CHAPTER 3
EFFECTS OF HIGH PRESSURE TREATMENT ON THE ACTIVITY OF PEROXIDASE AND POLYPHENOLOXIDASE

3.1 INTRODUCTION

3.1.1 PEROXIDASE

3.1.1.1 Generalities

Peroxidase (E.C.1.11.1.7, donor: hydrogen-peroxide oxidoreductase) are members of the group of oxidoreductase. They decompose hydrogen peroxide in the presence of a hydrogen donor. Peroxidases can be divided into two main classes : (1) iron-containing peroxidases, and (2) flavo-protein peroxidases. The enzymes belonging to class 1 can be subdivided into two groups : ferriprotoporphyrin peroxidases and verdoperoxidases. The former contain ferriprotoporphyrin IX (hematin) as prosthetic group, and are brown when purified. They occur in higher plants [horseradish (being one of the main enzyme sources), turnip, fig, sap, tobacco, potato and microorganisms (yeast cytochrome c). The prosthetic group of verdoperoxidases contain also an iron-protoporphyrin group different, however, from ferriprotoporphyrin IX. These enzymes are green in the purified state. They occur in animal organs and in milk (lactoperoxidase). The flavo-protein peroxidases contain flavine-adenine-dinucleotide as prosthetic group and occur in microorganism and animal tissues. The two groups of iron-containing peroxidases can be distinguished by acidic acetone treatment, which removes hematin from the protein moiety of ferriprotoporphyrin peroxidases, while it has no effect on verdoperoxidases (Vámos-Vigyázó,1981 and Wong,1995a).

In plants, peroxidase is located in the cell partly in soluble form, in the cytoplasm and partly in an insoluble, cell-wall bound form. Soluble POD can be extracted from tissue homogenates with a low ionic strength buffer. The particulate enzyme can be present in two forms : ionically bound and covalently bound. The ionically bound form can be extracted with buffers of higher ionic strength ; the covalently bound enzyme requires digestion with a pectolytic or cellulolytic enzyme

preparation to be liberated (Vámos-Vigyázó,1981 and Mclellan and Robinson,1981).

Plant peroxidases have been demonstrated to have a multiplicity of isoenzyme forms. Shannon *et al*. (1966 ; cited by Nagle and Haard,1975) identified seven isoenzymes in peroxidase isolated from horseradish root (HRP). These isoenzymes were fractionated and classified as anionic or cationic on the basis of ion exchange chromatography. In 1977, Hoyle (cited by Wong,1995a) demonstrated that horseradish peroxidase consists of 42 isoenzymes. The major isoenzymes are A, B and C and have pI's of 6.1, 6.9 and 8.9 respectively. The complete primary structure of isoenzyme C has been described by Welinder (1979). This isoenzyme consists of a prosthetic group (ferriprotoporphyrin IX, 550 daltons), 2 calcium atoms, a single polypeptide chain with 4 disulfide bonds (308 amino acids, 33,890 daltons) and eight chains of carbohydrate (18% of the total weight). The carbohydrate moieties are comprised of N-acetylglucosamine, mannose, fucose and xylose, attached to an AsnX-Ser/Thr sequence mostly in the C-terminal half of the molecule. The molecular weight of HPR-C is close to 44 kD. Six isoenzymes, E1 to E6, with extremely high pI values (10.6 for E1 and E2, and ≥ 12 for E3-E6) have also been isolated (Aibara *et al*.,1981). The molecular weights of these basic isoenzymes are slightly lower than the neutral isoenzymes because of the variation in the contents of carbohydrate moiety. The HRP-E5 whose crystal structure has been recently analysed, has a MW of 36 kD, consisting of 306 amino acid residues, 2 glucosamines and 8 other sugars.

The native enzyme (HRP-C) has the ferric iron coordinated to the four nitrogens of the pyrrole ring of the protoporphyrin IX (Fig 3.1). The fifth coordination is occupied by an imidazole ligand, the proximal His 170. The sixth coordination is vacant. The regions of the proximal His 170 and the distal His 42 residues are highly conserved not only in plants but also in microbial peroxidases (Wong,1995a).

HRP-C contains 2 moles of calcium per mole of enzyme with one of the calcium atoms being essential for correct folding conformation (Ogawa *et al*.,1979). This calcium at the high-affinity binding site is responsible for maintaining the protein structure around the heme group (Morishima *et al*., 1986). Studies on the recombinant enzyme also suggest that Ca^{++} is critical for correct folding and activity (Smith *et al.*,1990). Removal of Ca^{++} causes changes in the heme distal and proximal structures. These conformational changes affect the rate constant of the reduction process (HRP-

I + AH_2 → HRP II + AH), resulting in a decrease of enzyme activity (Shiro *et al*., 1986).

3.1.1.2 Reaction mechanism

Peroxidase catalyses four types of reactions : (1) peroxidatic, (2) oxidatic, (3) catalatic and (4) hydroxylation. The overall equation of the peroxidatic reaction can be given as follows :

$$ROOH + AH_2 \xrightarrow{POD} H_2O + ROH + A$$

where R = H^+,CH_3 or C_2H_5 ; AH_2 = hydrogen donor in the reduced form and A = hydrogen donor in the oxidised form.

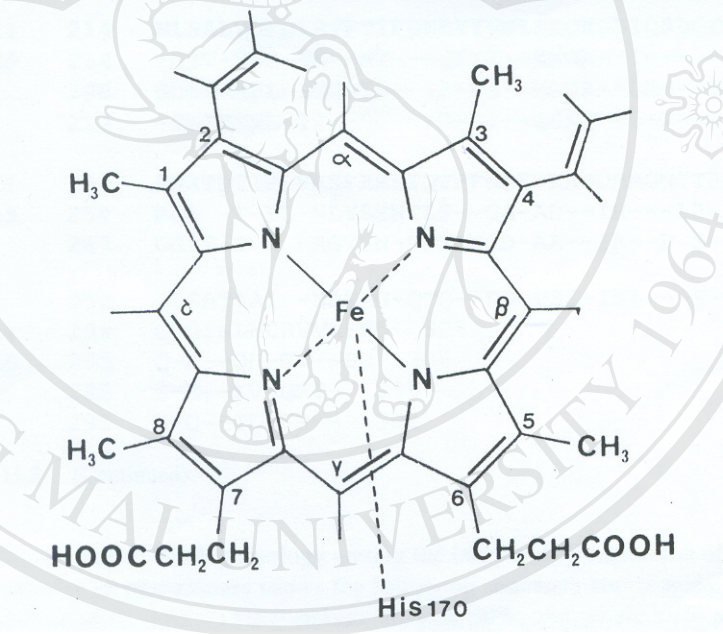


Fig 3.1 Horseradish peroxidase has the ferric iron coordinated to the four nitrogens of pyrrole ring of the protoporphyrin IX. From Wong (1995a).

