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MULTIPLE FORMS OF PEROXIDASE FROM *NARCISSUS PSEUDONARCISSUS*

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Key Word Index—*Narcissus pseudonarcissus*; Amaryllidaceae; King Alfred daffodil; identification; isolation; enzyme; peroxidase.

Abstract—Multiple forms of peroxidase from *Narcissus pseudonarcissus* were identified and separated by polyacrylamide gel electrophoresis. The enzyme forms were found to be particulate but could be solubilized in buffers of high ionic strength and high pH. Bulbs at different stages (dormant, early growth, flowering and post-flowering) were investigated and both the number and distribution of peroxidase forms were found to differ. The major peroxidase form in dormant bulbs was purified and displayed a number of notable properties including a MW of at least 10^5, a high isoelectric point and the apparent absence of a heme prosthetic group.

INTRODUCTION

The biosynthetic pathways leading to a number of alkaloids involve oxidative coupling of phenolic precursors [1, 2]. Three types of oxidoreductases in nature—laccases, tyrosinases, and peroxidases—are known to catalyse reactions of this type [3]. Although the Amaryllidaceae alkaloids appear also to be derived via phenolic coupling [4], the only enzymes which have been described are those catalysing earlier reactions in the proposed pathway [5, 6]. Because of the important role one or more of these enzymes may play in alkaloid biogenesis, we initiated a study of the oxidoreductases in one member of the Amaryllidaceae, *Narcissus pseudonarcissus*. Two model substrates, syringaldazine [7] and *o*-dianisidine [8], were employed to identify, classify and quantitate the appropriate enzyme activities.

RESULTS

Oxidoreductase activity in *Narcissus pseudonarcissus* was detected initially by the appearance of a characteristic pink-red coloration when fresh bulb slices were treated with a solution of syringaldazine [7] and hydrogen peroxide. When H_2O_2 was omitted no color was produced thereby identifying the major oxidoreductase to be a peroxidase (EC 1.11.1.7). A procedure for monitoring quantitatively and continuously syringaldazine oxidation by H_2O_2 and enzyme was developed and is described in the Experimental. The more common assay system using *o*-dianisidine [8] and H_2O_2 also provided an effective analysis of enzyme activity.

Grinding bulbs in a buffer of moderate ionic strength and neutral pH (buffer A), followed by removal of the pulp via filtration and centrifugation gave a soluble extract which contained little peroxidase activity. In contrast, high levels of peroxidase activity were obtained in the soluble fraction following the same procedure but substituting a solubilization buffer possessing high ionic strength, elevated pH and EDTA (buffer B). An effective purification of the peroxidase fraction was accomplished by taking advantage of this particulate character. Plant material homogenized in buffer A gave an aqueous fraction containing the bulk of the protein and soluble plant constituents but little peroxidase. After removal of this fraction the solids were rehomogenized with buffer B. Centrifugation produced an aqueous fraction containing most of the peroxidase activity in a substantially purified form. This activity was precipitated with ammonium sulfate, collected and stored at −10°. With minor modifications this procedure was used to recover peroxidase fractions from bulbs of this plant at several growth stages (dormant, early growth, flowering and post-flowering).

Fig. 1 shows the results of analysis by polyacrylamide disc gel electrophoresis of the peroxidase fractions from each growth stage. All gels were stained for peroxidase activity and exhibited a pair of bands having intermediate mobility. In the dormant bulbs the most prominent band exhibited very low mobility. This band was not observed in early growth bulbs; however, two additional forms were detected as minor bands of high mobility. Flowering bulbs also contained four enzyme forms, but their distribution was quite different from early growth bulbs. Most notable was the appearance of another major form (second from the top) and the disappearance of the two minor bands of high mobility seen in the early growth bulbs. In addition, a minor band of low mobility indistinguishable from the major form in the dormant case was now apparent. The pattern observed in post-flowering bulbs was virtually the same as that of flowering bulbs except the form of lowest mobility was now more prominent. It should be noted that bulbs in the latter three stages of growth yielded some peroxidase activity in the

