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CHANGES IN THE CHEMICAL COMPOSITION AND POLYPHENOL OXIDASE, PEROXIDASE ACTIVITIES ASSOCIATED WITH FRUIT DEVELOPMENT AND RIPENING IN *CAPSICUM ANNUUM* L. VAR. JWALAMUKHI

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Chilli pepper is a popular food additive and is economically important as a vegetable, spice and medicine. Pepper fruit undergoes changes in the level of lignin, free phenolics, soluble proteins, sugar and ascorbate during the maturation process. Maximum level of free phenolics and lignin were observed during early stage of development. A decrease of peroxidase (POD) activity was observed during maturation and was related with a decrease in other physiological parameters. POD activity increased after 49th day of fruit development may be responsible to capsaicinoids degradation. The level of glucose and reducing sugar gradually increased during fruit development and ripening. The content of soluble protein decreased during fruit development but increased in the stage of ripening. During the early stages of chilli fruit development, the PPO activity and the level of ascorbate was low, whereas in the post ripening stage the PPO activity increased. Ascorbic acid level increased after the green mature stage and peaked in red succulent fruit with about 80.5% moisture content in the fruit followed by a decrease in the post ripening period. Thus chilies are nutritionally balanced with respect to vitamin C. In breeding for high nutrient content, selection should be done at the red succulent, where the natural antioxidants present in high levels.

Keywords : Ascorbate; Capsaicin; *Capsicum annuum*; Chilli; Peroxidase; Polyphenol oxidase; Ripening.

Introduction

Chilli (*Capsicum annuum* L.) is a dark-green annual or short-lived perennial plant, belongs to the family solanaceae obtaining a height between one half to two meters. The fruits are long, cylindrical, and mostly ovoid indehiscent berry, and when ripe are either scarlet or yellow, with a smooth shiny surface. There are not enough studies on the chemical composition of the fruits. However, little information is available on the polyphenol oxidase and peroxidase activities and the chemical content during development and ripening of the fruits. Polyphenol oxidase has been found in all higher plants, and is responsible for enzymic browning of fruits and vegetables[1]. Polyphenol oxidase catalyzes the hydroxylation of monophenols to diphenols and which inturn converted to quinones by molecular oxygen. The generated unstable highly reactive quinone subsequently react with themselves, amino acids, proteins, evolving into brown, black or red heterogenous polymers responsible for quality loss in many fruits[1]. The considerable economic and nutritional loss induced by enzymic browning is a concern to food processors. Peroxidase, a hydrogen dependent haeme protein, exhibit high oxidative and hydroxylative activities besides the conventional peroxidase activity. The main feature of peroxidase catalysis is production of free radicals which participate in different post-enzymatic reactions. Oxidative destruction of coloured compounds is significantly stimulated by peroxidase and is of practical interest for decoloration processes used in food capable of oxidizing monolignols to free radicals. Apart from the role of POD in the last step of lignification POD has been implicated in numerous physiological processes including cross linking of cell wall polysaccharides, pathogen resistance, oxidation of fatty acid and phenols, phytohormone catabolism and fruit ripening[2,3]. POD activity has been used as an index of various operations associated with the processing of vegetables and fruits due to its stability. This enzyme has attracted the food industry because its ability to bring out desirable and sometimes undesirable changes. Ascorbic acid is an antioxidant that acts to slow senescence and to maintain biological integrity during ripening by oxidizing ascorbate to dihydro ascorbate, and several oxides may be involved in ascorbate degradation. Ascorbate acts as a natural inhibitor of PPO. The present study was undertaken

to find out the changes in the soluble proteins, sugars, ascorbate, total phenols, and lignin and in the activities of peroxidase and polyphenol oxidase during fruit ripening of the chillies.

Materials and Methods

Fresh seeds of *Capsicum annuum* var. jwalamukhi were collected from the Kerala Agricultural University, Vellayani and were grown in the green house in the department. After anthesis young pepper fruits were collected every 7 days from 14 (I stage) to 56 days (VII stage). The fruits were subjected for all analytical and biochemical analysis.

Estimation of total phenols: Total phenol content of fruit tissues were estimated by the method of Mayr *et al*[4].

Spectrophotometric determination of lignin: Lignin content of the fruit samples were estimated by acetyl bromide method by recording the absorbance at 280 nm[5].

Estimation of photosynthetic pigments: Total chlorophyll and carotenoids were estimated by the method of Arnon[6].

Estimation of ascorbate: Ascorbate content of the sample was estimated in mg per g tissue using the procedure of Sadasivam and Subramanian[7].