In the course of the reaction, intermediate compounds are formed :

$$POD\ (brown) + H_2O_2 \rightarrow Compound\ I\ (HRP\text{-}I)$$
$$Compound\ I\ (green) + AH_2 \rightarrow Compound\ II\ (HRP\ II) + AH$$
$$Compound\ II\ (pale\ red) + AH \rightarrow POD\ (brown) + A$$

whereby the last reaction is rate-limiting. As apparent from spectral changes, two further intermediates are formed in the reaction at high H_2O_2 concentrations ; these are, however, not thought to be directly involved in the main mechanism. A great variety of compounds may act as hydrogen donors including phenols (p-cresol,

guaiacol, resorcinol), aromatic amines (aniline, benzidine, o-phenylene diamine, o-dianisidine), reduced nicotinamide-adenine dinucleotide and reduced nicotinamide-adenine dinucleotide phosphate.

The oxidatic reaction of POD may take place in the absence of hydrogen peroxide. It requires O_2 and cofactors : Mn^{+2} and a phenol (usually 2,4-dichlorophenol). A wide variety of substrates can be transformed in this reaction such as oxalate, oxaloacetate, ketomalonate, dihydroxyfumarate or indoleacetic acid (IAA).

The catalatic decomposition of H_2O_2 occurs in the absence of a hydrogen donor, according to the following equation :

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

The rate of this reaction is negligible in comparison to the rates of the peroxidatic and the oxidatic reactions.

The hydroxylating reaction produces o-dihydroxy phenols from monophenols and O_2 similar to PPO. However, with POD, the reaction requires a hydrogen donor such as dihydroxyfumaric acid which provides the free radicals necessary for the action of the enzyme (Vámos-Vigyázó,1981 and Whitaker,1985).

3.1.1.3 Substrates of POD

(a) The Peroxide

POD is highly specific to the peroxide substrate. The main peroxide substrate is H_2O_2 . The enzyme is inactivated by high concentrations of H_2O_2. It was concluded that H_2O_2 attacked a non-heme as well as a heme site on the enzyme (Weinryb, 1966). Activity of grape POD was found to be maximal at a concentration of 6.37×10^{-2} M H_2O_2 with a plateau in the curve between 0.57×10^{-2} and 1.91×10^{-2} M H_2O_2 giving less than half of the maximum activity (Poux and Ournac, 1972 ; cited by Vámos-Vigyázó,1981). For POD in HRP homogenate, the concentration yielding maximum activity was 0.3×10^{-2} M H_2O_2 (Herzog and Fahimi, 1973). Hydroxymethyl-hydroperoxide was found to inhibit HRP, being at the same time a substrate of the enzyme. It has been concluded that POD requires a free HOO^- group in order to react with peroxides (Marklund, 1971).

(b) The Donor Substrate

POD has low specificity for the hydrogen donor substrate. This is interpreted as resulting from the different substrate specificities of the individual isoenzymes

present in plant POD. The number of the isoenzymes detected as well as heat stability and regeneration of a given POD varies with the donor substrate applied. This is of importance if the enzyme is used as a genetic marker or as indicator of the efficiency of heat treatment of fruits and vegetables (Vámos-Vigyázó,1981). Bound and soluble enzyme fractions were also found to differ in substrate specificity (Haard, 1973).

Some substrates are preferentially used for a given purpose such as guaiacol is traditionally applied to check thermal treatments, most often in a qualitative test. The concentration of guaiacol giving maximum reaction rate was found to be 1.4 X 10^{-2} M. Benzidine is applied in histochemical stains as well to detect isoenzymes after gel electrophoresis or isoelectric focusion. O-Dianisidine, o-phenylene diamine, o-tolidine, 3-amino-9-ethyl-carbazole, 3,3´-diaminobenzidine tetrachloride (DAB), p-phenylene diamine, N,N´-dimethyl-p- phenylene diamine and o-toluidine are also widely used substrates (Herzog and Fahimi, 1973 ; Nagle and Haard, 1975 ; Vámos-Vigyázó,1981) of which DAB was found to give a more sensitive assay for HRP than guaiacol or o-dianisidine and o-phenylene diamine to be the most suited to detect isoperoxidases of green bean. When using DAB as substrate, low concentrations of gelatin (0.1 %) should be incorporated into the reaction mixture to prevent precipitation of the oxidised donor. Eugenol (2-methoxy-4-alkylphenol) was recommended for activity measurements as a non-carcinogenic donor substrate. Pyridoxal, pyridoxal phosphate, pyridoxine and pyridoxamine in legume seedlings undergo oxidative destruction in the presence of POD and H_2O_2 and may therefore be considered as substrates (Vámos-Vigyázó,1981).

3.1.1.4 pH and Temperature optima of enzyme activity

The pH optimum of POD activity varies with the enzyme source, the isoenzyme composition, the donor substrate and the buffer applied. The broad pH optima observed with POD from some sources are due to the presence of isoenzymes of different pH optima (Nagle and Haard, 1975). Bound and soluble POD fractions from the same source may differ in pH optima as well (Haard, 1973).

Activity decreases at low as well as at high pH values. The loss of activity observed on acidification is attributed to the change in the protein from the native state to the reversible denatured state, brought about by detachment of the heme from the protein. The disturbance of the heme-protein interaction causes a loss of protein

stability. The transfer of the protein from the reversible denatured state to the irreversible denatured state is, therefore, influenced by pH (Lu and Whitaker, 1974). The activity changes occurring with changes in pH are related to structural changes in the enzyme molecule as shown by circular dichroic spectra of Japanese radish peroxidase. Acidification of the enzyme solution destroyed the α-helical structure and a spectrum characteristic of the β-structure was obtained (Vámos-Vigyázó, 1981).

Temperature optima for potatoes, kohlrabi and cauliflower POD (in homogenates) were found to be 55°C and 35-40°C respectively. Activity varied with temperature up to these values according to the Arrhenius equation. The apparent activation energies of the reaction as calculated from plots of mass-related activity vs. reciprocal temperature(K) were 40.6 kJ mol^{-1} and 21.9 kJ mol^{-1} for kohlrabi and cauliflower POD respectively (Vámos-Vigyázó *et al*., 1979 ; Vámos-Vigyázó *et al*., 1980).

3.1.1.5 Temperature stability of POD

Heat inactivation of POD from many sources, is in certain conditions, a biphasic and partly reversible process. The enzyme is composed of units or fractions of different heat resistance and part of its activity is restored during shorter or longer periods of storage at room temperature or lower temperature following thermal treatment. The biphasic inactivation curve were infact proven to be a mixture of isozymes of different heat resistance (heat stable and heat labile isozymes) and were attributed by some authors to the formation, during heat treatment, of a new compound of higher thermostability from heat denatured enzyme and groups of POD that remained active (Vámos-Vigyázó, 1981).

The processes found to be involved in the thermal denaturation of POD are : (1) the dissociation of the prosthetic group from the holoenzyme, (2) a conformation change in the apoenzyme and (3) the modification or degradation of the prosthetic group (Tamura and Morita,1975). The factors affecting heat inactivation of POD can be divided into two groups : (1) those connected with the source of the enzyme as the genus, species or cultivar and (2) those resulting from external parameters as time and temperature of the heat treatment (Vámos-Vigyázó, 1981).

