4$ fraction precipitate (30–50% saturation), prepared from GA_3-treated half-seeds, was fractionated on a Sephadex G-200 column for the separation of high and low MW multiple forms (peaks I and II). The separated multiple forms were concentrated by solid $(NH_4)_2SO_4$ (0–85% saturation) precipitation. The concentrated samples were refractionated on a Sephadex G-200 column (30 × 1.8 cm) for determining the pattern of multiple forms.

although its activation could be observed in the urea-treated enzyme fraction (Fig. 3). Other workers [6, 20] have suggested that the exposure of enzyme preparations to urea, acidic or alkaline pH brings about an activation of phenolases due to conformational alterations. Thus, activation of monophenolase by urea could also be ascribed to a conformational change.

Lack of association–dissociation of monophenolase multiple forms by enrichment of protein

An attempt was made to study the interconversion of the two multiple forms in GA_3-treated half-seeds. It was considered that if the multiple forms were completely separated by the gel filtration technique, the isolated multiple forms could reorganize into high and low MW multiple forms through the phenomenon of association–dissociation and thus attain a state of equilibrium. Enrichment of the physically separated multiple forms of monophenolase (5–6 mg protein/ml) by ammonium sulphate precipitation (0–85% saturation), followed by refractionation on Sephadex G-200, failed to bring about dissociation or even association of the isolated multiple forms of monophenolase. The low MW multiple form (peak II) showed no aggregation following concentration of the protein. Similarly, there was no dissociation of the high MW multiple form when it was no longer in equilibrium with the low MW multiple form (Fig. 4). Thus the phenomenon of association–dissociation of monophenolase multiple forms in GA_3-treated half-seeds was not merely dependent upon the concentration of the enzyme protein. This is in contrast to the behaviour of mushroom tyrosinase, where an increase in protein concentration (1 mg/ml) brought about a complete association of the low MW multiple form of the enzyme [16]. Spontaneous interconversions between different multiple forms of phenolases, apparently due to the association–dissociation phenomenon, have been reported in olives [21]. Such interconversions can be accelerated by various factors, viz. ionic strength, concentration or dilution of the protein, presence of certain ions and elevated temperature [7].

EXPERIMENTAL

Material. Wheat seeds (*Triticum aestivum* var. HD 2009) were procured from the Division of Seed Technology, Indian Agricultural Research Institute, New Delhi, 110012, India.

Imbibition of half-seeds. The transversely cut embryoless wheat half-seeds were prewashed in H_2O (1 g half-seeds/100 ml H_2O) for 6 hr at 20°, followed by surface steril-

ization with $HgCl_2$ soln (0.02%) for 10 min. The half-seeds were then imbibed in the presence and absence of GA_3 soln (10^{-5} M) under aseptic conditions in the dark at 25° for 45 hr. Chloramphenicol (50 μg/ml) was routinely added to half-seeds during imbibition.

Preparation of the enzyme fraction. The imbibed half-seeds (40 g) were homogenized in Pi buffer (200 ml; 50 mM; pH 6.6). Polyvinyl polypyrrolidone (PVP, 4% w/v) and acid washed sand (6 g) were added during grinding. The homogenate was filtered through two layers of cheese cloth and centrifuged at 40500 *g* for 15 min at 4°. The crude extract was enriched by $(NH_4)_2SO_4$ ppt (30–50% satn) and desalted either by passing through a Sephadex G-25 column (14 × 1.8 cm) or by dialysis against Tris–HCl buffer (20 mM, pH 7). The desalted fraction was frozen in liquid N_2 and stored overnight at −10°. This enzyme preparation was used for the assay of monophenolase activity.