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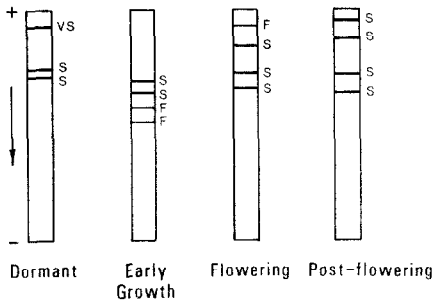

Fig. 1. Electrophoretic comparison of peroxidase forms at different stages of growth. Polyacrylamide disc gels were stained for peroxidase activity. Samples from dormant bulbs were purified via $(NH_4)_2SO_4$ precipitation (35–95%). Samples from non-dormant bulbs were precipitated between 40 and 80% $(NH_4)_2SO_4$ saturation in the presence of 2 M NaCl to avoid co-precipitation of plant pigments. 'VS' indicates a very strong or especially prominent band while 'S' denotes a strong band and 'F' a faint or minor band.

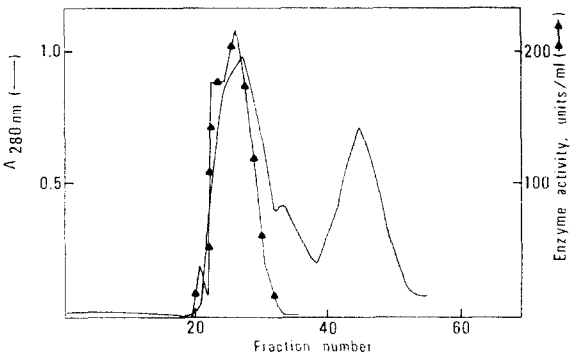

Fig. 2. DEAE-cellulose chromatography elution profile of the dormant bulb peroxidase from the $(NH_4)_2SO_4$ fractionation.

initial homogenized extracts prior to solubilization. The amount of activity, however, was always less than that which was subsequently released by solubilization.

The results of isolation and purification of the major peroxidase in dormant bulbs are summarized in Table 1. The isolation used 2.6 kg of dormant bulbs and began with the solubilization procedure discussed previously. Removal of highly colored, presumably phenolic, materials was aided by vacuum infiltration and homogenization in the presence of soluble PVP [10]. A broad range of ammonium sulfate concentrations (35–95% saturation) was necessary to precipitate fully the solubilized peroxidase activity, presumably because of the presence of multiple enzyme forms. DEAE-cellulose chromatography (Fig. 2) was used to remove contaminating proteins as well as remaining phenolic material. Under the conditions employed, peroxidase activity was not bound to the columns and eluted as a single peak. After elution from a second column, the enzyme preparation was virtually colorless and exhibited a substantially increased A_{280}/A_{260} ratio indicating the successful removal of phenolics from the preparation.

The peroxidase forms were further purified by Sephadex G-100 chromatography. Two regions of enzyme activity, designated fractions A and B, could be distinguished in the elution profile (Fig. 3). After the indicated fractions were pooled, polyacrylamide disc gel electrophoresis revealed that fraction A contained only

Table 1. Summary of peroxidase purification

Procedure	Total protein* (mg)	Total† enzyme (units)	Protein concentration (mg/ml)	Specific enzyme activity (units/mg)	Overall yield (%)	Fold purification
Extract after solubilization	34660	30500	22.6	0.88	100	1.0
$(NH_4)_2SO_4$ fractionation (35–90%)	1040	26500	5.0	25.5	87	29
DEAE-Cellulose No. 1	93	14040	1.4	151	46	172
DEAE-Cellulose No. 2	41	14300	1.5	347	47	394
Sephadex G-100, fraction A	3	6650	0.37	2165	22	2460
Sephadex G-100, fraction B	5	1400	0.58	302	4.5	343
Isoelectric focusing of fraction A	0.5	4800	0.26	9231	16	10490

* Protein was determined by the method of Kalckar [9]. Estimates of total protein prior to DEAE-cellulose chromatography are probably high due to the presence of phenolic materials in extracts.

