Phytochemistry, 1973, Vol. 12, pp. 1269 to 1272. Pergamon Press. Printed in England.

DIFFERENTIAL EXTRACTION OF ISOPEROXIDASES FROM *PISUM* SHOOTS

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(*Received* 4 *October* 1972. *Accepted* 8 *December* 1972)

Key Word Index—*Pisum sativum*; Leguminosae; pea; isoperoxidases; differential extraction.

Abstract—When green or etiolated Alaska pea shoots are extracted with water or 50 mM phosphate buffer, pH 6·5, or macerated without addition of buffer, the elution profile of the resulting supernatant lacks several isoperoxidase components which are readily extracted from the residue with 10 mM EDTA at pH 6·5. Subsequent extraction with 270 mM NaCl does not solubilize any further components. These results cast doubt on the validity of earlier reports on isoperoxidase patterns in *Pisum* and other plants.

INTRODUCTION

'SAP' FROM tissue macerates, or extracts made with water or phosphate buffer of low ionic strength have been used extensively in studies on plant peroxidase involving its total activity,[1] IAA oxidase function,[2,3] and isoenzymic composition as affected by ontogeny[4,5] and hormone treatment.[6–8] In most of these studies, which have considerable bearing on the physiological role of the enzyme, the experimental plant was the pea (*Pisum sativum* L.). I have found that the residue from such breis made from pea shoots yields, on appropriate mild extraction, considerable additional peroxidase of different isoenzymic composition. This may well invalidate some of the conclusions reached above.

Histochemical work[9,10] shows that some peroxidase is located in the cell wall. Peroxidase is also strongly adsorbed by cell wall material *in vitro*, elution requiring high ionic strength.[11,12] On the other hand, investigation of the association of peroxidase with the ribosomal fraction of plant homogenates[13] has led to the discovery[14] that the *de novo* synthesis of two ribosome-bound isoperoxidases is stimulated by treatment of lentil roots with IAA. Solubilization of these enzymes can be achieved with either low concentrations of EDTA or with salt solutions of moderate ionic strength.

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Extraction of the full spectrum of isoperoxidases from a plant tissue may therefore require not only high ionic strength but also the presence of EDTA. The present communication demonstrates that these factors are not equivalent and that the major pea shoot isoperoxidases, in addition to differing markedly in oxidase activity and tissue distribution,[15] must also differ in their intracellular localization.

RESULTS

Figure 1 compares the isoperoxidase patterns that result from exhaustive differential extraction at pH 6·5 of etiolated pea shoots. The numbering of the peaks is in order of decreasing electrophoretic mobility towards the cathode; it is shown elsewhere[16] that each peak contains a single, electrophoretically distinct peroxidase. As the assay of column fractions was done under standard conditions, the elution profiles are directly comparable with each other.

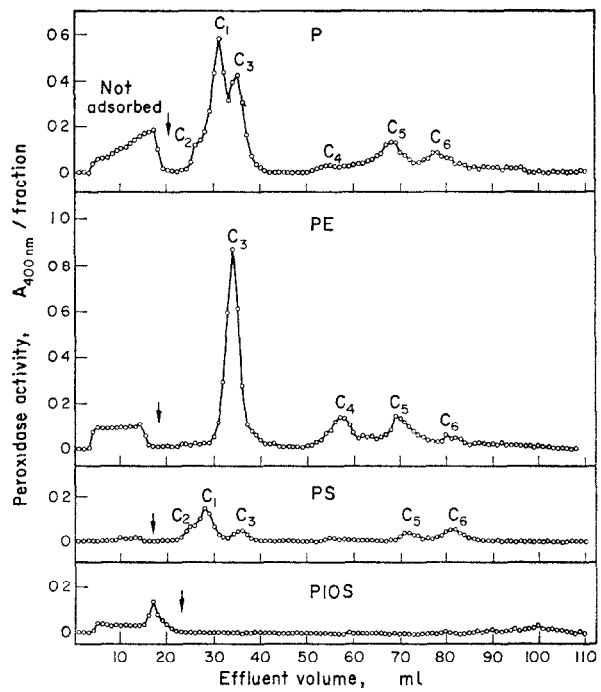

FIG. 1. ISOPEROXIDASE PROFILES RESULTING FROM EXHAUSTIVE EXTRACTION AT pH 6 5 OF ETIOLATED PEA SHOOTS WITH 50 mM Na PHOSPHATE BUFFER (P), FOLLOWED BY 10 mM Na_2 EDTA IN P (PE) THEN 270 mM NaCl IN P (P10S); OR FOLLOWED BY 20 mM NaCl IN P (PS). EXTRACTS WERE CHROMATOGRAPHED ON DOWEX 50 COLUMNS AS DESCRIBED IN THE TEXT, ARROWS DENOTE BEGINNING OF SALT GRADIENT.

Striking differences in the profiles are apparent. Phosphate buffer alone (P) extracts practically all of the C_1 and C_2, a small proportion of the C_3 and some C_5 and C_6. Subsequent extraction with 10 mM Na_2 EDTA in phosphate buffer (PE) solubilizes a large

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amount of C_3, accompanied by a little C_4 and some C_5. As a check that this effect is due to the EDTA and not the sodium ion, parallel extraction with NaCl at the same Na concentration (PS) yielded a small amount of activity with a profile like that of P. Finally, 'high salt' extraction of the PE residue with 270 mM NaCl (P10S) removed only a small amount of activity moving with the front. A similar result was obtained with green shoots; in this case the high activities of C_1 and C_2[15] made it difficult to ascertain whether C_3 was contributing to the P profile. If water was substituted for phosphate buffer as the first extractant, then no C_3 was extracted at all.

