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Biochemical Studies of Cocoa Bean *o*-diphenol O_2 Oxidoreductase (catechol oxidase)

Stuart G. Reeves, Ian McDowell,* Kevin Behn & Jane Dench

Tropical Development and Research Institute, 56–62 Gray's Inn Road, London WC1X 8LU, UK

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ABSTRACT

*Monophenol monoxygenase (E.C. 1.14.18.1 Mayer and Harel, 1979) was extracted from cocoa (*Theobroma cacao *L.) cotyledons. Activity was measured with an oxygen electrode using 4-methyl catechol as a substrate. The effects of pH, extraction time, presence or absence of salt, detergent, PVPP, sodium ascorbate and dithiothreitol on enzyme solubilisation were investigated. NaCl and Triton X-100 were found to give the optimum extraction activity and this activity was found to be considerably higher than that reported by previous workers.*

INTRODUCTION

The presence of a polyphenol oxidase activity in cocoa cotyledons is well established and changes in the activity of this enzyme or enzymes during cocoa fermentation have been described, together with accompanying changes in polyphenol composition (Rohan, 1963). The actual role and significance of the enzyme in the production of chocolate flavour is not well understood, although the occurrence of low enzyme activities or conditions which might be expected to reduce enzyme activity, have been associated with the production of poor flavour quality (Holden, 1959). The significance of the enzyme is often mentioned implicitly in the reduction of undesirable, astringent off-flavours (Holden, 1959).

* To whom all correspondence should be addressed.

More recently, a more direct role for polyphenol oxidase in flavour production has been suggested (Purr *et al.*, 1963; Purr, 1972). *In vitro* experiments have shown the formation of addition products from quinones and amino acids as a result of polyphenol oxidase and protease activities. The addition products were implicated in the oxidative deamination of amino acids to form keto acids which are important for flavour production (Purr, 1972). The formation of volatile aldehydes by a similar process has also been demonstrated in cocoa extracts (Motoda, 1979), using a microbial polyphenol oxidase.

MATERIALS AND METHODS

Ripe brown Ghanaian cocoa pods were obtained from the Cocoa Research Institute, Tafo, and stored at −15°C until required. All reagents used were AnalaR grade.

Preparation of acetone extracted cocoa cotyledons

Frozen pods were broken open and the beans quickly removed and peeled. The cotyledons were immersed immediately in liquid nitrogen then freeze-dried for 17 h. After grinding briefly in a pestle and mortar or coffee grinder (Moulinex), the freeze-dried powder was stored desiccated at −15°C.

Acetone-extracted powders were prepared by extraction with 80% aqueous acetone (6 times) and 100% acetone (3 times) in a Waring blender at −20°C using a sample:solvent ratio of 1:10. Residual solvent was removed overnight under vacuum at 20°C, the powder sieved to pass a 60 mesh sieve and stored desiccated at −15°C. This procedure removed most of the brown pigment in the beans leaving a cream- to buff-coloured powder, which could be stored for several months with little loss of enzyme activity.

Extraction of enzyme from acetone powder

An extract possessing enzyme activity was prepared from acetone powders using 0·02M citrate/0·04M phosphate buffer pH 5·5 (1 g powder:50 ml buffer) with continuous agitation (flask shaker) for 2·5 h. The extract was centrifuged at 12 000 rpm for 30 min and the supernatant assayed for activity. All operations were performed at 4°C.

In order to optimise the extraction of soluble enzyme from the acetone extract of cotyledons, the effects of pH, extraction time, ionic strength, detergent and other additives to the extraction medium, were investigated.

Buffer solutions were prepared by dilution of 1M stock solutions of

trisodium citrate and sodium dihydrogen orthophosphate and adjustment of pH with HCl or NaOH prior to volume adjustment.

