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SHORT REPORTS

DYE-SENSITIZED PHOTO-OXIDATION OF ACID RIBONUCLEASE FROM PEA COTYLEDONS

PAUL S. PHILLIPS*, JOHN A. BRYANT†‡ and HILARY J. SMYTH†

* Department of Chemistry and † Department of Plant Science, University College, P.O. Box 78, Cardiff, CF1 1XL, U.K.

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Key Word Index—*Pisum*; Leguminosae; pea; photo-oxidation; ribonuclease, histidine.

Abstract—In crude extracts, pea cotyledon acid ribonuclease is not inactivated by photo-oxidation, but after 150-fold purification, it is markedly inactivated when illuminated in the presence of methylene blue at pH 7.2. It is, however, still resistant to methylene blue-sensitized photo-oxidation at pH 5.4. These data suggest that photo-oxidation of methionine, cysteine and tryptophan has no effect on enzyme activity, whereas photo-oxidation of histidine markedly reduces catalytic acitivity. It is thus likely that the mode of action of acid ribonuclease from pea cotyledons is similar to that of pancreatic ribonuclease.

INTRODUCTION

It is well known that illumination of protein solutions in the presence of certain dyes leads to modification of certain amino acid residues [1]. Only 5 amino acids, cysteine, histidine, methionine, tryptophan and tyrosine are modified by photodynamic treatment, and there is no effect on peptide bonds or disulphide bridges [1]. By careful control of the reaction conditions and selection of an appropriate oxidizing dye, photo-oxidation may be limited to a single amino acid species [1, 2]. It is therefore possible to determine the importance of the amino acids which are susceptible to dye-sensitized photo-oxidation for the activity of the enzyme. For the great majority of enzymes, most of the amino acid residues are involved in the maintenance of catalytic activity, and modification of any amino acid species by photodynamic treatment, or by other means, leads to at least partial loss of activity. Thus, of over 50 enzymes investigated, only two (radish peroxidase and bacterial alkaline phosphatase) are resistant to dye-sensitised photo-oxidation [1].

In a previous paper [3], we demonstrated that a small fragment of pea cotyledon acid ribonuclease (EC 2.7.7.16), with a MW of 3100 (from a native enzyme of MW 17500) retains some catalytic activity. The retention of activity in such a small fragment is extremely unusual and suggests that a large part of the amino acid sequence may not be directly involved in maintaining the integrity of the active site. Acid ribonuclease from pea cotyledons is thus an interesting subject for study by photodynamic techniques. Further, the data from this work provide a means of comparison between an acid ribonuclease from a plant and bovine pancreatic ribonuclease, the structure and mode of action of which are known in detail [4–6].

‡ To whom reprint requests should be addressed.

RESULTS AND DISCUSSION

In a wide-ranging series of preliminary experiments, acid ribonuclease in crude supernatants prepared from pea cotyledons was shown to be completely insensitive to photo-oxidation in the presence of either methylene blue or crystal violet at pH 5.4 or 7.2. The enzyme in crude supernatants is also insensitive to γ-irradiation (1.8×10^6 rad/hr). In contrast, acid phosphatase in the same crude supernatants is extensively inactivated by both photodynamic treatment and by γ-irradiation.

Differential precipitation of acid ribonuclease with $(NH_4)_2SO_4$, followed by gel filtration on columns of Sephadex G-50 (fine) results in a purification of *ca* 150-fold (2.1 ± 0.2 μg RNA hydrolysed/mg protein/min in crude extracts; 312 ± 105 μg RNA hydrolysed/mg protein/min in partially purified extracts). Further, the purification procedure also removes substrate (RNA) from the enzyme preparations, since all native RNA molecules are excluded from the gel. Figs. 1a and 1b show the effects of irradiation of the partially purified enzyme preparations in the presence of methylene blue at pH 5.4 and 7.2. It is clear that at pH 5.4, irradiation in the presence of methylene blue has no effect on the activity of acid ribonuclease. In contrast, irradiation of the enzyme at pH 7.2 causes a marked decrease in activity during the first 15 min, although there is an apparent slight recovery of activity between 15 and 60 min. Further, at pH 7.2, the control samples incubated in darkness in the presence of dye show an increase in activity during a 60 min incubation and it is thus likely that the total extent of inactivation in the presence of methylene blue in the light at pH 7.2 is even greater than the net inactivation recorded in Fig. 1b.

