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PURIFICATION AND MULTIPLICITY OF CATECHOL OXIDASE FROM APPLE CHLOROPLASTS

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Abstract—Catechol oxidases were extracted from subcellular fractions of apples. Triton X-100 was efficient in extracting the enzymes from chloroplasts and digitonin from mitochondria. The enzymes present in the chloroplast extract were separated by starch gel electrophoresis into three bands which corresponded to fractions obtained by chromatography on DEAE-cellulose columns. The fractions were differentiated according to K_m, substrate specificity and sensitivity to inhibitors. A three-hundred-fold purification of the major fraction was obtained. The evidence for a real multiplicity and its possible implications are discussed.

INTRODUCTION

IN A previous paper it was shown that in apples, catechol oxidases (*o*-diphenol: oxygen oxidoreductase, 1.10.3.1[1]) are present in several subcellular fractions.[2] Walker[3] has also shown that apples contain catechol oxidase but did not definitely assign it to a single subcellular fraction, nor did he prove the presence of more than one enzyme. Although, a number of workers have shown that other plant tissues contain several distinct catechol oxidases,[4–11] most of these authors did not indicate the subcellular location of the different enzymes. It was also uncertain whether the apparent multiplicity might not have been an artefact caused by method of extraction and preparation. Catechol oxidase has been shown to be latent in certain cases,[12] and the activation of the latent enzyme has been related to changes in tertiary structure.[11]

Increase of activity of such enzymes by detergent treatment has also been reported.[13–16] Purification of catechol oxidase has been achieved by many workers. However since a number of such enzymes appear to be present in different subcellular fractions it seemed of

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considerable importance to relate the enzyme activity of a purified fraction to a definite subcellular structure. This is of particular importance if the physiological role of these enzymes is to be understood. This can only be deduced if accurate information on the nature and location of these enzymes is known.

In the following, the multiplicity of catechol oxidase in a single subcellular fraction of apples will be shown and evidence brought to indicate that these are not isoenzymes.

RESULTS AND DISCUSSION

As previously shown (Harel *et al.*[2]), the chloroplast and mitochondrial fractions, isolated from apples, appeared to contain different catechol oxidases. Although apparently two pH optima could be observed in each fraction, it seemed possible that these might have arisen

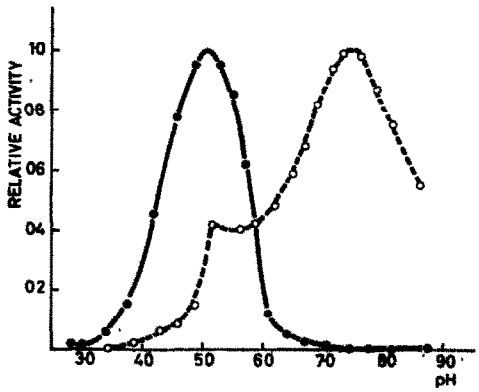

FIG. 1. CHANGE IN CATECHOL OXIDASE ACTIVITY WITH pH.

Citrate-phosphate buffer. Substrate: 4-methylcatechol 5×10^{-3} M. Activity determined with O_2 electrode.

●——● 1% Triton X-100 extract of chloroplasts.
○---○ 1% Digitonin extract of mitochondrial fraction.

from contamination of one fraction by the other. To clarify this point chloroplasts were prepared at a lower "g" value, as described in "Experimental", to reduce possible contamination by other fractions and then extracted with Triton X-100. Such an extract showed a single pH optimum at pH 5·1 (Fig. 1). It was impossible to prepare a mitochondrial fraction devoid of chloroplast fragments. However, when a "mitochondrial" fraction was prepared and treated with Triton X-100, the extract had an activity at pH 5·1 about 12 times higher than at pH 7·3, while in the residue the ratio was 0·8.

Triton X-100 appears to extract preferentially from chloroplasts, while digitonin extracts preferentially from mitochondria (Table 1). Thus it may be concluded that in fact the pH optimum at 5·1 is due to chloroplast enzymes and that at pH 7·3 due to mitochondrial ones. This was further confirmed by the fact that a digitonin–NaCl extract of the mitochondria shows a single pH optimum at pH 7·4 with a shoulder at pH 5·1 (Fig. 1). The bulk of the subsequent work to be described relates therefore to the more readily isolated chloroplasts. Some experiments with mitochondria will also be described.