Sephadex G-200 column chromatography. The $(NH_4)_2SO_4$ fraction ppt (30–50%, satn), prepared from control and GA_3-treated wheat half-seeds, was fractionated on a Sephadex G-200 column (70 × 1.8 cm) at 8°. An aliquot of enzyme fraction (2 ml) was loaded on the column and fractions (2.5 ml each) were collected immediately after the void vol. (V_0). Pi buffer (50 mM; pH 7) was used for equilibration of the column and elution of the protein fractions. The V_0 (60 ml) of the column was determined experimentally from the elution profile of Blue Dextran. Protein was determined in each fraction by the procedure of ref. [22]. The sp. act. of monophenolase enzyme was plotted against fraction No. The MWs of the individual multiple forms of monophenolase were estimated from its distribution coefficient (K_{av}) on Sephadex G-200 (70 × 1.8 cm). Ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin and aldolase (Pharmacia) were used for the calibration of the column.

The pH profiles of isolated multiple forms of monophenolase. The $(NH_4)_2SO_4$ fraction ppts (30–50%, satn), prepared from control and GA_3-treated half-seeds, were fractionated on Sephadex G-200 column (30 × 1.8 cm) to isolate the multiple forms of monophenolase. The enzyme activity of each multiple form was assayed at different pH values (6.0–9.6). The sp. act. of the individual multiple forms were plotted as a function of pH.

Effect of trypsin on multiple forms of monophenolase. The $(NH_4)_2SO_4$ fraction ppts (30–50%, satn), prepared from control and GA_3-treated wheat half-seeds, were fractionated on a Sephadex G-200 column (30 × 1.8 cm). The eluted multiple forms (peaks I and II) were assayed at pH 7 and 9 in the presence of active and inactivated preparations of trypsin (150 μg/incubation). The active trypsin was inactivated by the addition of trichloroacetic acid (TCA, 5%). The ppt protein was suspended in Pi buffer (50 mM, pH 7). This pretreatment of trypsin with acid completely abolished the enzyme activity.

Effect of urea on monophenolase activity. The $(NH_4)_2SO_4$ fraction ppts (30–50% satn), prepared from control and GA_3-treated half-seeds, were treated with 6 M urea for 30 min at 4°. The urea-treated samples were dialysed for 16 hr against Tris–HCl buffer (20 mM, pH 7). The activity of monophenolase was assayed both at pH 7 and 9. In another expt, the $(NH_4)_2SO_4$ fraction ppt (30–50% satn) prepared from GA_3-treated wheat half-seeds, was treated with 6 M urea for 30 min at 4°. The sample was directly loaded on a Sephadex G-200 column (70 × 1.8 cm) and the elution profile of monophenolase activity was compared with that of the untreated enzyme preparation.

Isolation and enrichment of individual multiple forms (peaks I and II) and their refractionation on a Sephadex G-200 column. The $(NH_4)_2SO_4$ fraction ppt (30–50% satn) from GA_3-treated half-seeds was loaded on a Sephadex G-200 column (30 × 1.8 cm). The eluted fractions containing peaks I and II were pooled separately and concd with solid $(NH_4)_2SO_4$ (0–85% satn). The ppt protein was suspended in Pi buffer (2 ml; 50 mM, pH 7), frozen in liquid N_2 and stored overnight at −10°. The individual multiple forms were reloaded on a Sephadex G-200 column (30 × 1.8 cm). Fractions (2.5 ml each) were eluted with Pi buffer (50 mM, pH 7). Monophenolase activity was assayed at pH 7 using 2 ml of each fraction.

Assay of monophenolase activity. The incubation mixture contained tyramine base (2 ml, 8 mM), enzyme fraction (1–2 mg protein) and Pi buffer (50 mM, pH 7) in a final vol. of 4 ml. The reaction mixture was incubated for 1 hr at 37° and the A_{420} was measured. The Pi buffer (50 mM, pH 7) was substituted by Tris–HCl buffer (50 mM, pH 9) when the enzyme was assayed at pH 9. Tyramine base was dissolved in Pi buffer (50 mM, pH 7) or in Tris–HCl buffer (50 mM, pH 9) as required. The enzyme activity was also assayed at different pH values using different buffers [23] for determining the pH optimum of the isolated monophenolase multiple forms.

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