Estimation of total protein, hexose, pentose and glucose: The protein content was estimated according to the method of Bradford[8]. The total hexose, pentose and glucose were extracted and quantified by Dubois *et al*[9].The amount of total glucose, hexose and pentose present in the extract were determined from the respective standard graphs.

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) of phenols: Quantitative fractionation of various phenolic acids occurs in fruit samples were studied by HPLC analysis[10]. Phenolic acids extracted from fresh fruit tissues in aqueous methanol were used for the study. Distribution of phenolic acids in tissues was studied by isolating phenols from these regions separately. The role of phenolic acids in lignin as well as capsaicin synthesis was also studied from their pattern of distribution.Standard phenolic acids such as gallic, vanillic, p-hydroxybenzoic, ferulic, chlorogenic sinapic, para coumarate and cinnamic acids were injected into the column separately. Comparing with the retention time of the standard identified phenolic acids in the sample. Height of the peaks was taken for quantification. Concentration of the standard and height of the standard peak were taken as the standard parameters.

Extraction and quantification of capsaicinoids by HPLC: Capsaicinoids were extracted from chilli fruits using the technique described by Estrada *et al.*[11]. Standards of capsaicin and dihydro capsaicin were obtained from Sigma Chemical Co. (St.Louis, MO) and were used for retention time, verification and instrument calibration. The mean retention time of capsaicin and dihydrocapsaicin under these conditions were 7.36 and 10.30 min respectively.

Isolation and assay of Peroxidase (POD): Peroxidase was isolated and assayed following the method of Goliber[12] and Ingham[13]. One unit of POD is the amount of enzyme required to oxidize 1 µM of guaiacol by H_2O_2 at test condition.

Isolation and assay of Polyphenol oxidase (PPO): Polyphenol oxidase was extracted and assayed according to the method of Oktay *et al*[14]. The activity of PPO was determined spectrophotometrically by recording the increase in absorbance at 420 nm for 10 min. One unit of the enzyme activity is defined as an increase in absorbance of the mixture at 420 nm of 0.1 per min and per milliliter of enzyme solution.

Results and Discussion

Composition and Physical Changes: Table 1 summarizes the physical and compositional characteristics associated with fruit maturity. The pH exhibited slight increases during maturation until the 4th stage, but declined rapidly in the last stage. Soluble proteins decreased during development, then increased during ripening may be due to the increased synthesis of enzyme proteins involved in ripening and senescence of the fruit. Pentose and hexose levels as well as the soluble protein content decreased during fruit development followed by an increase during maturation. The level of glucose showed a continuous increase during the ripening of chilli fruits. Kadiolglu and Aydin[15] observed that glucose content of medlar increased with maturation. The increase in glucose concentration may be due to the rapid growth phase of the fruit.

Table 1. The physical and compositional features associated with Fruit maturity.

	Stage I	Stage II	Stage III	Stage IV	Stage V
pH	5.72	5.8	5.89	6.1	5.3
Glucose (mg/g tissue)	35.4	37.5	40.64	44	46.9
Pentose (mg/g tissue)	229.5	239.1	242	286	516
Hexose (mg/g tissue)	22.9	24.3	27.4	29.5	30.9
Total Protein (mg/g tissue)	0.31	0.33	0.35	0.54	0.66

Pigments: The concentration of the chlorophyll and carotenoids pigments during maturation was quantified. The total carotenoid contents were increased, while the chlorophyll content was reduced to non-detectable level (Table 2).

Total Phenols: Total soluble phenolics was determined by Folin - Ciocalteau assay. Figure 1 shows the changes in free phenolics in chilli fruit during ripening. Phenolics were detected in five stages of maturity namely 14th day (stage -1), 21st day (stage - 2), 28th day (stage - 3), 35th day (stage - 4) and 42nd day (stage - 5). Predominance of free phenolics was found different in the levels associated with successive stages of fruit development. The pattern of free phenolics accumulation was totally different from that of capsaicinoids. In this study it is observed that a maximum level of phenols were observed in the first stage, and then decreased until maturation when the level has 1/4th the amount detected at the 5th stage approximately. The sink

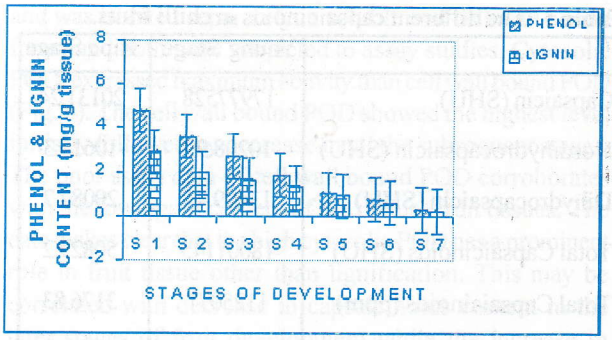

Fig.1. Total phenol and lignin content in different stages in chilli fruit.