TABLE 1. EXTRACTION OF CATECHOL OXIDASE FROM CHLOROPLAST AND MITOCHONDRIAL FRACTIONS OF APPLES BY VARIOUS SURFACE ACTIVE AGENTS

Treatment	Per cent solubilization		Type of extracting agent
	Chloroplasts	Mitochondria	
1% Digitonin in 0·5 N NaCl pH 7·0	8	24	Non-ionic
1% Deoxycholate pH 7·3	36	19	Anionic
1% Deoxycholate in 1 M KCl pH 7·3	42	25	Anionic
1% Na-Dodecyl Sulphate pH 7·3	4	0	Anionic
0·5% Manoxol IB pH 5·0	0		Anionic
3·6% Tween-80 pH 7·3	28		Non-ionic
1% Triton X-100 pH 7·3	77	11	Non-ionic
2% Butanol	0		Non-ionic

Solubilization expressed as per cent of activity of whole particles, remaining in the supernatant after 100,000 *g* for 1 hr.

Activity of Chloroplast catechol oxidase determined at pH 5·1 and of Mitochondrial catechol oxidase at pH 7·3—both with 5×10^{-3} M 4-methylcatechol.

Extraction of Enzyme from Chloroplasts

Although the above experiments show that the enzymes in chloroplasts have a pH optimum of 5·1, they do not indicate whether one or more enzymes are responsible for catechol oxidase activity. Preliminary experiments indicated the existence of several enzymes in the chloroplast. However multiplicity might also be caused by breakage of a single enzyme into subunits or by changes in tertiary structure. In order to investigate this question the chloroplasts were extracted by several different means. The efficacy of extraction was compared and the electrophoretic pattern of the extracts studied.

Artefacts arising from breakage or structural changes would be expected to differ in their electrophoretic behaviour, depending on the extracting agent used. The same would also be expected if the extracting agent forms complexes with the enzyme. Table 1 shows the amount of enzymes solubilized by the different agents; for most experiments, therefore, Triton X–100 was used subsequently. Although deoxycholate (DOC) was fairly effective, its tendency to form gels made it inconvenient for further use. Efficiency of extraction correlated with solubilization of chlorophyll and depended not only on pH and type of detergent but also on the detergent/protein ratio. The Triton X–100 extract has catechol oxidase properties similar to whole chloroplasts. Two changes could however be observed, an almost complete loss of activity towards *p*-cresol and a lowering of activity towards Dopa and Dopamine.

The electrophoretic patterns of different extracts was compared. Initially, agar gel electrophoresis was attempted but gave unsatisfactory results. Unless otherwise stated the results quoted were obtained using starch gel electrophoresis, as described in the experimental section. Best separation was obtained at pH 8·0, in 0·04 M phosphate buffer, using 7·5 V/cm at 1·37 mA/cm and running for 20 hr.

Characteristically three bands were observed; one moving towards the cathode, Zone I, having low activity, and two moving towards the anode, Zones II and III (Fig. 2). The highest activity was observed in Zone II. These bands appeared whether extraction was with Triton X–100, Tween 80, DOC or digitonin. The substrate specifity of the different zones was tested by spraying the electrophoretograms with different compounds. Zone II was active towards 4-methylcatechol, dopa, (−)-epicatechin and chlorogenic acid. Zone I reacted with 4-methylcatechol and epicatechin and gave very slight reaction with the other compounds,

while Zone III gave no reaction with chlorogenic acid and a very faint one with dopa. Although colour development is not an accurate measure of substrate specifity these results do indicate that the three zones differ in this respect.