Inactivation was further established to involve the aggregation into oligomers with an average molecular mass of about double the value (87 kD) of the native

enzyme, as well as the unfolding of the molecules with subsequent stacking of the unfolded molecules. As a result of unfolding, the exposure of the heme group increased and this led to an increase in the non-enzymatic lipid oxidising capacity of the hemoprotein, the heme being still attached to the protein moiety. This phenomenon was found to occur under time and temperature conditions prevailing in food processing. The higher the temperature, the greater the amount of the POD-inactive, lipid-oxidising macromolecule that was formed (Vámos-Vigyázó, 1981). It seems probable that off-flavour formation in blanched canned or frozen vegetables during storage is not a consequence of residual POD activity but the result of non-enzymatic lipid oxidation by the aggregates formed from the isoenzymes of lower heat resistance which have been deactivated during thermal treatment. The role of residual POD activity might indicate the insufficiency of the heat treatment applied in destroying the heme accumulated in the aggregates (Eriksson and Vallentin, 1973). This assumption can be supported by the fact that the constituents of the off-flavour formed were identified as lipid oxidation products (Zoueil and Essenlen, 1958 ; cited by Vámos-Vigyázó, 1981). This being a result of lipoxygenase action is not feasible, as this enzyme is not heat resistant nor does it show a tendency to regeneration (Svensson and Eriksson, 1974). On the other hand, carrots frozen unblanched and stored for a year at -18°C maintained their taste and flavour better than the blanched control in spite of the presence of POD activity which increase in the non heat-treated sample to a high value during storage (Vámos-Vigyázó, 1981). In the blanched sample no activity was restored.

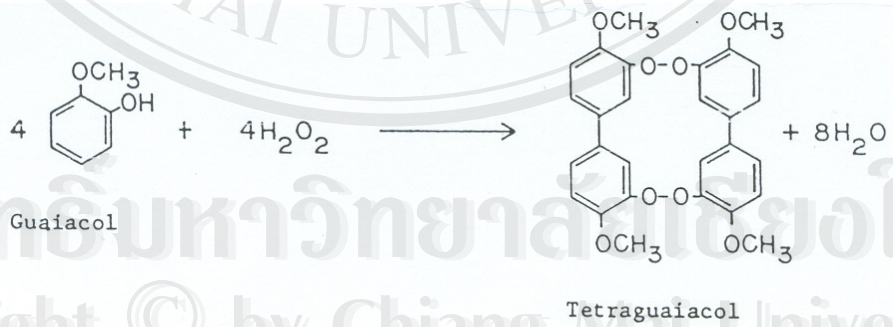
3.1.1.6 Determination of enzyme activity

a. Peroxidatic activity

Spectrophotometric methods based on the formation of a coloured compound from the hydrogen donor substrate during the reaction are generally applied to determine POD activity, and to estimate, by visual method or densitometry, residual activities after heat treatment, as well as isoenzyme composition. Care must be taken in selecting the concentrations of H_2O_2 and of the donor substrate, as well as the pH, so as to give maximum reaction rates, and thus, high sensitivity. The selection of the appropriate wavelength is also of importance, as the absorption maxima of the various

oxidised donors are different. The nature of the donor substrate might affect activity values as specificities of peroxidases from different sources are different.

Guaiacol is often used as a donor substrate. It is transformed by the enzyme into tetraguaiacol (Eq.1). It has been claimed to be the most sensitive donor for determining residual activity after heat treatment (Flurkey and Jen, 1978). However, o-phenylene diamine was found to be a more sensitive donor for assays of POD activity and detection of isoenzymes in vegetable extracts (Vámos-Vigyázó, 1981). Pyrogallol is one of the classical substrates. It is oxidised by POD to purpurogallin, hence the activity unit encountered in the early literature goes under the name of " Purpurogallinzahl" (Maehly and Chance, 1954 ; cited by Vámos-Vigyázó, 1981). O-Dianisidine is preferentially used with purified enzyme preparations as well to detect isoenzymes after gel electrophoresis or isoelectric focusing (Nagle and Haard, 1975). 2,3′,6-Trichloro-indophenol in its leuco form can be used below pH 6.0 as a hydrogen donor (Nickel and Cunningham, 1969). 3,3′-Dimethoxy-benzidine hydrochloride at pH 4.0 and 6.0 (banana POD) and 3,3′-diaminobenzidine tetrahydrochloride (pH 4.3, HRP) were found to be highly sensitive hydrogen donors (Haard and Timbie, 1973). Benzidine is used to detect POD in histochemical preparations (Vámos-Vigyázó, 1981).



Eq.1

a. Oxidatic activity

2-Methyl-1,4-naphthoquinone dissolved in 0.01 N HCl was used as substrate; the determination was carried out at pH 6.0, by measuring the increase in absorbance

at 262 nm (Klapper and Hackett, 1965). With oxaloacetate as substrate, oxygen consumption was followed polarographically with the use of a platinum oxygen electrode coupled to a millivolt recorder (Kay *et al*., 1967). The cofactors $MnCl_2$ and 2,4-dichlorophenol have to be included in the reaction mixture. Activity is calculated from the linear section of oxygen consumption which follows the 2- to 3-min induction period.

Indole-3-acetic acid is most often used to assess the oxidatic (IAA oxidase) activity of POD. The reaction mixture may contain, for example, 0.2 mM IAA, the necessary cofactors (e.g., 0.1 mM $MnCl_2$ and 0.1 mM 2,4-dichlorophenol) and the enzyme solution in 1 mM phosphate buffer (pH 6.1). Either the increase in absorbance at 261 nm is measured or a reagent (1% p-dimethylamino-cinnamaldehyde in 2 M HCl) is added to the mixture (1:1 v/v) and the absorbance of the purple reaction products is read, after 70 min in the dark, at 562 nm (Nagle and Haard, 1975). According to another method, the reaction mixture containing the substrate, the cofactors, and the enzyme is complemented with the Salkowski reagent ($FeCl_3$ + $HClO_4$). In this case, colour development takes 2 hr (Darbyshire, 1971). With the use of colour reagents and absorbance readings after 1 to 2 hr the induction period when this reaction starts does not appear, it seems more advisable to use kinetic measurements and eliminate the lag phase, if desired, by the addition of traces of H_2O_2.

3.1.1.7 Effects of high pressure on POD activity

POD is very pressure resistant, and pressure inactivation proceeds only when POD is subjected to very high pressures. Anese *et al*.(1995) found that POD extracted from carrots (pH 6-7), irreversible and complete loss of enzyme activity can only be achieved at 900 MPa (1 min) and the pH of the crude juice strongly affects the extent of enzyme inactivation, with a maximal effect at pH 6. POD from Satsuma mandarin juice retained 70 % of its residual activity after treatment at 600 MPa and 23°C for 10 min (Seyderhelm *et al*., 1996) The maximum extent of POD inactivation in green peas was obtained at 900 MPa for 10 min, amounting to an activity reduction of 88 %. Combination with thermal treatment enhanced the inactivation at 600 MPa, but no significant differences were noticed at 700 MPa. This may be due to the presence of isoenzymes, reacting in different ways to pressure as well as to heat. Moreover,

further increases in treatment time seemed to have no significant effect (Quaglia *et al*., 1996). Rovere *et al* . (1996 ; cited by Ludikhuyze *et al.*, 2001a) demonstrated that for POD from apples, a 5 min treatment at a pressure exceeding 1000 MPa resulted in inactivation. POD in guava puree was reported to be somewhat less pressure stable, and it could be inactivated at room temperature by treatment for 15 min at 600 MPa (Yen and Lin, 1996). For POD from horseradish, combinations of 800 to 900 MPa with temperatures in the range of 55°C to 70°C are required to induce any significant inactivation. However, the pressure stability can be manipulated by changes in pH, with the highest stability being observed at about neutral pH (Ludikhuyze *et al.*, 2001 a). Crelier *et al*. (1998 ; cited by Ludikhuyze *et al.*, 2001a) found that tomato POD was not affected by treatment at 500 MPa, even at 60°C. Furthermore, it was observed that the extent of POD inactivation did not vary much with the type of sample (i.e., crushed sample, whole vegetable or liquid extract).