† Peroxidase activity was measured using *o*-dianisidine as substrate.

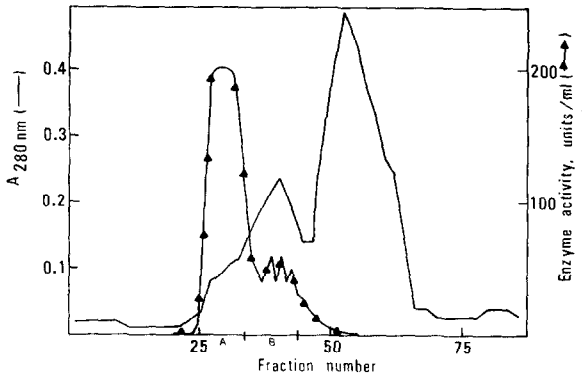

Fig. 3. Sephadex G-100 chromatography elution profile of pooled DEAE-cellulose chromatography fractions. The fractions indicated were pooled and designated fractions A and B.

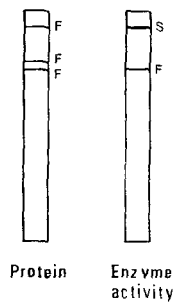

Fig. 5. Polyacrylamide disc gel electrophoresis patterns of pooled fractions after isoelectric focusing. Identical gels were stained either for protein or peroxidase activity as indicated.

the major peroxidase form of low mobility. Fraction B contained the other two peroxidases plus a small amount of the form in fraction A. This is the only technique which we found that effectively separated these enzyme forms on a preparative scale. Since the elution position of fraction A protein was near the void volume of the column, determined using blue dextran, we conclude that the major peroxidase form in the dormant bulbs has a MW of at least 10^5.

The final step in the purification scheme was isoelectric focusing of fraction A protein. The activity profile obtained (Fig. 4) is skewed suggesting the presence of more than one enzyme form. Indeed, polyacrylamide disc gels stained for peroxidase activity (Fig. 5) revealed the presence of two peroxidase forms in the pooled fractions. Although the form of low mobility accounted for *ca* 90% of the total peroxidase activity, a form of intermediate mobility was clearly evident. These results were unexpected since the material subjected to isoelectric focusing contained no detectable activity of intermediate mobility. Polyacrylamide disc gels stained for protein (Fig. 5) revealed the presence of only three protein bands in the final preparation, and two of the bands corresponded closely to the active peroxidase forms. Thus, although the peroxidase activity was purified more than 10000-fold relative to the solubilization extract, at least one contaminating protein remains in our purest preparations. The absorption spectrum of the final preparation showed no Sorèt band near 410 nm.

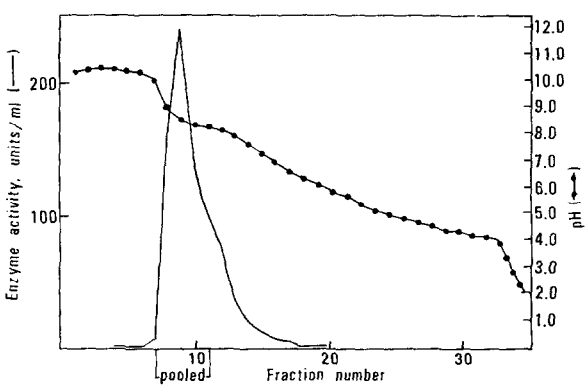

Fig. 4. Elution profile of isoelectric focusing at pH 3.5–10.0 for pooled Sephadex G-100 chromatography fractions (fraction A) of the dormant bulb peroxidase. The column was run at 400 V for 48 hr.