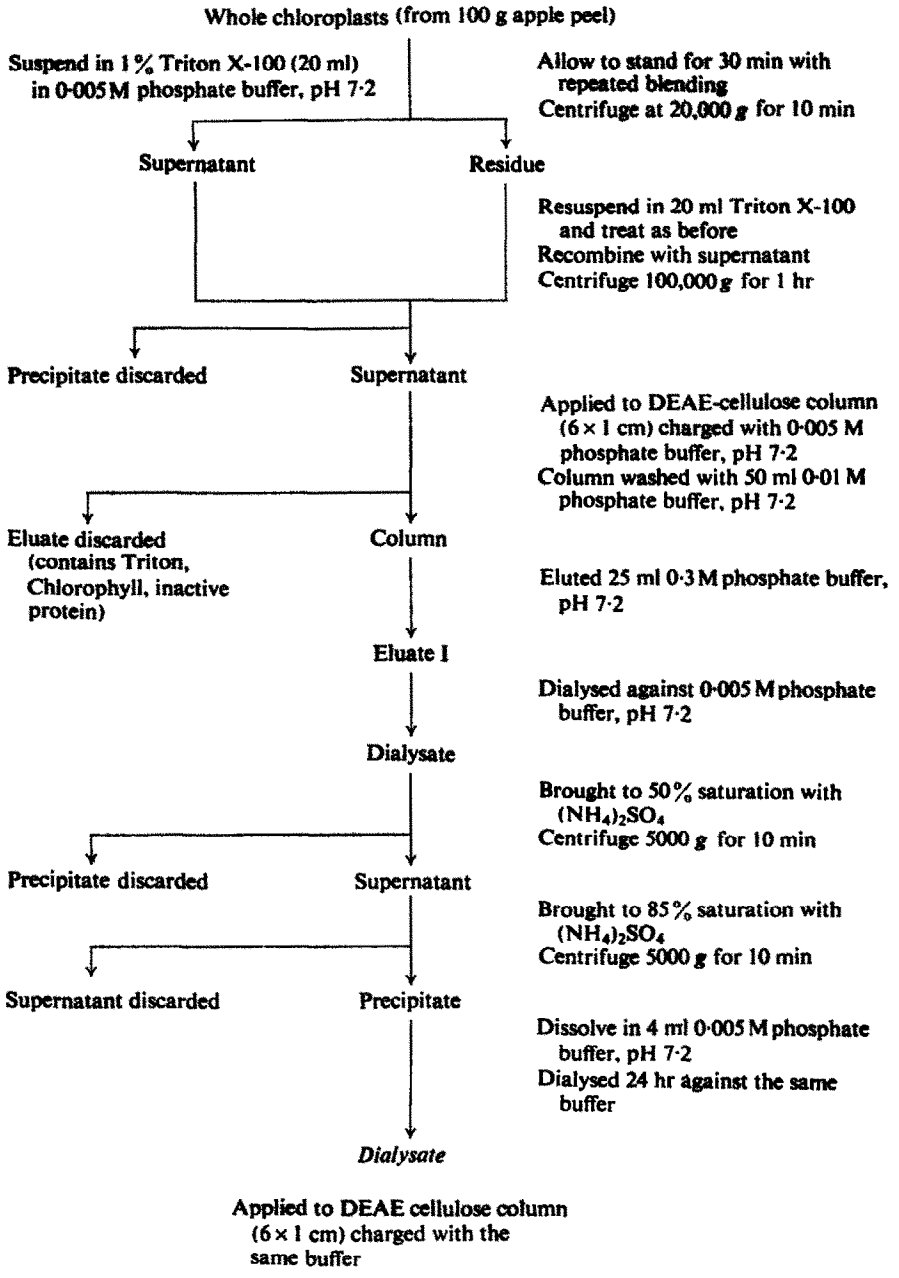

FIG. 3. FLOW SHEET OF PRELIMINARY PURIFICATION PROCEDURE OF TRITON X-100 EXTRACT OF CHLOROPLASTS.

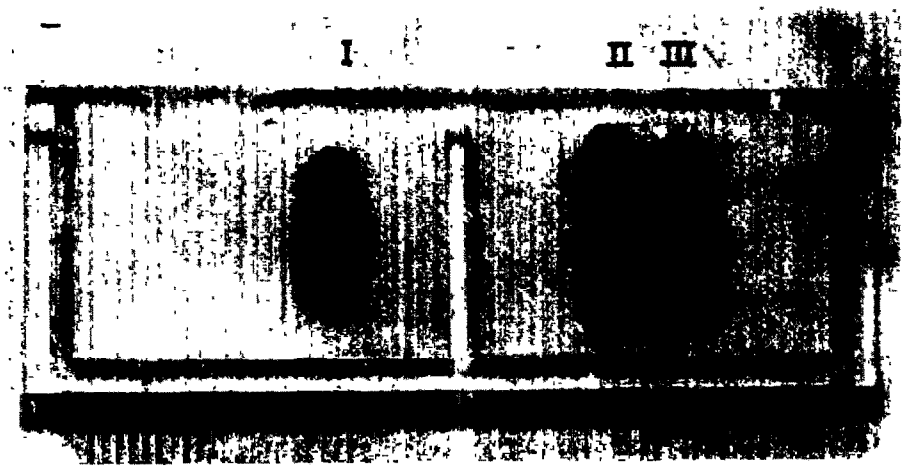

FIG. 2. ELECTROPHORETIC SEPARATION OF CATECHOL OXIDASES FROM TRITON X–100 EXTRACT OF CHLOROPLASTS ON STARCH GEL.

