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SUBSTRATE SPECIFICITY OF ISOPEROXIDASES IN THE SHOOTS OF TEA (*CAMELLIA* spp.) CLONES

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ABSTRACT

The substrate specificity of peroxidase was examined in flush shoots of four different tea clones, with partially purified enzyme. Peroxidase was separated using disc gel electrophoresis and subjected to activity stain by using substrates such as amine (dihydroxyphenylalanine), aromatic diamines (benzidine, o- dianisidine) and phenolics (catechol, pyrogallol, guaiacol, phloroglucinol, chlorogenic acid and gallic acid). Tea shoot peroxidases catalysed all the substrates indicating the capability of oxidizing a wide range of hydrogen donors; certain peroxidase fractions have multicatalytic properties. While the aromatic diamines were oxidized by more isoperoxidase fractions, the amine and phenolics were catalysed by a few fractions. This shows a greater affinity towards the aromatic diamines than that of amine and phenolics. Certain specific isoperoxidase fractions were also detected in the fast fermenting clone, UPASI-14, which may be used as a marker to identify clones which have fast fermenting ability. The implication of the results is discussed.

INTRODUCTION

Gel electrophoresis of enzymes in higher plants has shown that many of these molecules exist in multiple molecular forms (Cherry and Ory, 1973). The use of isozyme, particularly isoperoxidases as genetic markers at molecular level and its quantitative and qualitative variations have been utilized in biochemical (Dange and Reddy, 1984), physiological (Srivastava and Huystee, 197; Kay and Basile, 1987), pathological (Seevers *et. al.*, 1971; Ramachandra *et. al.*, 1981), morphogenetic (Goodall and Stoddart, 1989; Rao *et. al.*, 1993) and chemogenetic (Triest *et. al.*, 1989) investigations.

In tea, o-diphenol specific polyphenoloxidase (PPO) plays a vital role in oxidative reactions associated with fermentation (Coggon *et. al.*, 1973). On the other hand, peroxidase (PO) is not particularly specific to hydrogen donors, but extremely specific for the requirement of H_2O_2 to catalyze various substrates (Scandalios, 1974). Although the distribution of PO has been reported in tea (Gregory, 1966; Takeo and Kato, 1971), the exact role during tea processing is not yet understood

Dix *et. al.* (1981) reported that the nature and distribution of pigments responsible for quality of tea formed during fermentation are partly governed by the relative actions of PO and PPO. Similarly, the sum of PO and PPO activity may also give a better index to identify the potential quality tea clones (Gunasekar *et. al.*, 1994). The present investigation is undertaken to study the biochemical properties of peroxidase from different clonal tea shoots, in relation to fermentation.

MATERIALS AND METHODS

Four tea clones, namely, UPASI-14 (fast fermenting: 30.1 min), UPASI-3, CR-6017 (medium fermenting: 46.0 min) and SA-6 (slow fermenting : 94.8 min) with different fermenting abilities were selected for this study. About two kg each of tender shoots comprising a terminal bud and three expanded leaves were collected from the UPASI TRI experimental plots. Nearly 250 g of uniform shoots were taken at random,

cut into small pieces in cold condition and used immediately for enzyme preparation. All operations were carried out at 3± 1° C, unless otherwise stated.

Samples were homogenized with 100mM sodium phosphate buffer (pH 7.0) containing 20 mM ascorbic acid, 15mM β–mercaptoethanol, seven per cent PVP-40, one per cent poly-clar AT and five per cent tween-80. The homogenate was then centrifuged at 15,000 rpm for 30 min. The supernatant so obtained was brought to 35 to 90 per cent ammonium sulphate saturation. The precipitate was dissolved in 50 mM phosphate buffer (pH 7.0) containing 100 mM NaCl, then dialysed overnight against three changes of the same buffer devoid of NaCl. The dialysate was centrifuged at 10,000 rpm for 15min and the supernatant was used for electrophoretic separation of isoperoxidases.