However, the extreme pressure resistance of POD is rebutted by the results of Cano *et al*. (1997). They observed POD in strawberry puree to be increasingly inactivated by treatments up to 300 MPa at 20°C for 15 min. Above this pressure, POD activity was slightly increased. On the other hand, above 45°C, a decrease in POD activity was found at all pressure (50 to 400 MPa). In orange juice, POD activity decreased continuously up to 400 MPa (15 min processing time) at room temperature. At 32°C, a maximal inactivation rate was found. High pressure treatments at 32°C to 60°C adversely increased POD activity in orange juice.

In case of lactoperoxidase, the major peroxidase in milk, the barotolerance at temperatures ranging from 10°C to 30°C was strongly dependent on the surrounding medium. Pressure inactivation was much more pronounced in Tris buffer (pH 7) than in milk. In Tris buffer, initial activity was reduced by 70 % at 600 MPa and 25°C for 2 min while no inactivation was found in milk (Seyderhelm *et al.*, 1996). Ludikhuyze *et al*. (2001b) reported that pressures up to 800 MPa combined with temperatures up to 50°C did not result in any activity loss. Moreover, it has been noted that the application of pressure (100-700 MPa) at temperatures which would normally induce thermal inactivation at atmospheric pressure (69-75°C) completely inhibits the

thermal inactivation. In other words, pressure exerts a protective effect against thermal inactivation.

3.1.2 POLYPHENOLOXIDASE

3.1.2.1 Generalities

Polyphenoloxidases (monophenol, dihydroxyphenylalanine : oxygen oxido-reductase ; EC 1.14.18.1 ; 1,2-benzenediol : oxygen oxidoreductase ; EC 1.10.3.1) has been denoted in the early literature for two different enzymes (Whitaker, 1985 and Vámos-Vigyázó, 1981). They are, primarily different with respect to substrate specificity. One of them oxidises phenolic compounds with ortho and vicinal (3,4,5-trihydroxy) OH-groups and is able to act on monophenols by converting them into o-dihydroxy phenols (Vámos-Vigyázó, 1981). They are also known as tyrosinase, phenolase, catechol oxidase, monophenol oxidase, cresolase and catecholase, referring to the substrate acted on (Whitaker, 1995). The other enzyme oxidises o- and p-dihydroxy phenols and is most often referred to as laccase (Vámos-Vigyázó, 1981).

Polyphenoloxidase (PPO) was first discovered in mushrooms by Schoenbein (1856 ; cited by Whitaker, 1995) and was changed to tyrosinase (Hammer, 1993). The generally accepted molecular weight of mushroom PPO is 128.0 kD (Whitaker, 1995). However, the overall diversity of molecular weights of PPO is not known. PPO from most sources has been reported to be present in different molecular forms. The number of these forms depends on the enzyme source and on the methods applied to extract and separate them. Nagai and Suzuki (2003) reported that the molecular weight of PPO from bean sprout is 54 kD. Gandía-Herrero *et al*. (2004) found that the molecular weight of soluble and membrane-bound PPO from beet root are 55 and 54 kD respectively. Whereas the molecular weight of PPO in pear was estimated to be 70 kD by gel permeation chromatography and 65 kD by SDS-polyacrylamide gel electrophoresis (Asaka *et al.*, 1994).

PPO is widely distributed in nature. It is probably found in most plant tissues, with especially high concentrations in mushrooms, potato tubers, peaches, apples, bananas, avocados, tea leaves, coffee beans and tobacco leaves. It also can be found in microorganisms especially in fungi and some animal organs (Whitaker, 1985; Whitaker, 1995). The activity can vary markedly in different varieties of the same plant, and different stages of maturity, cultivation conditions, etc (Whitaker, 1985).

For a great number of fruits and vegetables olives were found to have the highest PPO activity (especially on catechol substrate) along with a slight laccase activity (Vámos-Vigyázó, 1981).

The localisation of this enzyme in most plant cell depends on the species, age and maturity (Vámos-Vigyázó, 1981). For instance, in green leaves, a considerable part of PPO activity is localised in the chloroplasts (Tolbert, 1973) whereas in potato tubers, they were found mostly in the subcellular fraction. In the stem of spinach beet, most of the activity was sedimentable and associated with plastid membranes and mitochondria (Vámos-Vigyázó, 1981). For freshly harvested apples, the enzyme obtained from these two particulate fractions differed slightly with respect to substrate specificity (Harel *et al.*, 1964).

In some species, e.g., spinach, alfalfa, wheat, oats, pea and sugarcane leaves, the enzyme presents in a latent form in the chloroplast, and needs trypsin or red light to be activated. The chloroplast enzyme from potatoes, mushrooms, beans, tomatoes and corn leaves did not exhibit latency (Tolbert, 1973), while PPO in the water extract of broad bean leaves required exposure to acidic or basic pH for activation (Asaka *et al*., 1994). Thus, latency does not seem to be related to the localisation of the enzyme in the cell nor to its solubility, and activators differ according to its source (Vámos-Vigyázó, 1981).

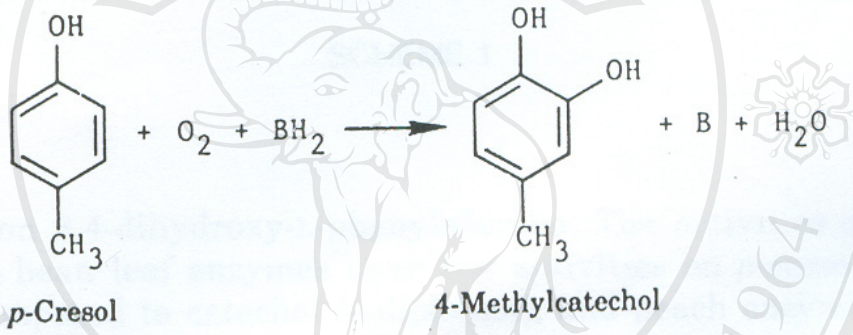
The distribution of PPO in the different parts of fruits and vegetables may be considerably different and the ratio of particle-bound and soluble enzymes varies with maturity. In grapes, the enzyme activity was found to be higher in the skin than in the flesh and will be decreased during ripening. Similar occurring as in 16 peach, 3 cherry and 3 plum cultivars : overall 77-99 % PPO activity presents as particulate-insoluble enzymes which decreased during ripening on the tree. In most fruits the insoluble part of the enzyme was found to be dominant. The activity of the soluble part amounted to 20-30 % in peaches, 15-17 % in sweet cherries, 13 % in apricots and 8-15 % in apples. In several plum and pear cultivars only water-insoluble enzyme activity could be detected (Vámos-Vigyázó, 1981).