Experimental conditions and method of identification of catechol oxidase activity are described in the text. Substrate: used 4-methylcatechol.

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When the Triton X–100 extract of chloroplasts was separated by starch gel electrophoresis in the presence of 6 M urea, only the three bands having the same electrophoretic mobility as before were obtained. Electrophoresis of the purified fractions, on starch blocks in the presence of 6 M urea, gave single bands of the same electrophoretic mobility previously noted. The same was found when such fractions were incubated with Triton X–100 and rerun on starch.

The possibility of separating these three fractions by other means was investigated. For this purpose column chromatography was attempted. The Triton X–100 extract from whole chloroplasts was used as starting material. This extract contained inactive proteins, chlorophyll and excess Triton X–100 which could not be adequately removed by dialysis and interfered in fractionation with ammonium sulphate. For this reason the crude dialysed extract was applied to a DEAE column and the inactive fraction eluted with a low concentration of buffer. The active fractions were now eluted with a higher buffer concentration, and the eluate treated as described in the flow sheet (Fig. 3). Eighty per cent of activity was recovered between 50–80 per cent saturation with ammonium sulphate (Table 2).

TABLE 2. PURIFICATION OF CATECHOL OXIDASE FROM APPLE CHLOROPLASTS

Step	Specific activity	Purification	Yield (%)
Whole chloroplasts	6·3		
Triton extract after 100,000 g	8·5	1·3	80
1st eluate from DEAE-Cellulose	32·5	5·1	72
50–85 per cent Ammonium Sulphate precipitate	170·3	27·0	58
2nd DEAE-Cellulose eluate (0·09 M phosphate fraction)	2142·0	340·0	35

Specific activity as μl O_2/mg protein/min at pH 5·1 using Warburg technique at 26°, 4-methylcatechol 5×10^{-3} M.

The dialysate, after fractionation with ammonium sulphate, was applied to a DEAE cellulose column charged with 0·005 M phosphate buffer at pH 7·2. This column was eluted with a continuous gradient, using phosphate buffer pH 7·2. As can be seen from Fig. 4 this elution gave a separation into three distinct peaks. Attempts were made to compare the separation by using columns of TEAE-cellulose, ECTEOLA, or DEAE-Sephadex A–50 with phosphate buffer gradient pH 7·2 and also DEAE-cellulose using either KCl pH 7·2 or pH gradients. TEAE-cellulose gave results identical to those obtained with DEAE-cellulose. ECTEOLA did not bind the enzymes sufficiently strongly to permit adequate fractionation. DEAE-Sephadex binds enzymes more strongly so that higher buffer concentrations were required for elution. Attempts using a KCl gradient gave results essentially the same as those obtained with a buffer gradient. The pH gradient was unsuccessful, and no fractionation was obtained, elution occurring between pH 4·0–4·5.

In order to obtain the fractions in greater purity, a stepwise elution procedure was used. Fractions I, II, and III were eluted at concentrations of 0·04 M, 0·09 M and 0·20 M respectively (Table 2). Each fraction was now studied using starch gel electrophoresis. The fraction eluted with 0·04 M buffer moved to the cathode and corresponded to Zone I (Fig. 2), that eluted at 0·09 M corresponded to Zone II and that eluted at 0·20 M to Zone III.

The three fractions obtained from the column were compared for substrate specificity,

K_m for O_2 and 4-methylcatechol, pH optima and their sensitivity to inhibitors. The results appear in Tables 3 and 4. These show that as far as substrate specifity is concerned fraction II is distinctly different from fractions I and III which in this respect show considerable resemblance. However the difference between fraction I and III is shown by the difference in the K_m for O_2 and especially by the degree of inhibition by 2,3-naphthalenediol and *N*-vinyl-2-pyrrolidone.