The procedure as outlined by Davis (19 64) for anionic system was followed for polyacrylamide gel by electrophoresis. About 175 to 200 µg proteins (Bradford, 1976) were loaded on top of the gels. The activity staining was performed at 25° ± 1°C using 50 mM sodium acetate buffer (pH 5.5) and H_2O_2 at a concentration of 0.1 per cent for all substrates. Benzidine (Seevers *et. al.*, 1971), o- dianizidine, guaiacol (Kay and Basile, 1987), catechol, dihydroxyphenylalanine (Miller *et. al.*, 1990) were used as substrates. However, the concentration of pyrogallol and phloroglucinol was reduced to 10 mM after the method of Dange and Reddy (1984). Chlorogenic (5mM) and gallic (10 mM) acids were also tried. The relative migration (Rm) of each band was calculated. The stable banding patterns were scanned in a Beckman Du-64 spectrophotometer and others were recorded on graph.

RESULTS AND DISCUSSION

The relative mobility (Rm) values, for anionic isoperoxidase of four clones obtained by polyacrylamide gel electrophoresis using various substrates i.e., amine, aromatic diamines and phenolic compounds are presented in Table 1 and Table II. The banding patterns and gel scans are illustrated in Fig. 1 and 2. A total of nine peroxidase fractions were observed with the Rm values ranging from 0.07 to 0.57.

The results showed that four slow migrating isozyme fractions having the relative mobility values of 0.11, 0.16, 0.21 and 0.27 resolved with aromatic diamines (benzidine and o-dianizidine) in all the clones, but in varied proportions (Table I). However, the isozyme fraction No.1 could be detected only in UPASI-3 clone with benzidine, while four additional bands were resolved only in UPASI-14. More isoperoxidase fraction could be detected, on overall comparison, when the aromatic diamines were used as substrates.

The substrate specificity of the four clonal tea shoots showed similarity in selecting the dihydroxyphenyl-alanine as hydrogen donor, except in the clone UPASI-14 where the isozyme band No. 7 having the Rm value of 0.47 was unique to it (Table I).

Among the phenolic compounds as hydrogen donors tried, catechol and guaiacol retained the bands for longer time as compared to other phenolics studied. Although, four isozyme fractions could be detected using phenolic compounds, at least two major fractions were capable of catalysing all phenolic compounds, irrespective of the clones (Fig. 2 and Table II). However, the fast migrating isozyme fraction No. 7 of UPASI -14 (Rm 0.47) catalyzed all phenolic compounds except guaiacol and chlorogenic acid.

A number of studies have demonstrated the presence of peroxidase enzyme in all parts of tea plant (Bokuchava, 1950; Bokuchava *et. al.*,

Table I. Relative mobility (Rm) values of anionic isoperoxidase with aromatic diamines and amine as hydrogen donors.

Isozyme Number	Benzidine				O-Dianisidine				Dihydroxyphenylalanine (DOPA)			
	UPASI-3	UPASI-14	CR-6017	SA-6	UPASI-3	UPASI-14	CR-6017	SA-6	UPASI-3	UPASI-14	CR-6017	SA-6
1	0.07	–	–	–	–	–	–	–	–	–	–	–
2	0.11	0.11	0.11	0.10	0.10	0.09	0.09	0.09	0.09	0.09	0.10	0.09
3	0.16	0.15	0.16	0.15	0.16	0.16	0.16	0.16	0.15	0.15	0.16	0.16
4	0.21	0.21	0.22	0.20	0.21	0.21	0.20	0.20	0.20	0.21	0.21	0.21
5	0.27	0.28	0.28	0.27	0.29	0.27	0.27	0.27	–	–	–	–
6	–	0.33	–	–	–	0.33	–	–	–	–	–	–
7	–	0.47	–	–	–	0.47	–	–	–	0.47	–	–
8	–	0.52	–	–	–	0.52	–	–	–	–	–	–
9	–	0.57	–	–	–	–	–	–	–	–	–	–

Table II. Relative mobility (Rm) values of anionic peroxidase isoenzymes with phenolic compounds as hydrogen donors.