PPO is of particular importance in food science and technology because of the brown (usually), red and blue discolouration of bruised fruit and vegetables resulting from the action of this enzyme. Estimates of up to 50 % loss of fresh tropical

fruits can occur due to this enzyme. Not only is there undesirable colour formation, but browning results in loss of nutrient quality and undesirable taste. On the other hand, PPO browning is a desired activity in tea, coffee, cocoa, prunes, dates and dark raisin production (Vámos-Vigyázó, 1981 and Whitaker, 1985).

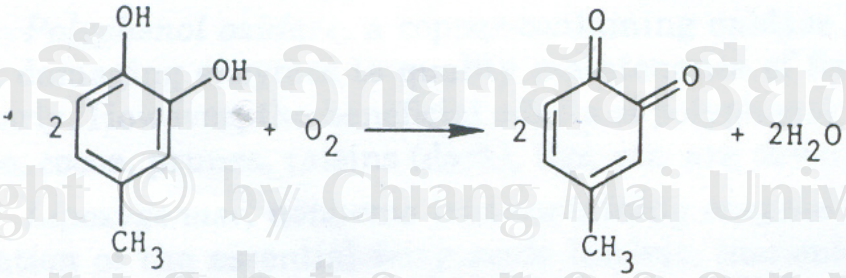
3.1.2.2 Reaction mechanism

PPO is a copper-containing enzyme which catalysed two quite different types of reactions, the hydroxylation of monophenols to form o-dihydroxyphenols (monophenol, dihydroxyphenylalanine : oxygen oxidoreductase, EC 1.14.18.1 ; mono-phenol monooxygenase ; cresolase-type activity ; Eq.2)



Eq.2

and further oxidation of o-dihydroxyphenols to benzoquinone (1,2-benzenediol : oxygen oxidoreductase, EC 1.10.3.1; catecholase-type activity ; Eq.3) (Vámos-Vigyázó, 1981 ; Hammer, 1993 ; Whitaker, 1985 ; Whitaker, 1995). The reaction requires molecular oxygen.



Eq.3

All PPO's have activity on o-diphenols. PPO from banana, tea leaf, tobacco, mango, pear, sweet cherry and clingstone peach were reported to oxidise o-diphenols only while the enzymes from potato, apple, sugar beet leaf, broad bean leaf

mushrooms and *Neurospora crassa* present both types of activity.(Whitaker, 1985 and Vámos-Vigyázó, 1981).

The initial reaction rate of PPO with monophenols is quite slow and shows hysteresis (lag phase) in the absence of catalytic amounts of BH_2 as a cofactor (Eq.3). Addition of catechol eg. BH_2 as little as 1×10^{-7} M gives normal kinetics.

The amount of prosthetic group in the enzyme was found to be 4 atoms of Cu per molecule in mushroom PPO and 1 atom per molecule in *Vicia faba* PPO (Bendall and Gregory, 1963; Robb *et al.*, 1965). The concentrations of copper in the enzyme extract from mushroom, potato, sweet potato and tobacco were 0.2-0.3 %, 0.2 %, 0.27 % and 0.32 % (w/w) respectively. The hydroxylation requires Cu in the cuprous form which is provided by the dehydrogenation step. Total or partial removal of copper from the protein moiety, leads to inactivation or reduction of activity which can be restored by the addition of Cu (Vámos-Vigyázó, 1981).

3.1.2.3 Substrates of PPO

The substrate specificity of plant PPO is quite broad and there are individual differences among PPO from different sources (genus, cultivar and the part of the fruits or vegetables). For example, peach PPO has only 51.5 % of the activity on 4-methylcatechol as on catechol, whereas broad bean leaf PPO has 200-225 % of the activity on 4-methylcatechol as on catechol. Potato, peach, broad bean leaf and pear PPO have 140, 22.2, 8 and 71.8 % respectively, the activity on chlorogenic acid is the same as on catechol (Whitaker, 1995).

Fruits and vegetables contain a wide variety of phenolic compounds. However, only a relatively small part of these serve as substrate to PPO. The most important natural substrates of PPO in fruits and vegetables are catechins, cinnamic acid esters, 3,4-dihydroxy phenylalanine (DOPA) and tyrosine (Walker, 1975; Vámos-Vigyázó, 1981). The extent to which naturally occurring phenolic substrates contribute to enzymatic browning of individual fruits or vegetables depends on the localisation and concentration of the phenol as well as on the colour intensity of the macromolecular pigments obtained from the different quinones. In most but not all fruits and vegetables, the phenol concentration is higher in the outer layers. In apples and pears, skin contained higher amounts of phenol than flesh, small fruits had a higher content of phenolic than large ones and phenol concentration was higher in

winter apples than in summer or autumn cultivars. In carrot, radish, horseradish and tomato tissue, phenol was higher in the outer (surface) layers, whereas no differences between inner and outer layer were found in this respect in celery and red beet (Vámos-Vigyázó, 1981).

In some fruits and vegetables, the main substrate of PPO is a compound not commonly occurring as a phenolic constituent of plant material. For example, the principal substrate in bananas and yam-tuber tissues was identified as dopamine (3,4-dihydroxy phenylethylamine) and catecholamine respectively (Griffiths, 1959;Martin and Ruberté , 1975).

3.1.2.4 pH and Temperature optima of PPO activity

The optimum pH of PPO activity varies with the source of the enzyme and with the substrate over a relatively wide range, in most cases between pH 4.0-7.0 (Aylward and Haisman, 1969). PPO preparations from several sources were reported to be inactive below pH 4.0 (Thomas and Janave, 1973). The enzyme in potato homogenates was found to be inactive, on both pyrogallol and chlorogenic acid at pH 5.0, while pyrogallol was not oxidised by PPO from upripe Satsuma mandarins at pH values below 6.0 (Vámos-Vigyázó, 1981). On the other hand, a preparation from grapes retained over 50 % of its maximum activity at pH 3.4, the normal pH of grape juice (Cash *et al*., 1976). The enzyme from plums was almost fully active at its natural pH (3.8), while practically all its activity was lost in neutral medium (Vámos-Vigyázó, 1981). The type of buffer and the purity of the enzyme affect the pH optimum as well. Particulate and soluble enzymes seem to be essentially different in this respect (Stelzig *et al*., 1972). Isoenzymes may have distinctly different pH optima. Enzymes obtained from the same fruit or vegetable at various stage of maturity have been reported to differ in optimum pH of activity. Not only the optimum, but the relationship between activity and pH over a wide range of pH values was found to differ according to genera, cultivars and substrates (Vámos-Vigyázó, 1981). Lanzarini *et al.* (1972) reported that most of the enzyme preparations have a single pH optimum of activity, a second optimum found in some cases was due to insufficient purification (Vámos-Vigyázó, 1981)..