Finally it must be mentioned that it has till now proved impossible to extract catechol oxidase from mitochondria. When the mitochondrial fraction was treated with Triton X–100, much of the contaminating activity due to chloroplasts was removed. When the residue was treated with digitonin, solubilization resulted. However, the enzyme so extracted failed to move from the starting line in starch or agar gel electrophoresis at various pH. It

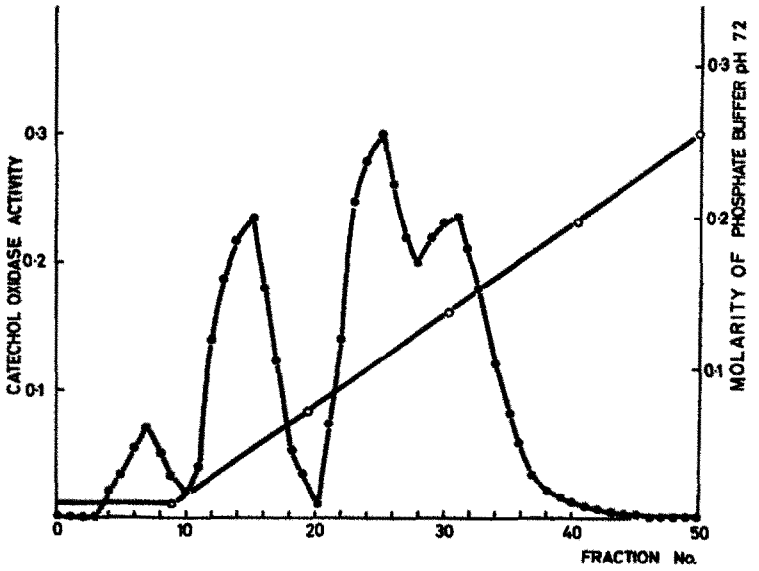

FIG. 4. COLUMN CHROMATOGRAPHY OF PARTIALLY PURIFIED TRITON X–100 EXTRACT USING DEAE CELLULOSE COLUMN ELUTED WITH PHOSPHATE BUFFER pH 7·2.
●——● Activity. ○——○ Molarity of buffer.

seems likely that no true solubilization was obtained. Clearly this enzyme is quite distinct from those occurring in the chloroplast.

The results described show that four distinct catechol oxidases are present in apples tissues, three of them in the chloroplasts and at least one in the mitochondria. The fact that these differ in electrophoretic mobility, movement on columns, substrate specifity, inhibition by different inhibitors and K_m towards O_2, no matter how separated or extracted, further stresses this point. Such enzymes could be regarded as artefacts arising from the extraction of an enzyme from its structure, breakage of a high molecular weight aggregate into smaller units or from changes in tertiary structure. However the evidence brought above makes such an interpretation unlikely. Equally it seems unlikely that iso-enzymes are involved. More probably each of these enzymes is bound to a specific site within its subcellular structure where it fulfils a definite physiological role. Apparently the amount of the enzymes differs. The complexity of catechol oxidase in plant tissues and consequently the difficulty of ascribing

any single function in cellular metabolism is therefore increasingly obvious. Any explanation of their function must take into account the multiplicity which has been demonstrated.

TABLE 3. SUBSTRATE SPECIFICITY OF CATECHOL OXIDASE FROM APPLE CHLOROPLASTS

Substrate*	Whole chloroplasts	DEAE cellulose column eluted with phosphate buffer pH 7·2		
		Fraction I 0·04 M	Fraction II 0·09 M	Fraction III 0·20 M
4-Methylcatechol	100	100	100	100
Chlorogenic acid	98	62	168	77
β-(3,4-Dihydroxyphenyl)alanine	88	10	33	15
(−)-Epicatechin	54	55	96	44
Caffeic acid (saturated solution)	26	12	38	13
3,4-Dihydroxybenzoic acid	34	4	22	0
p-Cresol	20	0	5	0

* Substrate concentration 5×10^{-3} M; activity determined using oxygen electrodes at pH 5·1 and expressed as per cent of activity towards 4-methylcatechol.