Clone	Isozyme number	Phenolic compounds					
		Catechol	Pyrogallol	Guaiacol	Phloroglucinol	Chlorogenic acid	Gallic acid
UPASI - 3	2	0.11	0.11	0.11	0.10	0.10	0.11
	3	0.16	0.15	0.15	–	–	0.16
	4	0.22	0.20	0.20	0.2	0.22	0.22
	5	0.27	–	–	–	–	–
UPASI - 14	2	0.11	0.10	0.11	0.10	0.10	0.11
	3	0.16	0.16	0.16	–	–	0.16
	4	0.21	0.20	0.22	0.22	0.22	0.21
	5	–	–	0.28	–	–	–
	7	0.47	0.47	–	0.47		0.47
CR - 6107	2	0.11	0.11	0.10	0.11	0.11	0.11
	3	0.16	0.16	0.16	–	–	0.16
	4	0.21	0.21	0.19	0.19	0.20	0.22
	5	0.27	0.27	–	–	–	–
SA - 6	2	0.11	0.11	0.11	0.10	0.10	0.11
	3	0.16	0.16	0.16	–	–	–
	4	0.21	0.21	0.21	0.22	0.22	–
	5	–	–	–	–	–	0.27

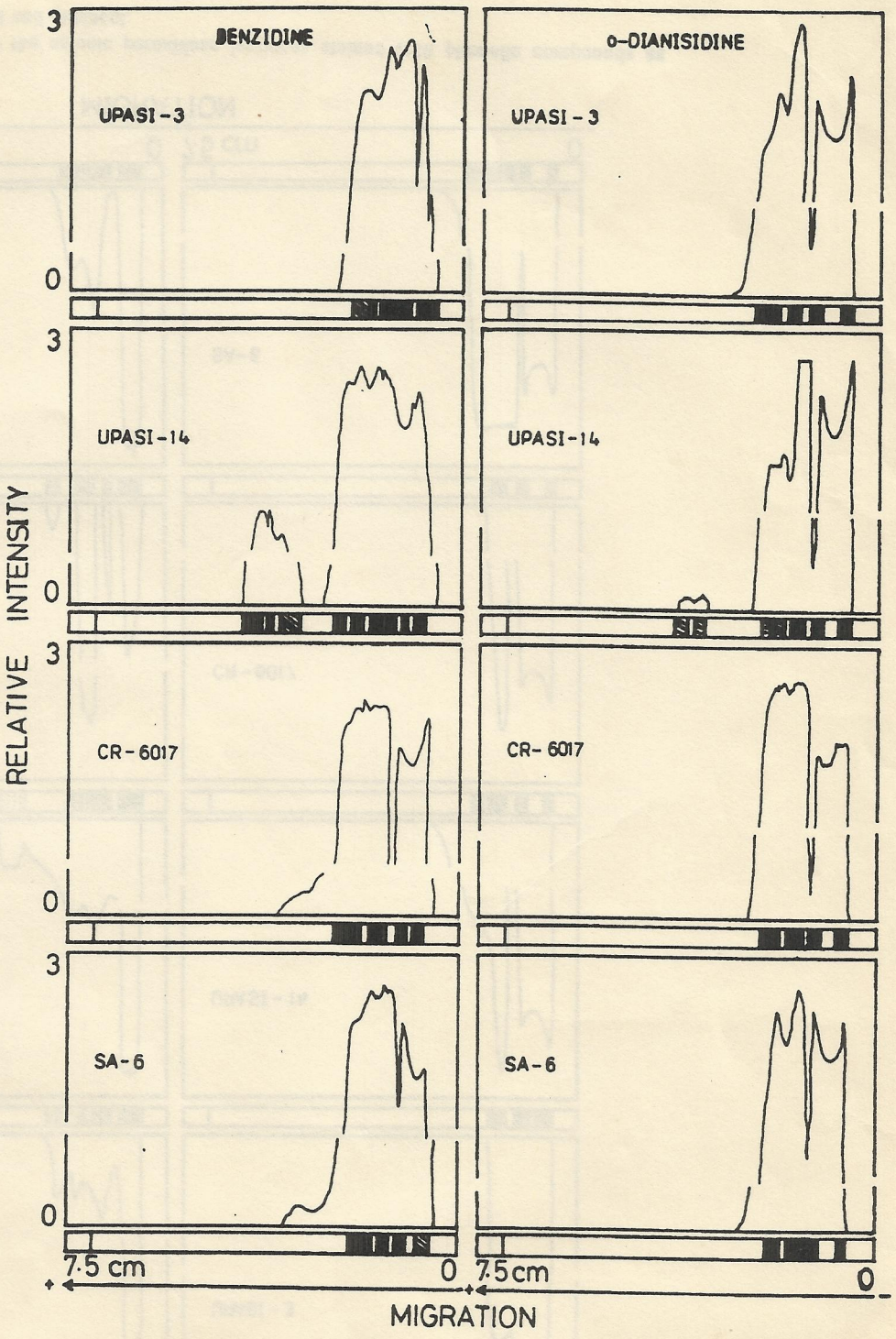

Fig. I. **Scans and Zymograms of the anionic peroxidase isozymes stained with aromatic diamines as hydrogen donors; benzidine and o-dianizidine.**

1966). The enzyme peroxidase was characterized by several biochemists and it was found to exist in the form of several isoenzymes (Tririmanna, 1972; Takeo and Kato, 1971). The present results also reveal the existence of multiple molecular form of peroxidase in the clones UPASI-3, UPASI-14, CR-6017 and SA-6, irrespective of the substrates used. However, Takeo and Kato (1971) reported that six isozyme fractions were present in peroxidase enzymes of tea. The difference in number of fractions may be due to the nature of the material used and the method adopted for detection.

The slow migrating behaviour of the major fraction on seven per cent polyacrylamide gel indicates that nearly 90 to 95 per cent of peroxidase are high molecular weight proteins (100 ~ 200 kD) with a marginal difference in their molecular weights. However, the catalytic property showed variation among them. It has also been demonstrated that the substrate specificity of the anionic peroxidase isozyme with chosen hydrogen donors differ structurally in number, type and position of the substance mainly in the phenyl ring (Dange and Reddy, 1984). The activity of peroxidase isozyme depends on the nature of hydrogen donors (Macko and Novacky, 1966) although substrate specificity varies considerably among the individual isozymes (Evans and Alridge, 1965).