Assay for catechol oxidase

Enzyme activity was measured by following the uptake of oxygen using an oxygen electrode (Rank Brothers Ltd) with 4-methyl catechol as substrate at 30°C. Routinely, enzyme (0·1–0·3 ml) was incubated in 0·1M citrate/0·2M phosphate buffer, pH 5·5 (3 ml), until equilibrated and the reaction started by the addition of 0·66M catechol solution to give a 20 mM concentration in the cuvette. The initial rate of oxygen uptake was measured and enzyme activity calculated using a value of 0·237mM for the concentration of O_2 in air-saturated buffer at 30°C. Initial rates were calculated within approximately the first 4 min of the reactions, during which the rate of oxygen uptake remained linear. Activity was expressed as moles O_2 consumed per second (K_{at}).

Incubation of enzyme extract with catalase prior to assay did not reduce the oxygen uptake and peroxidase-dependent oxidation of phenol was considered to be absent.

RESULTS

Extraction of catechol oxidase

In Fig. 1 the combined effect of extraction at pH values from pH 3 to pH 9, together with the sequential addition of sodium chloride and Triton X-100 to the extraction mixture, is shown. Two distinct observations are apparent from these data.

First, analysis of the uncentrifuged extracts indicates that catechol oxidase activity is maximised by extraction in detergent plus NaCl at pHs between 5 and 6. Secondly, analysis of the supernatant liquids, after centrifugation, shows that the amount of enzyme solubilised is also dependent on pH and is enhanced by the addition of detergent and detergent plus NaCl.

Within this range, the optimum pH for enzyme solubility is dependent upon the composition of the extraction medium (Fig. 1). In the presence of detergent a broad peak is observed around pH 6 whilst addition of sodium chloride gives a lower pH optimum around pH 5·1.

In order to assess the effect of ionic strength on activity and solubilisation of catechol oxidase, further extractions were made in buffer containing 0·05M, 0·25M and 0·5M NaCl (Table 1). Both enzyme activity and solubility