TABLE 4. COMPARISON OF PROPERTIES OF CATECHOL OXIDASES OBTAINED FROM DEAE CELLULOSE COLUMN

Property*	Whole chloroplasts	Fractions from DEAE cellulose column eluted with phosphate buffer		
		Fraction I 0·04 M	Fraction II 0·09 M	Fraction III 0·20 M
K_m for 4-methylcatechol	$5{\cdot}96 \times 10^{-3}$ M		$1{\cdot}54 \times 10^{-2}$ M	$4{\cdot}87 \times 10^{-3}$ M
K_m for oxygen (in presence of 1×10^{-2} M 4-methylcatechol)	25·8%	6·4%	39·2%	15·4%
pH maximum	5·1	5·1	5·1	5·1
Per cent inhibition by 5×10^{-3} M 2,3-naphthalenediol**	64	+59†	43	9
Per cent inhibition by 2·5% *N*-Vinyl-2-pyrrolidone**	60	21	68	55

* Reactions for K_m and inhibition determinations carried at pH 5·1. † Stimulation. ** 4-methylcatechol concentration 5×10^{-3} M.

EXPERIMENTAL

Apples, variety "Grand Alexander" purchased at a local supermarket and stored at 2–4° were used throughout the experiments. The subcellular fractions were prepared as previously described,[17] except that the chloroplast fraction was collected by centrifuging at 300 *g* for 20 min after an initial centrifugation for 3 min at 300 *g*, the precipitate of which was discarded. Oxygen uptake was either measured by conventional Warburg technique or by the use of a polarographic electrode, as previously described.[2] Michaelis constants for oxygen were determined using the polarographic electrode—the reaction mixture containing 2·5 ml phosphate citrate buffer 0·1 M pH 5·1, 0·1 ml enzyme and 1·0 ml 4-methylcatechol, 2×10^{-2} M. No gas phase was present.

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Extraction of Phenolase from Particles

After separation of the particulate fractions by centrifugation, resuspension in buffer and recentrifugation, the particles were suspended in the appropriate solutions of detergents in 0·01 M phosphate or phosphate citrate buffer. After allowing to stand for 30 min with occasional shaking, the suspensions were centrifuged at 100,000 *g* for one hr. Catechol oxidase activity and protein content of both the supernatant and precipitate were determined. The particulate precipitate suspended in buffer and treated as above was used as a control.

Column Chromatography

The most satisfactory treatment for solubilizing catechol oxidase from chloroplasts was with Triton X–100. The experimental procedure adopted for concentration and partial purification of the enzyme is described in Fig. 3.

The active fraction thus obtained was applied to columns of DEAE cellulose or similar compounds prepared in the usual way. The enzymes were eluted from the columns using either stepwise gradient or continuous gradient elutions, using a flow rate of 0·4–0·8 ml/min. The fractions were collected using a Shandon Automatic Fraction Collector AF/260. The gradient was determined by conductivity measurements. The activity of the eluates was estimated semi-quantitatively. To 1 ml eluate, 4 ml phosphate citrate-buffer 0·4 M, pH 5·1 and 0·5 ml 4-methylcatechol, 2×10^{-2} were added. The mixture was incubated for 20 min at room temperature and the intensity of the colour read at 390 mμ. The optical density was used as measure of activity. Protein was determined according to Lowry *et al.*[18]

Electrophoresis

The extracts obtained from chloroplasts, as described in Fig. 3, or fractions obtained by column chromatography were applied to $13{\cdot}5 \times 4{\cdot}0 \times 0{\cdot}3$ cm blocks consisting either of 1% Noble agar (Difco) or starch, prepared according to Smithies.[19] The samples were placed in a slit at the centre of the block. Electrophoresis was carried out at various buffers in the cold room, at 2°, in a "Shandon Universal Electrophoresis apparatus after Kohn" MK II, connected to a suitable d.c. power supply. A method was developed for detecting the enzymes in the blocks. The blocks were sprayed with a solution of the desired substrate (2×10^{-2} M) containing 0·05% *p*-phenylenediamine. This compound gives a coloured zone at the site of catechol oxidase activity due to its reaction with the quinones formed by the activity of the catechol oxidase. The colour varied depending on the phenolic substrate used. After 10 min the blocks were sprayed with 1×10^{-3} M ascorbic acid, to bleach the background colour formed by auto oxidation of phenylenediamine.

Chemicals

Chemicals were obtained commercially. *N*-Vinyl-2-pyrrolydone was purchased from Light & Co. Hydrolysed starch was obtained from Connaught Medical Research Laboratories, Toronto, the modified celluloses from Serva Entwicklungslabor and DEAE Sephadex A–50 (medium) from Pharmacia. Solutions of digitonin were prepared according to Kaplan.[20]

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