The temperature optimum of PPO depends essentially on the same factors as the pH optimum. In peach (cv. Redhaven), PPO activity was found to increase from

3°C to 37°C and then decline up to 45°C. At 3°C activity was about 50% of the maximum value. The activity of apricots and bananas reached their maximum activity at 25 and 37°C respectively (Vámos-Vigyázó, 1981). Cash *et al*. (1976) found that the temperature optima of two cultivars grapes were 25-30°C and 10-15°C. Mihályi *et al*. (1978 ; cited by Vámos-Vigyázó, 1981) reported that apples of the cvs. Jonathan and Starking reached their maximum activity on chlorogenic acid at 30°C and 25°C respectively, while on pyrogallol substrate, activity rose steeply with temperature, but no maximum was reached up to 35°C. In potatoes, PPO had its maximum activity on catechol at 22°C ; on pyrogallol, a nearly linear increase in activity was noticed between 15°C and 35°C (Vámos-Vigyázó, 1981).

3.1.2.5 Temperature Stability of PPO

PPO does not belong to the extremely heat-stable enzymes. Short exposures, in the tissues or in solution, to temperatures of 70°C to 90°C are, in most cases, sufficient for partial or total irreversible destruction of its catalytic function. Exposures to temperatures below zero may also effect activity. PPO in fruit or vegetable tissues is generally inactivated by heat or by chemicals, prior to exposure to low temperatures to prevent enzymatic browning, which otherwise would take place rapidly upon thawing as a result of cell rupture occurring during freezing and access of the enzyme to its endogenous substrates.

Thermotolerance of PPO depends, as with substrate specificity, and pH- and temperature optima of activity, to a considerable extent, on the source of the enzyme. When considering the enzyme as present in tissues , heat penetration connected to particle size might influence the efficiency of thermal inactivation.

Apple PPO was found to be markedly inactivated (at pH 5.0) at temperatures above 70°C and completely destroyed at 80°C. In one cultivar, activity could be destroyed at 75°C in 3 min. In different stone fruits, of 22 cultivars, peaches were found to possess the least, and plums the most heat stable PPO. The higher heat stability of the enzyme in plums and cherries was accompanied by higher activity levels as compared to peaches. No relationship could be established between pH and heat tolerance for PPO in stone fruits. However, the enzyme proved more heat stable in unripe than in ripe fruit (Vámos-Vigyázó, 1981).

The enzyme in apricots was reported to be relatively heat stable and the efficiency of thermal treatment was found to be dependent on pH. The hydroxylating activity (as determined on p-cresol substrate) was more stable, in 5 min heat inactivation experiments, at pH 5 than at pH 6.0. At both pH values, complete inactivation of both activities occurred at 100°C. At 90°C, under similar conditions, 15, 4 and 5 % of the original oxidising activity (catechol substrate) were retained at pH 4, 5 and 6 respectively. (The hydroxylating activity was unstable at pH 3 and the oxidising activity was most stable at pH 5, during 24 hr in the temperature range from 0°C up to 40°C, or during 17 hr from 0°C up to 60°C.) No essential difference was found between the thermal resistance of apricot PPO in solution or in the fruit tissue. Even very low residual activities were found to cause discolouration in canned fruit on storage (Vámos-Vigyázó, 1981). A 10 min heat treatment at 90°C ensured complete inactivation of the enzyme in apricot puree, and prevented browning and off-flavour formation in the product on thawing after frozen storage (Ponting *et al*., 1954).

Grape juice PPO was found to lose activity relatively slowly up to 60°C, 65°C may be regarded as the critical temperature above which rapid inactivation starts. A heat treatment of 5 min at 65°C destroyed 74.5 % of the original activity at pH 3.3, the natural pH of the juice. Inactivation at pH 3.0 was 81 %, but at pH 4.0, only 59 % inactivation occurred under the same conditions. Total inactivation could be achieved in 5 min only above 70°C.

PPO in strawberries was reported to lose its activity upon prolonged storage of the fruit in the cold ; when kept at -18°C, enzyme activity decreased at first relatively slow, then more rapidly so that, after 7 ½ months it was 16 % of the initial value. After 11 months of cold storage the samples occasionally contained traces of PPO activity.

Studies into the heat inactivation of banana PPO revealed, in the range of 42 to 80°C, that below 42°C the enzyme was very stable at pH 6.0 ; a 1-hr exposure to this temperature brought about 8 % inactivation. Complete inactivation of banana PPO could be achieved by exposure to 80°C for 15 min, while Satsuma mandarins PPO retained more than 50 % of their original activity after 30 min exposure to 80°C.

PPO extracts of green beans and green peas were completely inactivated at -20°C. Inactivation was, however reversible as activity reappeared after thawing. At -15°C some activity could be detected. The resistance to cold of the enzyme of these two vegetables was found to be remarkable : after thawing, activity remained essentially unaltered after up to 395 and 310 days respectively, of storage at -20°C, whereafter a slow diminution or, with some string bean cultivars, even a slight increase was observed. The time-course of inactivation was found to approximate linearity for enzyme extracts of either vegetable. This, however, did not apply to the enzyme in the tissues (Vámos-Vigyázó, 1981).

3.1.2.6 Determination of Enzyme Activity

PPO activity can be determined by measuring (1) the rate of substrate disappearance, or (2) the rate of product formation. With either method, care must be taken to restrict measurement to the initial phase of the reaction, as reaction inactivation of the enzyme soon slows down o-dihydroxyl phenol oxidation.

When determining the rate of substrate disappearance, generally O_2 absorption is measured, either manometrically in a Warburg respirometer, or polarographically with an oxygen electrode. The two methods do not give identical results. The linear section of the time course of the reaction is considerably longer, and the O_2 adsorption values obtained under identical conditions are higher with the polarographic method (Mayer *et al*.,1966). The latter is considered by several authors the method of choice for determining PPO activity (Walker, 1975 ; Mayer *et al*., 1966).

The rate of product formation can be determined spectrophotometrically by measuring the optical density of the coloured compounds formed from the quinones. These methods are very simple and lend themselves to routine analysis. Linearity is maintained for a relatively long period as with the polarographic method (Mayer *et al*., 1966). However, some authors are definitely against the use of spectrophotometric activity determinations, as these measure the secondary reaction products of PPO, and the secondary reactions are influenced by many factors which are difficult to control : the presence of ascorbic acid lowers the values obtained ; while amino acids, protein degradation products, heavy metal ions, endogenous substrate of fruits and autooxidation products of polyphenols may increase the levels of enzyme activity.

Spectrophotometric methods may be recommended whenever relationships between enzyme activity and enzymatic browning are to be established (Vámos-Vigyázó, 1981).

A wide variety of substrates can be used with spectrophotometric methods e.g. catechol (Kahn, 1977), pyrogallol or natural substrates such as chlorogenic acid (Vámos-Vigyázó, 1981). It has to be taken into account that the coloured compounds formed from the oxidation products of the various phenols have their absorption maxima at different wavelengths, that the substrates may undergo autoxidation, especially at alkaline pH values, and that excess of some substrates (e.g. chlorogenic acid) causes strong inhibition of the enzyme (Vámos-Vigyázó, 1981).