In the present investigation, tea shoots showed greater substrate affinity towards the aromatic diamines than that of amines and phenolic acids. Thus, the aromatic diamines in tea may play an important role as hydrogen donors by influencing the rate of reaction. The four major fractions of peroxidase having Rm value of 0.11, 0.16, 0.21 and 0.27 catalyzed most of the substrate tried, which may be due to its multiple catalytic property. Besides varied substrate specific nature due to its diverse metabolic functions, peroxidase enzyme in tea may play an important role in the evolution of species as reported in other crops (Dange and Reddy, 1984).

Since the major components of young tea shoot are phenolics and their derivatives, the multisubstrate specificity of peroxidase enzyme fractions play an important role during fermentation which is in line with the earlier reports (Dix *et. al.*, 1981). Dix *et al.* (1981) also postulated that the pigments essential for quality in made tea, formed during fermentation are partly governed by the relative actions of polyphenoloxidase and peroxidase. Moreover, the hydrogen peroxide required for the activity of peroxidase is also formed by the action of tea catechol oxidase on certain flavanols (Gregory, 1966).

It is also interesting to note that the clone, UPASI-14, which is a fast fermenting clone (Thanaraj and Seshadri, 1990), had higher number of isozymes. Of these, the isozyme No. 7 (Rm 0.47) is specific to the clone UPASI-14, irrespective of the substrates used as hydrogen donors except, guaiacol and chlorogenic acid. Thus, the particular fraction of peroxidase enzyme could be used as a marker to screen the clones for their fermenting abilities. The present investigation further supports the earlier view that the combined action of PO and PPO may decide the quality of tea through fermentation (Gunasekar, *et al.* 1994).