The contrast between the total insensitivity of acid ribonuclease to photodynamic treatment in crude extracts, and its selective sensitivity in partially purified preparations, clearly suggests that the enzyme is afforded a great deal of protection in crude homogenates. The

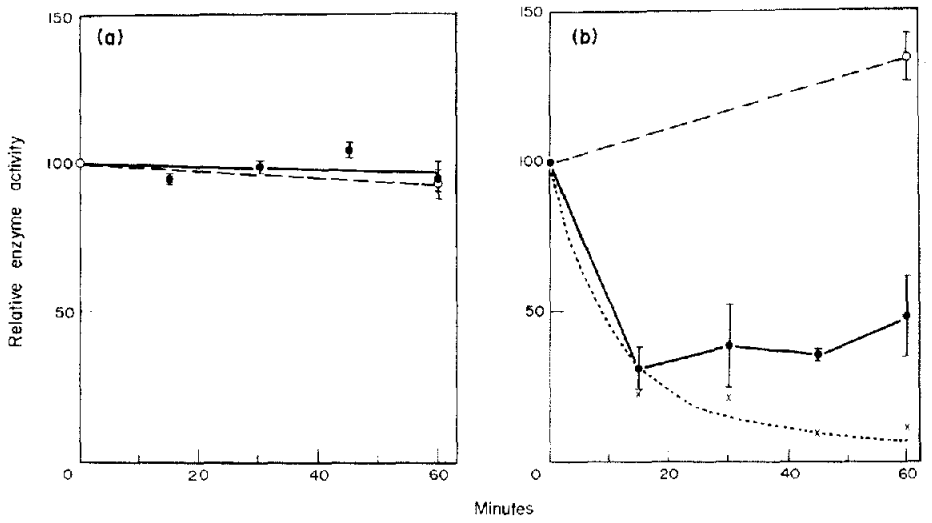

Fig. 1. Effect of irradiation in the presence of methylene blue on the activity of acid ribonuclease. (a) At pH 5.4; (b) at pH 7.2. Data are the mean of 8 determinations. Vertical bars are standard errors of the mean. Symbols: ●——● Experimental; ○– – –○ dark controls; × ····· × experimental values corrected for dark controls, with fitted first-order curve (i.e. log activity $1/t$ time).

most probable source of protection for the enzyme is the substrate, RNA. Cotyledons of pea seedlings are very active in RNA degradation [7] and it is thus likely that much of the ribonuclease is associated with RNA molecules in crude homogenates.

The resistance of the enzyme to photo-dynamic treatment at pH 5.4 is interesting. Methionine, cysteine, and to a lesser extent, tryptophan, are photo-oxidized at this pH [1, 2], and the results therefore suggest that these amino acids do not contribute directly to the enzyme activity. This contrasts with the behaviour of pancreatic ribonuclease. Firstly, in pancreatic ribonuclease, photo-oxidation of methionine residues lying on or near the surface of the native enzyme molecule causes a small, but significant reduction of activity [8]. The fact that this does not happen with pea cotyledon ribonuclease supports our earlier finding that a small fragment of the native enzyme retains catalytic activity [3]. Secondly, in pancreatic ribonuclease, photo-oxidation of the methionine residue at position 13 leads to an 80% reduction in activity [8]. This is because this particular methionine residue lies in the active site, adjacent to a histidine residue which participates in the enzymic reaction [4, 5, 8, 9]. Our data thus suggest that methionine does not occupy such a position in pea ribonuclease.

The acid ribonuclease from pea cotyledons is very markedly inactivated by photo-oxidation at pH 7.2. At this pH, histidine is by far the most vulnerable amino acid, and cysteine, methionine and tryptophan are also photo-oxidized to a significant extent. The marked inactivation at pH 7.2 (taken with the lack of inactivation at pH 5.4) may thus be attributed to photo-oxidation of histidine. This suggests that histidine may be directly involved in the active site. The mode of action of pea cotyledon ribonuclease is thus likely to be very similar to that of pancreatic ribonuclease. In pancreatic ribonuclease, the histidine residues at positions 12 and 119 lie on opposite sides of the groove which forms the active site [4, 5, 9] and actually participate in the reaction. Modification of either of these residues by photo-oxidation [6], or by iodo-acetate [9], causes a marked loss of enzyme activity.

The inactivation curve for pea cotyledon ribonuclease, irradiated in the presence of methylene blue at pH 7.2, departs markedly from the theoretical first-order plot [10]. Further, in control samples incubated in the presence of the dye in darkness, there is an increase in activity. The reason for this is not known, but the result suggests that methylene blue itself may interact with the enzyme at alkaline pH, but not at acid pH. If the data for the irradiated samples are corrected for the activation of the enzyme by methylene blue in darkness, the inactivation curve approximates much more closely to a first-order plot (Fig. 1b).

EXPERIMENTAL

Pea seeds (*Pisum sativum* L., cv Feltham First) were surface-sterilized in Na hypochlorite soln (2% available Cl_2) washed in running tap H_2O for 4 hr, planted in moist vermiculite and allowed to germinate at 22–25°. The seedlings were harvested after 5 days. The cotyledons were homogenized in 0.2 M NaOAc buffer, (pH 5.4) with a pestle and mortar at 3°. The homogenate was centrifuged at 34000 *g* for 15 min at 4°. Ribonuclease was purified 150-fold from the supernatant by differential precipitation and gel filtration as described earlier [3]. Samples of enzyme prepns were irradiated with visible light from an Osram 1500 W tungsten bulb (120 mm from the sample), in the presence of methylene blue (10^{-5} M). During irradiation, the samples were maintained at 1–5°. Aliquots were removed at 15 min intervals for assay of acid ribonuclease [11]. Control samples containing dye were kept in darkness. Aliquots from control samples were taken at zero time and after 60 min. For expts in which irradiation was performed under alkaline conditions, enzyme samples were prepared in 0.05 M Tris–HCl buffer (pH 7.2). The pH was adjusted to 5.4 after irradiation.