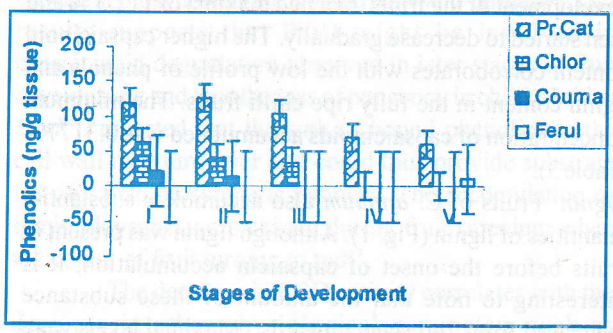

Fig.2. Phenolic acid profile during chilli fruit development.

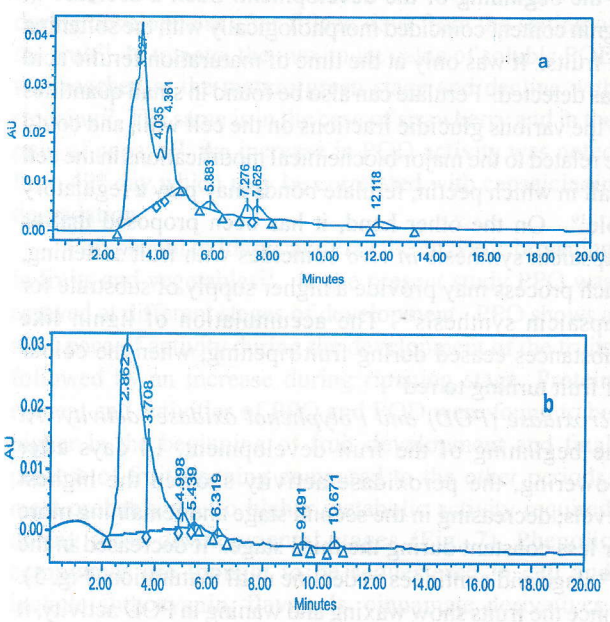

Fig.3a & b. HPLC chromatogram showing different types of phenolic acids in green and ripe fruits of chilli.

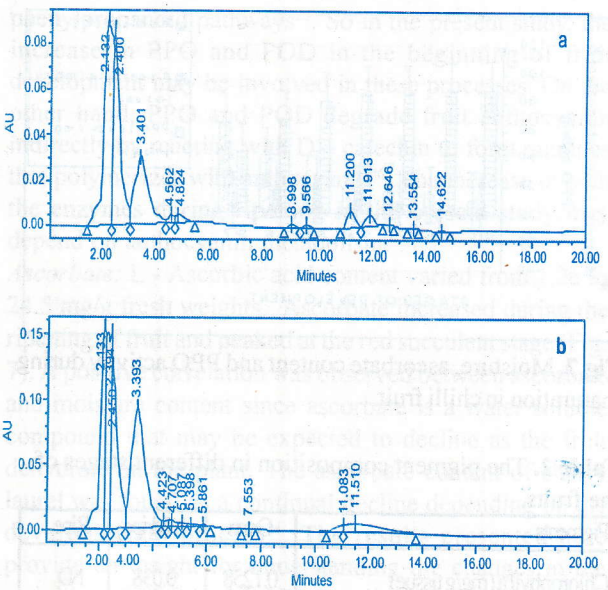

Fig.4a & b. HPLC chromatogram showing different types of capsaicinoids in green and ripe fruits of chilli.

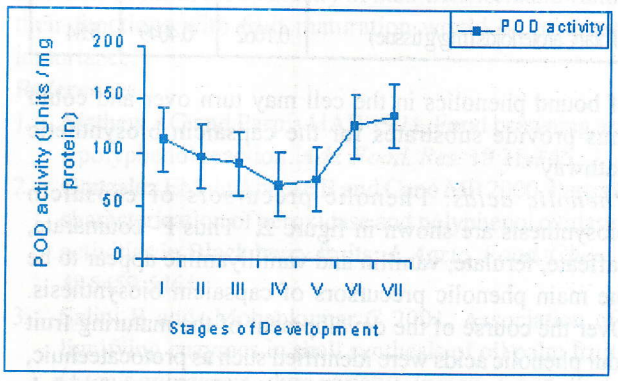

Fig.5. Changes in POX activity in chilli fruit maturation.