The hydroxylating activity has to be determined by the O_2 absorption method. When pure enzymes are tested using a monophenol substrate (most often p-cresol or tyrosine) in the absence of o-dihydroxy phenols, the lag phase of the reaction is not taken into account in calculating the activity value : the end of the induction period is considered as the beginning of the reaction, and the slope of the rising section of the O_2 absorption vs. time plot is taken as the enzyme activity (Long and Alben, 1969 ; cited by Vámos-Vigyázó, 1981).

In order to delay reaction inactivation of PPO during activity measurements, coupled reactions are made use of. The reaction mixture contains, beside the enzyme and the substrate, one or more compounds of more negative oxidation-reduction potential (e.g. hydroquinone and ascorbic acid) which immediately reduce the quinones formed. Thus catalytic amounts of the substrate are sufficient, as the concentration of this reactant remains practically constant. The oxidation-reduction potential of the system can be measured polarographically (Vámos-Vigyázó, 1981). Other versions based on this principle use only ascorbic acid as the easily oxidisable compound in the reaction mixture and measure the decrease of its concentration at 265 nm, its absorption maximum (El-Bayoumi and Frieden, 1957). The rate of disappearance of ascorbic acid is directly proportional to enzyme activity. The rate limiting step in the reaction system is the substrate ↔ quinone transformation. Ascorbic acid oxidase interferes with the results. According to another version of this method, the time required for the complete consumption of a certain amount of ascorbic acid added to the reaction mixture, i.e., the appearance of the coloured

quinone products, is measured. This is called the chronometric method . The use of such a method is not recommender, as there is no means of checking the linearity of the process. In fact , the time course of this reaction has been found to be non-linear (Mayer *et al*., 1966).

Ascorbic acid can be replaced by $K_4[Fe(CN)_6] \bullet 3\ H_2O$ and the absorbance read at 420 nm. In this case the presence of ascorbic acid oxidase or plant material absorbing in the region of 265 nm does not interfere. Other advantages of the method are that ferrocyanide is less sensitive to changes in pH than ascorbic acid, especially in the acid range and is also stable in solution. The drawback is that its sensitivity is less than half that of the ascorbate procedure when using the same substrate concentration (Vámos-Vigyázó, 1981).

Several procedures have been devised to overcome reaction inactivation. These are based on the elimination of the quinones from the reaction mixture. Besthorn's hydrazone (3-methyl-2-benzothiazolone hydrazone hydrochloride) forms condensation products with the quinones, which are subsequently extracted from the reaction mixture with chloroform and then the absorption of the organic phase is read at 500 nm (Pifferi and Baldassari, 1973). An improvement of sensitivity consists in dissolving the condensation product in acetone instead of extracting it (Mazzocco and Pifferi, 1976). Another method makes use of the yellow compound 2-nitro-5-thiobenzoic acid anion, which consumes 1 mol of quinone per mol of thiol and yields almost colourless adducts. Enzyme activity is assessed by following spectrophotometrically the decrease in absorbance of the yellow compound at 412 nm (Esterbauer *et al*., 1977).

3.1.2.7 Effects of high pressure on PPO activity

The effect of pressure on PPO activity from a variety of origins have been carried out by many researchers. The available information is often fragmentary and little detailed kinetic data have been determined. To date, researchers have found that, upon pressurisation, PPO may display either inactivation or activation (enhanced catalytic activity). It can be concluded that the pressure level needed for PPO inactivation at ambient temperature is strongly dependent on its origin and the pH of the medium. In addition, microenvironmental conditions such as the presence of salts,

sugars and other additives have been proven to influence the pressure stability of PPO.

Weemaes *et al*. (1998a) attempted to combine some methods (such as different intrinsic and extrinsic stress factors) to reduce the pressure stability of PPO. In this context, they examined whether changes in pH, addition of antibrowning agents and temperature increase could be used to alter the pressure stability of mushroom and avocado PPO. For both mushroom PPO and avocado PPO, the threshold pressure for inactivation decreased when the pH was lowered below the optimal pH value. Moreover, acidification brought about an increase in the pressure sensitivity of the inactivation rate constant. That is, the absolute value of the activation volume increased with decreasing pH. Reducing the pH to 4 resulted in a change in the pressure inactivation behaviour of avocado PPO. This change in inactivation was suggested to be due to a distinct pressure stability of two avocado PPO isozymes at this low pH (Weemaes *et al*., 1998b).

Whereas the effect of pH on avocado and mushroom PPO was quite similar, the enzymes were differently affected by the presence of antibrowning agents [benzoate, ethylenediaminetetraacetic acid (EDTA), glutathione, sodium chloride (NaCl) and 4-hexylresorcinol]. The pressure stability of mushroom PPO at pH 6.5 was dramatically reduced in the presence of 4-hexylresorcinol and not affected or only slightly affected in the presence of EDTA, glutathione, NaCl or benzoate. For avocado PPO at pH 5, pressure stability was reduced by addition of EDTA and the effect was completely attributable to the pH decrease brought about by EDTA. However, upon addition of benzoate, NaCl or glutathione, a higher pressure was required to induce inactivation, indicating an increased pressure stability. The effect of 4-hexylresorcinol was dependent on the pressure. At pressures below 700 MPa, the pressure stability was decreased ; at higher pressures, it was increased (Weemaes *et al*., 1999b, 1999c).

3.2 EXPERIMENTAL

3.2.1 Materials

Lychees (*Litchi chinensis* Sonn.) were purchased from a commercial orchard at Chiangmai province of Thailand and stored in polyethylene bag at 2°C for 2 to 3 days prior to high pressure processing. Some lychees were kept at -18 °C up to 2 months for enzyme activity measurements.

Solution for enzyme assay are as follows :

0.05 M potassium phosphate buffer (Sigma, USA), pH 6.2, containing 1M potassium chloride (Fisher, UK) and 2% polyvinylpolypyrrolidone (Fisher, UK).

0.01 M sodium acetate buffer, pH 6.0, containing 0.5% guaiacol, (Sigma, USA).

0.1 % hydrogen peroxide (BDH, UK).

0.01 M sodium acetate buffer, pH 2.0-11.0 (0.5 pH unit intervals), containing 0.5% guaiacol (Sigma, USA).

0.1 M potassium phosphate buffer, pH 6.5 (Sigma, USA).

0.1 M potassium phosphate buffer, pH 3.0-9.0 (0.5 pH unit intervals) (Sigma, USA).

0.2 M catechol (Sigma, USA).

3.2.2 Methods

3.2.2.1 High Pressure Processing

The lychees were peeled, destoned and sealed in polyethylene bags (Cryovac Ltd., UK), taking care to exclude as much air as possible. Each bag contained 3 lychees so that the total weight was about 45 g. The bags were processed at pressure of 200, 400 and 600 MPa and temperature of 20, 40 and 60°C for 10 or 20 min in a prototype Stansted "Food-Lab" model 900 high pressure rig (Stansted Fluid Power Ltd., Stansted, UK). Three or four bags were treated at each pressure/ temperature regime.

Further sets of 3 lychees were mixed with the syrup which consisted of 300 g sucrose,1.3 g citric acid and 700 g deionised water in the ratio of fruit to syrup as 1:1 and were subjected to the pressure/ temperature regimes described above.

