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SHORT COMMUNICATION

STUDIES ON THE ENZYMIC BROWNING OF APPLES—III

PURIFICATION OF APPLE PHENOLASE*

J. R. L. WALKER† and A. C. HULME

Agricultural Research Council, Ditton Laboratory, Larkfield, Maidstone, Kent

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Abstract—Phenolase has been extracted from apple peel preparations by the use of Triton X-100, and separated into two fractions on a DEAE-cellulose column. Since each fraction was homogeneous by starch gel electrophoresis and exhibited similar substrate specificities it was considered that they were isozymes.

EARLIER attempts to fractionate and purify the phenolase (*o*-diphenol:O_2 oxidoreductase, E.C. No.: 1.10.3.1.) of apple peel and fruit[1] were only partially successful because of the restrictions imposed by the need to use highly alkaline media to solubilize the enzyme. However, Harel *et al.*[2] have shown recently that the phenolase in chloroplast preparations from apple peel is soluble in dilute (1 %) solutions of Triton X-100 (iso-octyl-phenoxy-polyethoxy-ethanol; Rohm and Haas Co., U.S.A.). We have, therefore, investigated the effect of this nonionic detergent on the phenolase present in the mitochondrial and polyethylene glycol (PEG) precipitate fractions[3] obtained from the peel of Cox's Orange Pippin apples.

The extraction procedure was similar to that used by Harel *et al.*[2] The mitochondrial or PEG-precipitate fractions, prepared in the presence of polyvinylpyrrolidone as previously described,[3] were extracted with Triton X-100 (1 %, final concentration) for 30 min and then centrifuged at 100,000 *g* for 30 min. The viscous clear green supernatant was applied to a column, 90 × 15 mm, of DEAE-cellulose powder (Whatman P/DE. 100.F.2) which had been previously equilibrated with 0·01 M phosphate-citrate buffer, pH 5·0. The column was washed with 50 ml of 0·01 M buffer which removed the Triton X-100 and chlorophyll, then eluted stepwise with 100 ml 0·05 M buffer followed, in order, by 50 ml 0·1 M buffer and 50 ml 0·2 M buffer; the eluate being collected in 6 ml fractions. Further elution with 0·2 M buffer, pH 7·5, yielded no more active fractions. Phenolase activity was estimated manometrically in a Warburg respirometer (as in Table 1) or by means of a Clark oxygen electrode, using 0·5 ml eluate fraction, 1·6 ml 0·1 M buffer, (pH 5·0) containing 0·1 M Manoxol OT (to release maximum activity[4]) and 4 μmole substrate. As can be seen from Fig. 1 the bulk of the phenolase activity was readily eluted by 0·05 M buffer (Peak I) whilst a second, much smaller, amount of enzyme was eluted by 0·1 M (Peak II). Two similar enzyme fractions

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† A.R.C. Underwood Research Fellow: on leave from the Cawthron Institute, Nelson, New Zealand.

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Table 1. Specific activities of phenolase fractions obtained during purification procedure

Fraction	O_2 uptake, μl/min/mg protein*	
	From mitochondria†	From PEG-precipitate
Original sample	2·16	2·08
Triton X-100 extract	2·26	2·38
Peak I	47·6	76·6
Peak II	29·1	24·9

* Determined manometrically, 4-methylcatechol as test substrate.

† Electron microscopy showed that these mitochondria contain a small amount of debris including parts of a rudimentary type of chloroplast much smaller than the normal chloroplast of leaves.

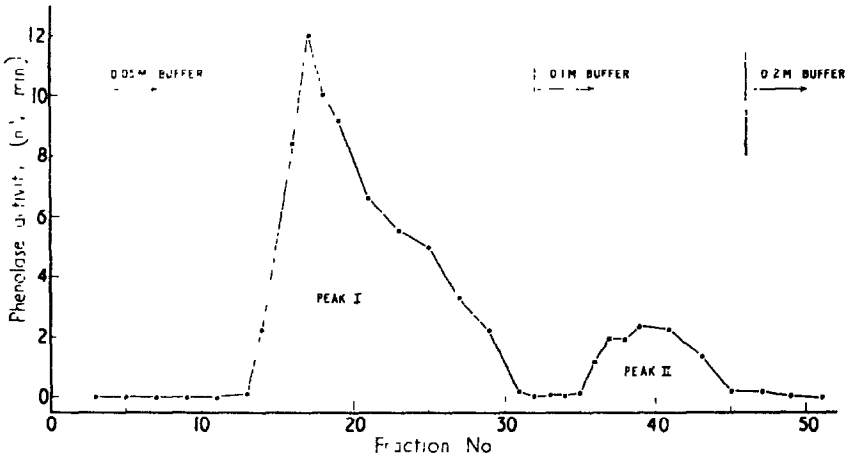

Fig. 1. Elution of Triton-solubilized phenolase from PEG-precipitate from DEAE-cellulose column (phosphate-citrate buffer pH 5·0).