DISCUSSION

A major finding of this work is the identification of multiple peroxidase forms in the daffodil. Similar findings have been reported for a number of plants including horseradish [8, 11], Japanese radish [12], sweet potato [13], wheat germ [14], pea [15] and turnip [16, 17] with the number of enzyme forms varying between two and eight. Our data indicate that there are at least six forms in the daffodil which are electrophoretically separable. Although these enzyme forms could have arisen as artefacts during purification, the widespread occurrence of multiple forms in plants as well as the fact that the daffodil forms were present at all stages of purification including the crudest fractions argues against this hypothesis. In addition, the fact that the number and distribution of daffodil peroxidase forms varied with growth stage suggests strongly that multiple forms are present *in vivo*. Similar shifts have been reported during development of pea cotyledons [15] and maize [18]; following indoleacetic acid application to dwarf peas [19], *Avena* [20] and cultures of *Nicotiana tabacum* [21]; and after pathogen infection of the sweet potato [18] and tobacco leaves [22]. It should be noted, however, that the reappearance of a second peroxidase form following isoelectric focusing of a highly purified daffodil peroxidase may suggest that one peroxidase form can be generated from another.

Although many peroxidases are thought to be cytoplasmic and appear in aqueous extract of plant tissues, a number of sources such as pine [23], banana [24], oat coleoptile [20], cotton plant [25], red algae [26], cultured tobacco tissue [27], and wheat embryo [28], in addition to the daffodil have been shown to contain particulate peroxidase activities. The conditions used in this work to solubilize the peroxidase forms were derived from procedures developed by Albertsson [29] to release phospholipase A from the membrane of *E. coli*. The fact that the peroxidase forms could be rendered soluble by these mild conditions, involving no detergents, suggests that these enzymes may be peripheral membrane proteins [30] and/or are organelle-associated. The appearance of some activity in

later growth stages suggests that these enzymes may be released during growth.

Gel filtration with Sephadex G-100 separated the most active form of peroxidase from the two less active forms in the dormant stage. Since it eluted near the void volume on Sephadex G-100, the major peroxidase form in the dormant stage appears to have a MW of at least 10^5. This value is unusually high for a plant peroxidase but compares with MWs reported for some mammalian enzymes [31]. The isoelectric focusing data show that the major peroxidase in dormant bulbs is a strongly basic protein as are the two most abundant of the seven peroxidase forms in horseradish [32], a peroxidase form isolated from pineapple [33] and the major forms isolated from turnip [16].

The absorption spectra of the most purified dormant peroxidase form indicated no Sorèt band near 410 nm and thus, the absence of ferriprotoporphyrin IX. This prosthetic group has been found in all purified peroxidases from higher plants [34]; however, no Sorèt band was observed in impure peroxidase forms in green and red algae [26, 35].

Although complete purification of a daffodil peroxidase form was not achieved, the major peroxidase from dormant bulbs was purified over 10000-fold to near-homogeneity. The amount of enzyme activity present in the daffodil is at least two orders of magnitude less than that present in horseradish, the source of the best-studied plant peroxidase [8].

This study constitutes the first identification and purification of a peroxidase from *Narcissus pseudonarcissus* and to our knowledge the first from a plant producing alkaloids derived from phenols by phenyl–phenyl oxidative coupling. Although present evidence implicating the enzyme in alkaloid biosynthesis is indirect, further support for this hypothesis comes from recent work showing that *p*-cresol is coupled efficiently by daffodil peroxidase [36]. Finally, syringaldazine has been shown to be useful in qualitative tests to distinguish laccase, tyrosinase and peroxidase activities in plant tissues. The spectrophotometric procedure described here extends the utility of this method by allowing quantitative measurements of activity to be made as well.

EXPERIMENTAL

Bulbs. Narcissus pseudonarcissus or King Alfred daffodil, bulbs were purchased locally from Davids & Royston Bulb Co., Inc., Gardena, California.