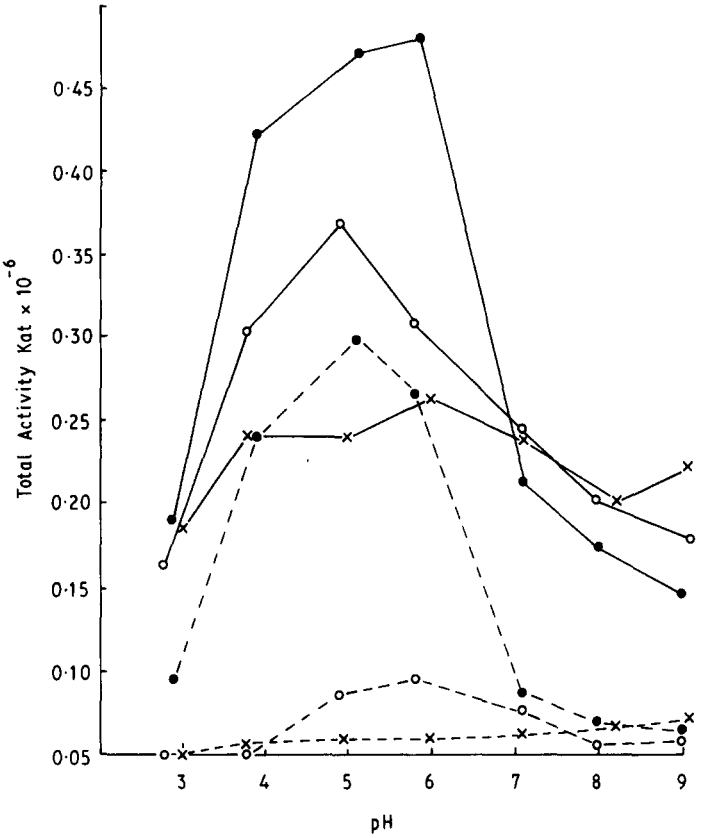

Fig. 1. Effect of NaCl and Triton X-100 on enzyme activity and extractability at different pHs. Acetone powder 1 g was extracted in 0·02M citrate/0·04M phosphate buffer (50 ml) adjusted to the required pH with 1M HCl or 2·5M NaOH for 30 min, then centrifuged (3000 rpm, 15 min). The pellets were resuspended (50 ml of 0·02M citrate/0·04M phosphate buffer) and Triton X-100 added to 1% (v/v) and stored overnight at 5°C. The following day, extracts were centrifuged and stored overnight at 5°C. Pellets were then resuspended (0·02M citrate/0·04M phosphate, 50 ml) and NaCl solution added to a final concentration of 0·5M. After stirring for 30 min, extracts were centrifuged. Samples of uncentrifuged extracts: buffer (— × —), buffer + Triton X-100 (—○—), buffer + Triton X-100 + NaCl (—●—) and supernatants: buffer (– – × – –), buffer + Triton X-100 (– –○– –), buffer + Triton X-100 + NaCl (– –●– –) were assayed at each stage.

are enhanced by increasing the level of NaCl in the extract. Addition of Triton X-100 (i.e. reversing the order of addition carried out in Fig. 1) further enhances enzyme activity and solubilisation.

The effects of other additives on enzyme activity and solubilisation were also investigated (Table 2). The addition, to the extraction medium, of acid-washed, insoluble polyvinyl polypyrrolidone (PVPP) subsequently removed by centrifugation, reduced the measurable activity in the supernatant and also the amount of enzyme solubilised compared with the control. This may

TABLE 1
Effect of Varying Levels of Sodium Chloride on Enzyme Activity and Solubilisation

Conditions	*Concentration of NaCl* (M)	*Total activity in extract* ($K_{at} \times 10^{-6}$)		*% enzyme solubilised*
		Uncentrifuged extract	*Supernatant*	
Buffer alone	0·05	0·485	0·022	4·5
	0·25	0·597	0·093	15·6
	0·50	0·768	0·180	23·4
Buffer + 1% Triton X-100	0·05	0·834	0·396	47·5
	0·25	1·07	0·722	67·5
	0·50	1·20	0·915	76·2

Acetone powder was extracted in 0·02M citrate/0·04M phosphate buffer, pH 5·0, containing NaCl for 1 h. Extracts were centrifuged at 3000 rpm for 15 min and stored overnight as pellets, then resuspended and Triton X-100 added. After extracting for 30 min, extracts were recentrifuged.

be due to binding and precipitation of the enzyme during extraction. Addition of a reducing agent, sodium ascorbate, increased the enzyme activity slightly. However, since the increased solubility was less than 10%, it was not included in routine extractions. The presence of another reducing agent, dithiothreitol (DTT), reduced the enzyme activity and did not affect solubilisation of the enzyme.

An experiment in which extraction time was varied showed an increase in both the enzyme activity (30% in the uncentrifuged extract, 50% in the

TABLE 2
Effect of PVPP, DTT and Sodium Ascorbate on Enzyme Activity and Solubilisation

Extraction medium	*Activity* ($K_{at} \times 10^{-6}$/*mg protein*)		*% enzyme solubilised*
	Uncentrifuged extract	*Supernatant*	
Control (0·1M citrate/0·1M phosphate buffer, pH 5, 0·5M NaCl, 1% Triton X-100)	0·081	0·066	81·5
Control + 2% PVPP	ND	0·052	64·2[a]
Control + 10mM sodium ascorbate	0·099	0·089	89·9
Control + 1mM DTT	0·023	0·02	87·0

[a] Calculated as percentage of control.
Acetone powder was extracted for 1 h, then centrifuged at 3000 rpm for 15 min.
ND, not determined.