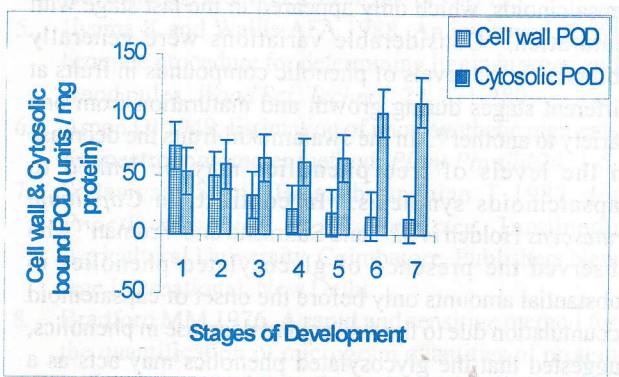

Fig.6. Activity of cell wall and cytosolic POX fractions in different stages of chilli fruit development.

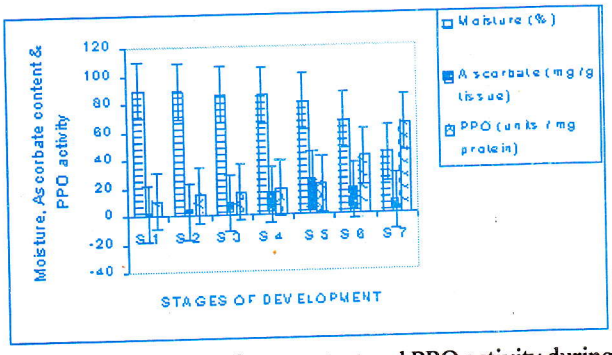

Fig.7. Moisture, ascorbate content and PPO activity during maturation in chilli fruit.

Table 2. The pigment composition in different stages of the fruits.

Pigments	Green	Midripe	Ripe
Chlorophyll a (mg/g tissue)	0.1258	0.038	ND
Chlorophyll b (mg/g tissue)	0.0511	0.0106	ND
Total Chlorophyll (mg/g tissue)	0.1769	0.0416	ND
Total Carotenoids (mg/g tissue)	0.1062	0.4049	5.84

of bound phenolics in the cell may turn over and could thus provide substrates for the capsaicin biosynthetic pathway[16,17].

Phenolic acids: Phenolic precursors of capsaicin biosynthesis are shown in figure 2. Thus P- coumarate, caffeate, ferulate, vanillin and vanilliyamine appear to be the main phenolic precursors of capsaicin biosynthesis. Over the course of the development of the maturing fruit four phenolic acids were identified such as protocatechuic, chlorogenic, coumaric and ferulic acid. Figure 3 a & b portraits the chromatogram of phenolic acids during mature green stage (2nd stage) and 5th stage of capsicum fruits by RP - HPLC. They are exhibiting a decrease in content with development except for ferulate, the precursor to capsaicinoids, which only appeared in the last stage with maturation. Considerable variations were generally observed in the levels of phenolic compounds in fruits at different stages during growth and maturation from one variety to another[18]. In the Jwalamukhi fruits the decrease in the levels of free phenolics may be linked to capsaicinoids synthesis. In contrast, in *Capsicum frutescens* Holden *et al.*[16] and Sukrasno and Yeoman[17] who observed the presence of glycosylated phenolics in substantial amounts only before the onset of capsaicinoid accumulation due to the subsequent decrease in phenolics, suggested that the glycosylated phenolics may acts as a source of intermediates for capsaicinoids synthesis.

Fig. 4 a & b portraits the peak of different capsaicinoids

Table 3. The different capsaicinoids in chilli fruits.

	Young Stage	Ripe Stage
Capsaicin (SHU)	17977528	20131.24
Nordihydrocapsaicin (SHU)	10208.99	10615.3
Dihydrocapsaicin (SHU)	13409.44	20082.7
Total Capsaicinoids (SHU)	18001145	50829.2
Total Capsaicinoids (ppm)	1125071	3176.83
Total Capsaicin (%)	112.51	0.3177

present in the green and fully ripe stages of chilli fruits. The different capsaicinoids quantified from the HPLC chromatogram by computing with appropriate standards. Capsaicinoids progressively accumulated during the development of the fruits, reached maxima of 112.5 % and then started to decrease gradually. The higher capsaicinoid content corroborates with the low profile of phenols and lignin content in the fully ripe chilli fruits. The minimum concentration of capsaicinoids accumulated was 0.3177% (Table 3).