Enzyme assay procedures. Peroxidase activity was measured routinely by monitoring the change in *A* at 460 nm due to the oxidation of *o*-dianisidine in the presence of H_2O_2 and enzyme at 30° [8]. Peroxidase activity could also be measured from the change in *A* at 530 nm due to the oxidation of syringaldazine in the presence of H_2O_2 and enzyme. The standard assay soln (3.0 ml) contained 2.6 ml 0.05 M acetate buffer pH 5.5, 0.23 ml 95% EtOH, 0.02 ml 0.1% w/v syringaldazine in 95% EtOH, 0.02 ml 0.01 M (0.03%) H_2O_2 and 0.1 ml enzyme. For both assays, a unit of activity was defined as the amount of enzyme giving an *A* change at the appropriate wavelength of 0.1/min at 30°.

Polyacrylamide gel electrophoresis. Samples were resolved on 5% polyacrylamide gels using acidic buffers in a discontinuous system [37]. Gels were stained for peroxidase activity using *o*-dianisidine and H_2O_2 according to the procedure of ref. [8]. Gels were stained for total protein using Coomassie Brilliant Blue G-250 [38].

Buffers. Buffer A was 0.04 M potassium phosphate, 0.01 M KCl (pH 7.2). Buffer B contained 0.01 M Tris–HCl, 2 M NaCl, 0.001 M EDTA (pH 9.2).

Enzyme purification. All manipulations were performed at 4° unless otherwise indicated.

Extraction and solubilization. After removal of roots, stems, and basal plates, the bulbs were diced and placed in buffer A containing 1% PVP. The extract was vacuum-infiltrated for 10 min and then homogenized in a Waring blender for 2 × 1 min during which time the temp. of the soln remained between 15 and 25°. The solids were collected with a juicerator and washed with fresh buffer. Solubilization of peroxidase activity was accomplished by resuspending the collected ppt. in buffer B. This suspension was stirred overnight. The extract was again collected with a juicerator and the ppt. discarded.

Ammonium sulfate fractionation. Centrifugation was performed at 10400 *g* for 15 min. The crude extract which had been dialysed vs. buffer A was made up to 1.37 M $(NH_4)_2SO_4$ (35% saturation) by adding solid $(NH_4)_2SO_4$ slowly over 1 hr and then stirred for an additional hr. The suspension was centrifuged and the supernatant was made 3.71 M $(NH_4)_2SO_4$ (95% saturation) as described above. The suspension was then centrifuged and the ppt. dissolved in 250 ml buffer A and dialysed against 3 × 1 l. 0.02 M potassium phosphate buffer, pH 7.0. The resulting soln was centrifuged and concd to 30 ml by ultrafiltration using an Amicon apparatus (CDS-10) and Amicon PM-10 membrane under N_2 (35 psi). The conc soln was then further dialysed against 2 × 1 l. 0.02 M potassium phosphate buffer, pH 7.0. The ppt. that formed was removed via centrifugation for 15 min at 12000 *g* and discarded.

DEAE-cellulose chromatography. Dialysed protein samples were applied to a DEAE-cellulose column (2.5 × 40 cm) which had been equilibrated with 400 ml 0.02 M potassium phosphate buffer, pH 7.0. Maintaining a 1.5 ml/min flow rate, 3 ml fractions were collected. The pooled fractions were concd by ultrafiltration.

Sephadex G-100 chromatography. The protein samples from the DEAE-cellulose column were dialysed against 1 l. buffer A. The dialysed protein was chromatographed on a 2.5 × 75 cm Sephadex G-100 column which had been previously equilibrated with buffer A. Maintaining a 0.5 ml/min flow rate, 6.5 ml fractions were collected. The pooled fractions containing the peroxidase activity were concd by ultrafiltration and dialysed against 0.4 mM potassium phosphate, pH 7.0.

Isoelectric focusing at pH 3.5–10. Density gradient isoelectric focusing was performed using an LKB 8100 column (110 ml) with 1% Ampholine and sucrose for the density gradient according to the manufacturer's instruction manual. The column was eluted by gravity at a flow rate of 1.5 ml/min and 3 ml fractions were collected. Fractions were dialysed against 3 × 1 l. buffer A prior to analysis.

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