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MYOPORONE AND RELATED KETO ALCOHOLS FROM STRESSED SWEET POTATOES

LEO T. BURKA and JEANNETTE ILES
Department of Chemistry, Vanderbilt University, Nashville, TN 37235, U.S.A.

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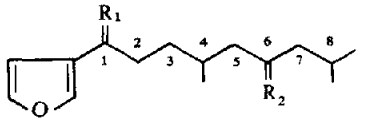
Key Word Index—*Ipomoea batatas*; Convolvulaceae; sesquiterpene; stress metabolite.

Our investigation of stress metabolites of the sweet potato has led to the isolation of myoprone (**1**) and two related keto alcohols (**2** and **3**) from this plant. The compounds were obtained from a methanol–chloroform extract of mercuric chloride treated sweet potatoes by a combination of column chromatography on Si gel and HPLC. By this method 39 mg of **1**, 109 mg of **2** and 53 mg of **3** were isolated from each kg of treated sweet potatoes.



1 $R_1 = R_2 = O$
2 $R_1 = O$; R_2 = H, OH
3 R_1 = H, OH; $R_2 = O$

Myoporone was initially isolated from Si gel chromatography as a mixture with ipomeamarone. The mixture was separated by HPLC on a reversed phase column. Compound **1** $[\alpha]_D^{25}$ −8.5°, c = 1.54, MeOH) was identified by comparing its ^{1}H NMR and mass spectra to those reported by Blackburne *et al.* [1].

The isomeric keto alcohols were isolated from the Si gel column as a mixture with 7-hydroxymyoporone [2]. This mixture was also resolved by HPLC on a reversed phase column. Compound **2** contained a keto–furan moiety as evidenced by the UV λ_{max} (MeOH) 251 nm (ε = 3000), IR ν_{max} (neat) 1670 cm^{-1}, and the MS fragments at *m/e* 95 and 110 corresponding to cleavage of C-1, C-2 and McLafferty cleavage of C-2, C-3. The broad absorption from 3700 to 3100 cm^{-1} in the IR verified the presence of a hydroxyl group. The MS fragment at *m/e* 195 is consistent with placing the hydroxyl group at C-6. The ^{1}H NMR spectrum of the compound is consistent with structure **2**: δ 0.91 and 0.93 (9H, superimposed *d*, CH_3), 1.34 (4H, *m*, 5- and 7-CH_2), 1.45–2.2 (5H, *m*), 2.80 (2H, *t*, J = 7 Hz, 2-CH_2), 3.88 (1H, *m*, 6-CH), 6.80 (1H, *m*, 4-furyl), 7.46 (1H, *m*, 5-furyl) and 8.07 (1H, *m*, 2-furyl). The product of oxidation of **2** with pyridinium had chlorochromate [3] ^{1}H NMR and mass spectra identical to those of **1**.

Unlike most of the furanosesquiterpenoid stress metabolites from sweet potatoes, C-1 in compound **3** ($[\alpha]_D^{25}$ −6.4°, c = 1.34, MeOH) is not in the ketone oxidation state. This is obvious from the coincidence of signals in the ^{1}H NMR for the 2- and 5-furyl protons, as is seen in the case of 1-ipomeanol and 1,4-ipomeadiol [4], and from the major fragment in the MS at *m/e* 97. The carbonyl absorption at 1705 cm^{-1} in the IR spectrum indicates that the carbonyl group is unconjugated. The fragment in the MS at *m/e* 195 resulting from cleavage at C-6, C-7 and the fragment at *m/e* 100 resulting from McLafferty cleavage of C-4, C-5 places the carbonyl group at C-6. The ^{1}H NMR of **3** is consistent with the proposed structure: δ 0.90 (9H, *d*, J = 7 Hz, CH_3), 1.34 (2H, four line *m*, 3-CH_2), 1.5–2.2 (CH, *m*), 2.26 (4H, *m*, 5- and 7-CH_2), 4.66 (1H, *t*, J = 7 Hz, 1-CH), 6.42 (1H, *m*, 4-furyl) and 7.42 (2H, *m*, 2- and 5-furyl). Oxidation of **3** with pyridinium chlorochromate [3] gave **1** as the major product.

Myoporone has been isolated from *Myoporum bontioides* A. Gray [5], from other *Myoporum* species and *Eremophila* species [1], and from *Eumophia sericea* and *E. prostata* [6]. Thus, myoporone joins epingaione [7] (ipomeamarone [8]), dehydroepingaione [7] (dehydroipomeamarone [9]), and athanagrandione [10] (4-hydroxymyoporone [11]) as furanosesquiterpenes which are stress metabolites of the sweet potato and are also normal secondary metabolites of other plants.

Toxicity studies on compound **2** indicate that it is