Table 2. Comparison of relative rates of O_2-uptake for different phenolic substrates by enzymes of peak I and peak II*

Substrate	From mitochondria				From PEG-precipitate			
	Peak I		Peak II		Peak I		Peak II	
	(mV/min)	(%)	(mV/min)	(%)	(mV/min)	(%)	(mV/min)	(%)
4-Methylcatechol	3·05	100	0·80	100	2·95	100	1·30	100
Chlorogenic acid	1·55	51	0·38	48	1·75	59	0·68	52
(−) Catechin	0·88	29	0·22	27	0·73	25	0·26	20

* Measured by O_2 electrode.

were obtained also from Triton extracts of mitochondria. The specific activities of the various fractions are given in Table 1, and it will be seen that the purification procedure achieved almost a forty-fold increase in specific activity. Protein was estimated by a micro-kjeldahl method[5] or from absorptivity measurements.[6] Table 2 gives a comparison of the relative reaction rates for different phenolic substrates.

Since attempts to fractionate further the two enzyme peaks by ammonium sulphate precipitation were unsuccessful the enzyme solutions were concentrated by treatment with Sephadex G-25 and examined by horizontal starch gel electrophoresis at pH 5·0 (phosphate-citrate buffer) and at pH 8·6 (borate buffer). The enzymes were located by spraying the gel blocks with the appropriate phenolic substrate dissolved in pH 5·0 buffer. Similar separation patterns, as shown in Fig. 2, were obtained at each pH value for both the mitochondrial and PEG precipitate enzymes. The amounts of protein involved were too small to enable the enzymes to be located by staining with amido black 10B. Electrophoresis experiments on polyacrylamide gel[7] also confirmed the above findings. Unlike the results of Harel *et al.*[2] no differences in substrate specificity were observed between the various enzyme fractions and, in confirmation of our earlier results,[4] the purified enzyme showed maximal activity only at pH 4·8–5·0.

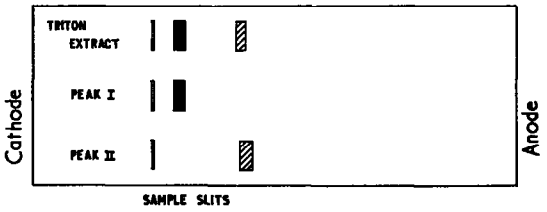

FIG. 2. ELECTROPHORETIC PATTERNS OF PHENOLASE FRACTIONS OBTAINED FROM PEG-PRECIPITATE; RUN ON STARCH GEL FOR 17 hr AT pH 5·0 (11 V/cm, 2 mA/cm WIDTH).

Recent reports by Bouchilloux *et. al.*[8] and other workers[9] indicate that mushroom tyrosinase (phenolase) can exist in several forms and that under certain conditions these forms are interconvertible[10] whilst Bendall and Gregory[11] consider that the various phenolase fractions they obtained from green tea leaves are modified forms of the one native enzyme. On the basis of the electrophoresis experiments described above and because of the close similarity of the relative reaction rates for chlorogenic acid and catechin, which are both key substrates for apple phenolase but of markedly different chemical constitution, it appears likely that the phenolases of the mitochondria and PEG-precipitate are identical and that Peak I and Peak II are probably isozymes. Present evidence, such as detergent activation[4, 12] or the frequent need to use organic solvents, detergents[2, 13] or high pH buffers[1, 11] in the extraction procedures, suggests that phenolase may be intimately associated with lipid material in the plant cell. However, in view of Jolley and Mason's[10] recent observations

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on the interconversion of the isozymes of mushroom tyrosinase and in the light of the above results it is pertinent to question the exact state and location of phenolase in the *intact* cell and to ask just how much the observed distribution is an artefact of the preparative procedure.

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