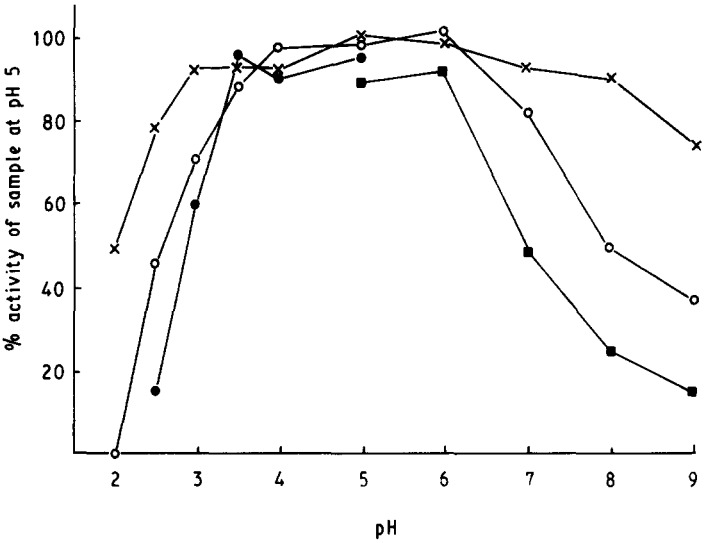

Fig. 2. Catechol oxidase stability on storage at various pH values. Following extraction in 0·01M citrate/0·02M phosphate buffer, pH 5, 0·5M NaCl and 1% Triton X-100, aliquots of centrifuged extract were adjusted to the required pH with 1M HCl or 2·5M NaOH and stored overnight at 5°C. Samples were assayed immediately after adjustment (— × —) and after 1 (—○—), 4 (—●—) and 5 days (—■—) incubation and the activity expressed as a percentage of the initial activity in the pH 5 sample.

supernatants) and solubilisation of the enzyme (12%) when the extraction time was increased from 20 min to 3 h. No further increase in activity or solubilisation was observed when extraction time was increased to 5 h. The studies described above indicated that the optimum conditions for enzyme activity and solubility were extraction for 3 h in 0·02M citrate/0·04M phosphate buffer, pH 5·5, containing 0·5M NaCl and 1% Triton X-100. Although initial studies utilised a low speed of centrifugation (3000 rpm), centrifugation at higher speed (17 000 rpm) did not significantly reduce the level of soluble enzyme. A ratio of 1:50 acetone powder to solvent was used. Under these conditions, activities of around $0{\cdot}09 \times 10^{-6}$ K_{at}/mg protein and 85–95% solubilities were routinely achieved.

Stability of catechol oxidase

The stability of the enzyme at various pH values was investigated to define the conditions necessary for enzyme fractionation. The results are shown in Fig. 2. A decline in activity is observed almost immediately following incubation of the enzyme at values below pH 3 and above pH 8. On storage overnight, the range over which the enzyme is stable is reduced to pH 3·5–6. In order to check whether this inactivation is reversible, aliquots of extract which had been incubated for 1 day were adjusted from pH 2 and 3 to

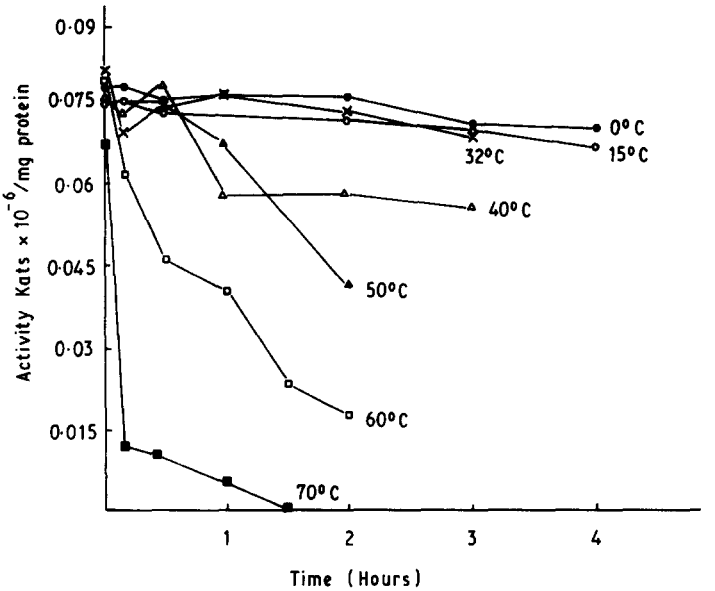

Fig. 3. Thermal stability of enzyme. Sample was extracted for 3 h in 0·02M citrate/phosphate buffer pH 5, 0·5M NaCl and 1% Triton, centrifuged at 17 000 rpm for 20 min and the supernatant stored overnight. Aliquots of supernatant (containing 2 mg/ml protein) were incubated in water baths at various temperatures and timing commenced as soon as the extract reached the required temperature (1–2 min).

pH 4 and from pH 7, 8 and 9 to pH 6. The activity of the pH-adjusted extracts was not increased to the same level as that in unadjusted extracts at pH 4 and pH 6, indicating that, after storage for one day, the pH-dependent inactivation is not reversible.