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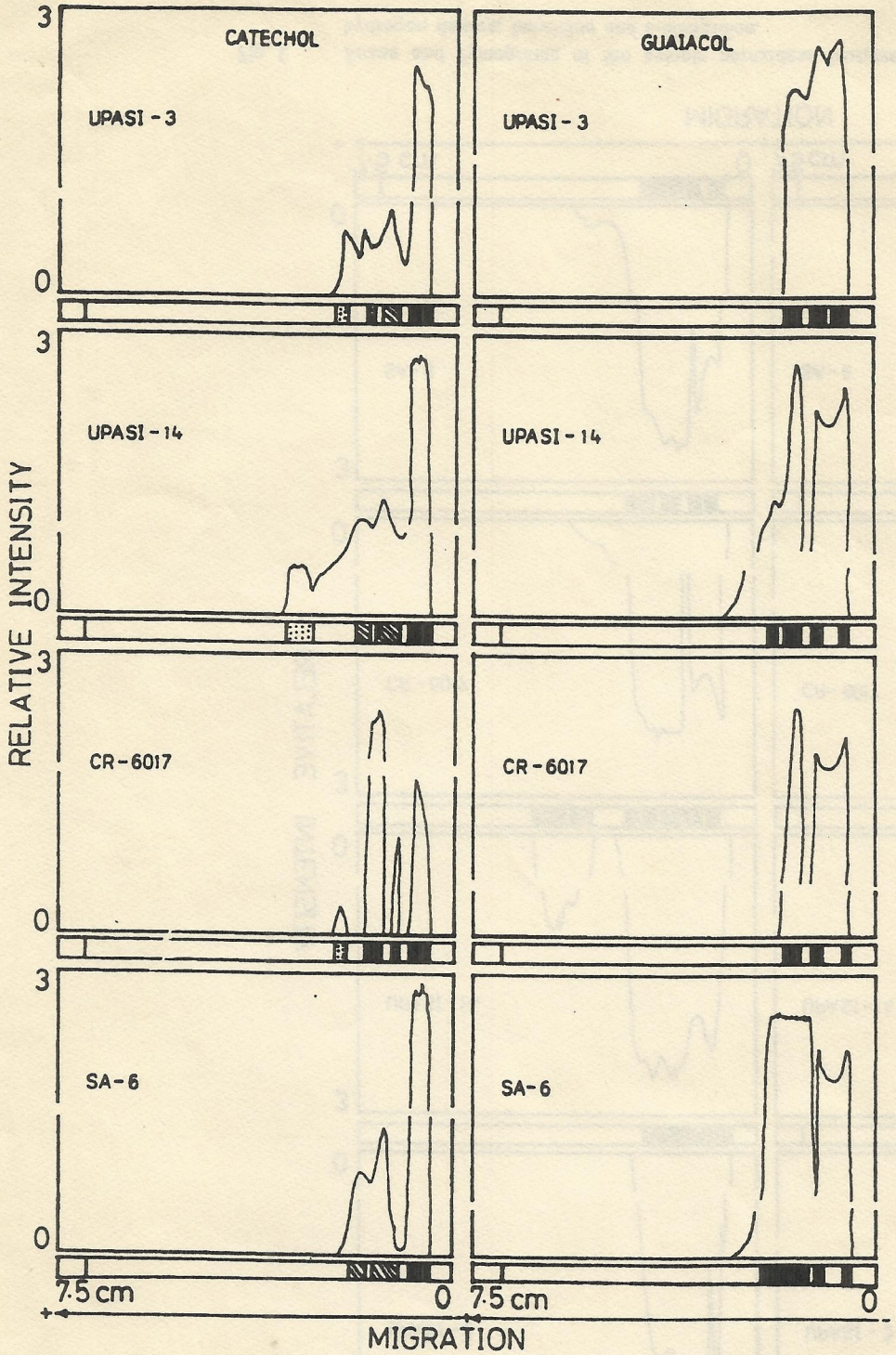

Fig. II. **Scans and Zymograms of the anionic peroxidase isozymes stained with phenolic compounds as hydrogen donors; catechol and guaiacol.**

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