Lignin: Fruits of *C. annuum* also accumulate substantial quantities of lignin (Fig. 1). Although lignin was present in fruits before the onset of capsaicin accumulation, it is interesting to note that the amount of these substance decreased from 2nd stage onwards. Maximal levels were observed in the first stage, and then decreased until maturation when the level was 1/3rd the amount detected at the beginning of the development. Such a decrease in lignin content coincided morphologically with the softening of fruits. It was only at the time of maturation ferulic acid was detected. Ferulate can also be found in small quantities in the various glucidic fractions on the cell wall, and could be related to the major biochemical modifications in the cell wall in which pectin, ferulate bonds may play a regulatory role[19]. On the other hand, it has been proposed that as capsaicin synthesis *in vivo* coincides with fruit softening, such process may provide a higher supply of substrate for capsaicin synthesis[16]. The accumulation of lignin like substances ceased during fruit ripening, when the colour of fruit turning to red[17].

Peroxidase (POD) and Polyphenol oxidase activity: At the beginning of the fruit development, 14 days after flowering, the peroxidase activity showed the highest levels; decreasing in the second stage and remaining more or less constant during the third stage. It decreased in the 4th stage and continues to decline until maturation (Fig. 5). Since the fruits show waxing and waning in POD activity, it is necessary to establish the specific function of POD in *Capsicum* fruit development. The POD was fractionated

and was isolated separately in soluble and cell wall bound forms and both were subjected to assay studies. Cytosolic POD, expressed maximum activity than cell wall bound POD (Fig. 6). The cell wall bound POD showed the highest level initially followed by decrease in all the subsequent stages. The poor assay data of cell wall bound POD corroborates with the low lignin content of the mature fruit tissues. The data makes clear that the high cytosolic POD has a prominent role in fruit tissue other than lignification. This may be correlated with decrease in capsaicinoids content in the later stages of fruit development while the increase in capsaicinoid content started before the increase in POD activity. These results basically indicate an inverse relationship between capsaicinoid content and POD activity that might indicate an involvement of this enzyme in capsaicinoid degradation. Chilli POD, especially POD isoenzyme – 6, oxidized the phenolic precursors of capsaicin biosynthesis such as caffeate and ferulate[20]. Our results supports that POD might be involved in capsaicinoid degradation observed in later stages of fruit development and thus the loss of pungency in chilli. Holden *et al*[16]., reported that the sink of bound phenolics in the cell wall may turn over and could thus provide substrate for capsaicin biosynthetic pathway. The accumulation of lignin like substances ceased during fruit ripening, when the colour of fruit turning to red[17].

The decrease in POD activity correlates with the decrease in other physiological parameters such as chlorophyll and pH, which define fruit maturity. Similar changes in POD activities have been reported in other ripening fruits like *C. chinensis*. POD activity decreased during development after fifteen days from the setting of the fruit[21]. In tomato, the maximum value of soluble POD was reached at the mature green stage and decline with ripening[22]. The same is in the case of strawberry and in the pulp of papaya[23]. An increase in POD activity was noted from 49th day which may be correlated with capsaicinoid degradation.

Polyphenol oxidase catalyzes the enzymic browning in fruits and vegetables[24]. In the present study PPO was assayed at different stages of development. PPO shows a slow pace of activity during the development of the fruits followed by an increase during ripening stage. Protein content and activities of PPO and POD were found to be higher in the beginning of fruit development and final process of fruit ripening compared to the other periods, most probably due to higher metabolic activity required during these developmental stages (Fig. 7). Phenolic compounds are thought to be sequestered in cell and include anthocyanin, flavonols, cinnamate derivatives, soluble phenols and catechin. Most of these phenolics are intermediates and derivatives of the shikimate and phenylpropanoid pathways[25]. So in the present study, the increase in PPO and POD in the beginning of fruit development may be involved in these processes. On the other hand, PPO and POD degrade fruit anthocyanin indirectly by reacting with D – catechin to form quinines that polymerizes with anthocyanin[26]. The increase in both the enzymes during ripening in the present study may depend on anthocyanin metabolism.

Ascorbate: L - Ascorbic acid content varied from 1.26 to 24.5 mg/g fresh weights. Ascorbate increased during the ripening of fruit and peaked at the red succulent stage (Fig. 7). A positive correlation was observed between ascorbate and moisture content since ascorbate is a water soluble compound that may be expected to decline as the fruit dehydrate on the plant. The ascorbate content of cherry laurel was found as a continual decline depending on the developmental process[24]. The results presented here provide an insight for understanding the changes in the activities of PPO and POD as well as in the contents of protein, sugar, ascorbate, lignin and capsaicin. In addition, understanding of the biochemical changes and enzyme activities in chilli, the chemistry of their transformation and their functions with fruit maturation would be of great importance.

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