The thermal stability of the enzyme extract is shown in Fig. 3. Storage for up to 4 h at 0–32°C has little effect on enzyme stability. Stability decreases rapidly in the range 40 to 70°C.

Ammonium sulphate fractionation and desalting by dialysis or gel filtration, was associated with reduced enzyme activities. Activity and stability were particularly impaired following prolonged dialysis (20 h). Subsequent addition of Cu^{2+} ions or increasing the ionic strength did not restore activity. Enzyme instability following dialysis had also been observed with the cocoa leaf enzyme (Adomako, 1967).

Fractionation and partial purification of catechol oxidase

Enzyme extraction in the presence of sodium chloride and Triton X-100 was followed by fractionation with ammonium sulphate. Addition of $(NH_4)_2SO_4$ in steps from 20–60% gave a progressive precipitation of both total protein and enzyme over the range. Addition of $(NH_4)_2SO_4$ to 25%

clarified the extract accompanied by a loss of 10–20% in enzyme activity. Further addition to 60% $(NH_4)_2SO_4$ precipitated the remaining enzyme activity with a recovery of 70–90% of the total activity. On resuspension a cloudy suspension was obtained which flocculated during dialysis, necessitating a short centrifugation with a slight loss of activity.

Properties of catechol oxidase

The activity of unfractionated catechol oxidase, assayed at various pH values is shown in Fig. 4. The pH optimum of about 5·4 compares with

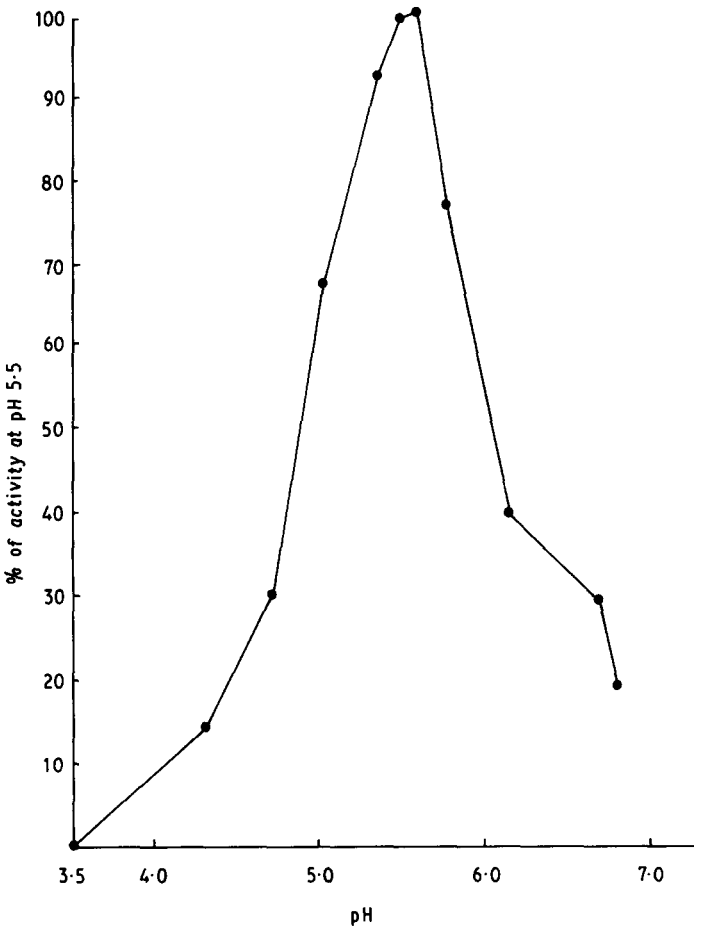

Fig. 4. Enzyme activity at varying pH. Acetone powder was extracted for 30 min in 0·1 M citrate buffer (pH 5·0) containing 0·5 M NaCl and 1% Triton X-100, then centrifuged at 3000 rpm, 15 min. 0·3 ml enzyme was assayed in 0·1 M citrate/0·05 M phosphate buffer at the desired pH, at constant ionic strength. Activity is expressed as a percentage of the activity at pH 5·5.