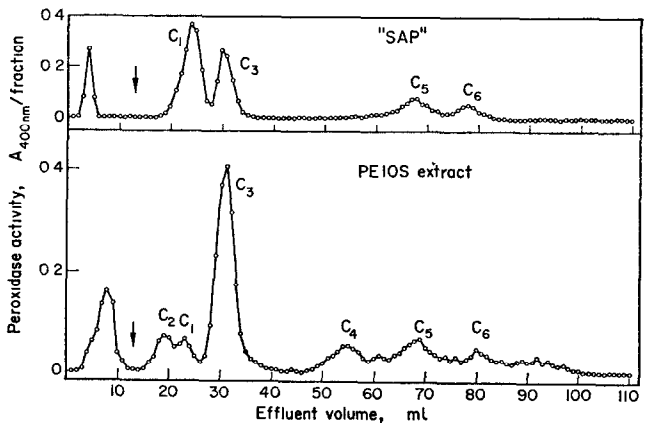

FIG. 2. ISOPEROXIDASE PROFILES OF 'SAP' FROM MACERATE OF ETIOLATED PEA SHOOTS AND OF PE10S EXTRACT OF MACERATE RESIDUE. (PE10S = 50 mM Na PHOSPHATE–10 mM Na_2 EDTA–270 mM NaCl, pH 6·5.)

Figure 2 contrasts the isoperoxidase pattern of 'sap' from a macerate of 10 etiolated shoots, prepared by grinding them with sand in a mortar and centrifuging, with that of a PE10S extract of the macerate residue (3 extractions with 10 ml, pooled). The components C_2 and C_4, and most of the C_3, are missing from the 'sap'.

DISCUSSION

Clearly neither the phosphate buffer extract nor the 'sap' from a macerate are representative of the isoperoxidase composition or total activity in the pea shoot. Because the relative IAA oxidase activity of C_3 is 10-fold greater than that of C_1[15] these types of extraction will also cause serious underestimation of IAA oxidase activity, depending on the part of the shoot being extracted. Rapid extraction of all 6 isoperoxidases is, however, achieved with PE10S extractant, which is therefore suitable for physiological studies. Although the low activity in the 'high salt' extract indicates the absence of significant amounts of ionically bound peroxidase in the residue, covalently bound cell wall peroxidase requiring cellulase digestion for liberation[17] could still be present.

It may be concluded that isoperoxidases C_3 and C_4 (possibly C_5 as well) are solubilized from some cellular structure by EDTA, acting as chelator rather than salt, whereas C_1 and C_2, being almost quantitatively extracted by buffer alone, are located in the cytosol or in a

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very labile structure. The similar behaviour of C_3 and C_4 towards extraction supports the evidence presented elsewhere[16] that their synthesis is controlled by alleles of the same gene.

Other examples of proteins selectively solubilized from cellular structures by EDTA are a coupling factor in thylakoid membranes[18] and the axoneme protein of *Chlamydomonas* flagella.[19] The association of peroxidases with pea shoot and lentil root ribosomes[13,14] may be an extraction artefact due to the binding of these basic proteins to the phosphate groups of the RNA.[20] The *in vivo* location of these enzymes and of the *Pisum* components C_3 and C_4 may rather be in the membranes of the endoplasmic reticulum, as is the case with thyroid peroxidase.[21]

EXPERIMENTAL

'Alaska' pea seedlings were grown as described elsewhere.[15,16] Etiolated shoots had the third internode about half elongated; green shoots had the third leaf partly expanded. Isoperoxidase patterns were determined by chromatography at pH 6·2 on 10 × 1 cm columns of Dowex 50 X2 resin in the Na^+ form,[15] using a 100 ml concave gradient from 10 mM Na phosphate buffer to 0·8 M NaCl in the same buffer. Peroxidase activity was assayed at 25° with guaiacol as H-donor, using a modified Maehly-Chance assay for extracts and a modified Jermyn–Thomas assay for column fractions.[15]

The following extractants were used: P—50 mM Na phosphate, pH 6·5; PE—P + 10 mM Na_2 EDTA + NaOH (7 mM) to pH 6·5; PS—P + 27 mM NaCl, pH 6·5; P10S—P + 270 mM NaCl, pH 6·5; PE10S—PE + 270 mM NaCl, pH 6·5. All operations were performed at 0–5°. Centrifugations were at 20 000 *g* for 10 min

For the experiment shown in Fig 1, 10 etiolated seedlings were ground in a chilled mortar with sand and 2 ml of P extractant to give a smooth hire, which was transferred to a polypropylene tube with a further 8 ml of P and centrifuged. The residue, after washing twice by resuspension in 10 ml of P was resuspended again and distributed equally between two tubes. Following centrifugation, the combined supernatant (i.e. fourth P extract) contained only 3% of the total activity (24·9 units) extracted by P.

One of the above pellets equivalent to half of the original material and extracted four times with P was now extracted four times with 10 ml of PE extractant; the fourth extract contained 3% of the total activity (5·0 units) extracted by PE. The other residue was extracted four times with PS; the fourth extract contained 12% of the total activity (1·6 units) extracted by PS. Finally the residue from PE extraction was extracted four times with P10S, the fourth extract containing 10% of the total activity (2·2 units) extracted by P10S.

Glycerol to a concentration of 10% was dissolved in aliquots of these pooled extracts equivalent to 2 shoots, and they were stored for several days at −20° prior to chromatography.

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