TABLE 3
Effect of Ionic Strength on Enzyme Activity

Assay buffer	*Ionic strength*	*Activity* ($K_{at} \times 10^{-6}$/*mg protein*)
0·05M citrate/phosphate	0·2	0·054
0·10M citrate/phosphate	0·4	0·078
0·20M citrate/phosphate	0·8	0·092
0·10M acetate	0·1	0·033
0·20M acetate	0·2	0·054
0·40M acetate	0·4	0·082

Acetone powder was extracted in 0·02M citrate/phosphate buffer pH 5·0 containing 0·5 M NaCl and 1% Triton X-100, then centrifuged at 16000 rpm for 20 min. Supernatant was stored overnight at 4°C. Enzyme activity in the supernatant was assayed in buffers at pH 5·5 and varying ionic strength.

previous reports of pH 6·0 for acetone powder extracts of fermented and unfermented seed (Quesnel & Jugmohunsingh, 1970) and pH 6·2 for acetone-extracted cocoa husk enzyme (Chaplin, 1978) both with catechol as substrate.

The effect of varying ionic strength on enzyme activity is shown in Table 3. Increasing the ionic strength results in an increase in enzyme activity similar to that obtained on enzyme extraction using high levels of sodium chloride.

Preliminary experiments to assess the substrate specificity of cocoa bean catechol oxidase have shown that the enzyme can oxidise two endogenous substrates, (+)catechin and (−)epicatechin and also 4-methyl catechol. Product inhibition was reduced with 4-methyl catechol and was almost absent with (−)epicatechin and (+)catechin, unlike oxidation of catechol in which it is pronounced.

DISCUSSION

Solubilisation of polyphenol oxidases from cocoa tissues has, in the few studies made, proved difficult. In the only detailed study of the bean enzyme, Quesnel & Jugmohunsingh (1970) found that solubility in buffered aqueous solution decreased when beans were fermented and attributed this to tanning during fermentation. Enzyme preparations from unripe and ripe cocoa husks were of high molecular weight since urea, SDS or Triton X would not solubilise the enzyme (Chaplin, 1978). In the extraction of cocoa leaf enzyme, extraction at pH 10 was necessary to give an adequate yield and resulted in a viscous yellow solution (Adomako, 1967). In all three cases, extraction in organic solvents was used. In the present study, acetone

TABLE 4
Residual Activity of Polyphenoloxidase (expressed as % initial activity) during the Course of a Fermentation (compared with other authors)

Day	*% initial activity*		
	Result[a]	*Villeneuve (1985)*[b]	*Holden (1959)*[c]
1	104	66	36
2	94	28	20
3	93	13·5	8
4	22	8	5
5	13	5·6	0

Reagents used for solubilisation.
[a] 0·02M citrate/0·04M phosphate, pH 5·5, containing 0·5M NaCl and 1% Triton X-100.
[b] 0·1M citrate/phosphate, pH 5·2, containing 4% PVPP, 1·2% polyethylene glycol 600 and 0·02% mercaptoethanol.
[c] 0·01M sodium acetate.

extraction of the unfermented beans was used as a means of removing the large quantitities of endogenous phenols (up to 15% of the dry weight) and as much as possible of the lipid present (about 30–50% of the dry weight) (Rohan, 1963). Subsequent solubilisation of the enzyme in aqueous buffer was low but was increased by the addition of sodium chloride and neutral detergent.

Table 4 shows the results of extracting polyphenol oxidase during the course of the fermentation. All the results show that the activity of the enzyme decreases with fermentation time but previous workers (Holden, 1959; Villeneuve *et al.*, 1985) reported a much greater decline in activity than that shown in the present work, perhaps because of the use of NaCl and Triton X-100 in the present study.

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