The pressure treated samples (and controls) were stored at -18°C for 2 months for enzymatic activity. Vámos-Vigyázó (1981) had shown that storage at -4°C and -20°C for up to 18 months has no effect on the total enzyme activity.

3.2.2.2 Assay for Peroxidase(POD) activity

Ten grams of lychee were homogenised at 4°C with a mixture of 40 ml of 50 mM potassium phosphate,1M KCl, 2% polyvinylpolypyrrolidone (PVPP), pH 6.2. The homogenate were centrifuged at 16,000 x g for 30 min at 4 °C (Sorvall® RC5 C Plus) and filtered through Whatman No 41 paper,the filtrate were used for the enzyme assays (modified from Huang *et al*., 1990). A 0.1 ml aliquot of crude enzyme extracted was added to 2.15 ml of 0.01 M sodium acetate buffer, pH 6.0 containing 0.5 % guaiacol, 0.25 ml of 0.1 % hydrogen peroxide and subsequently its absorbance at 470 nm was followed for at least 5 min with a Perkin Elmer UV/VIS Spectro-photometer Lambda 20. One unit of POD activity was defined as an increase of 0.1 unit of absorbance per min at 470 nm (Flurkey and Jen,1978).

The pH optima for POD was determined using the above incubation mixture by adjusting the pH from 2.0 to 11.0 (0.5 pH unit intervals) with 1 % acetic acid or 1 % NaOH and assayed as described above.

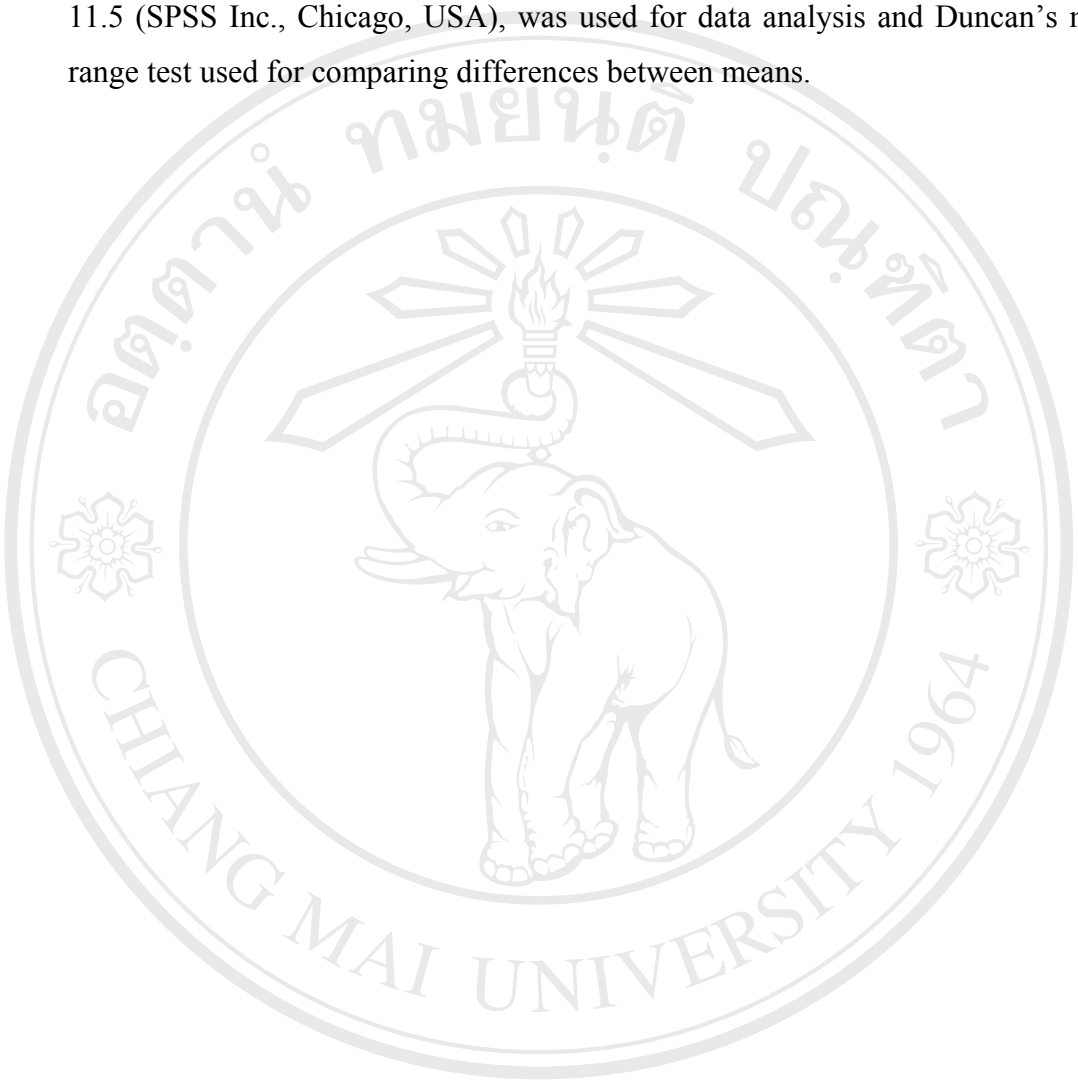
3.2.2.3 Assay for Polyphenoloxidase (PPO) activity

Extraction of the enzyme and measurement of its activity was carried out according to the procedure described by Huang *et al*. (1990) and Flurkey and Jen (1978). A 0.05 ml aliquot of crude enzyme extracted was added to a mixture of 2.2 ml of 0.1 M potassium phosphate buffer, pH 6.5 and 0.25 ml of 0.2 M catechol, and measured its absorbance at 420 nm followed for at least 5 min. One unit of PPO activity was defined as an increase of 0.1 unit of absorbance per min at 420 nm. The pH optimum was determined using the above incubation mixture by adjusting the pH values from 3.0 to 9.0 (0.5 pH unit intervals) with 1 % H_3PO_4 or 1 % NaOH and assayed as described above.

3.2.2.4 Experimental Design

Study of POD and PPO activities pressure treated at 10 min or 20 min, two variables were studied, these were 3 levels of pressure (200, 400, and 600 MPa) and 3 levels of temperature (20, 40, and 60°C), i.e., a 3x3 factorial in a completely

randomised design (CRD). Each treatment was determined twice for the activities and all treatments were carried out in at least 3 replicates. The statistical program, SPSS v 11.5 (SPSS Inc., Chicago, USA), was used for data analysis and Duncan's multiple range test used for comparing differences between means.



3.3 RESULTS AND DISCUSSIONS

3.3.1 pH Optima for POD

The pH optimum for lychee POD was 5.0-8.0 with maximal activity at 6.0 (Fig 3.2) which is similar to the value found by Nagle and Haard (1975), Baardseth and Slinde (1980) and Fujita *et al.* (1995) for POD from banana, carrot, swede, broccoli and cabbage. The broad pH optima observed is probably due to the presence of isoenzymes of different pH optima. The loss of activity observed on acidification is attributed to the change in the protein from the native state to a reversible denatured state, brought about by detachment of the heme from the protein (Vámos-Vigyázó,1981).

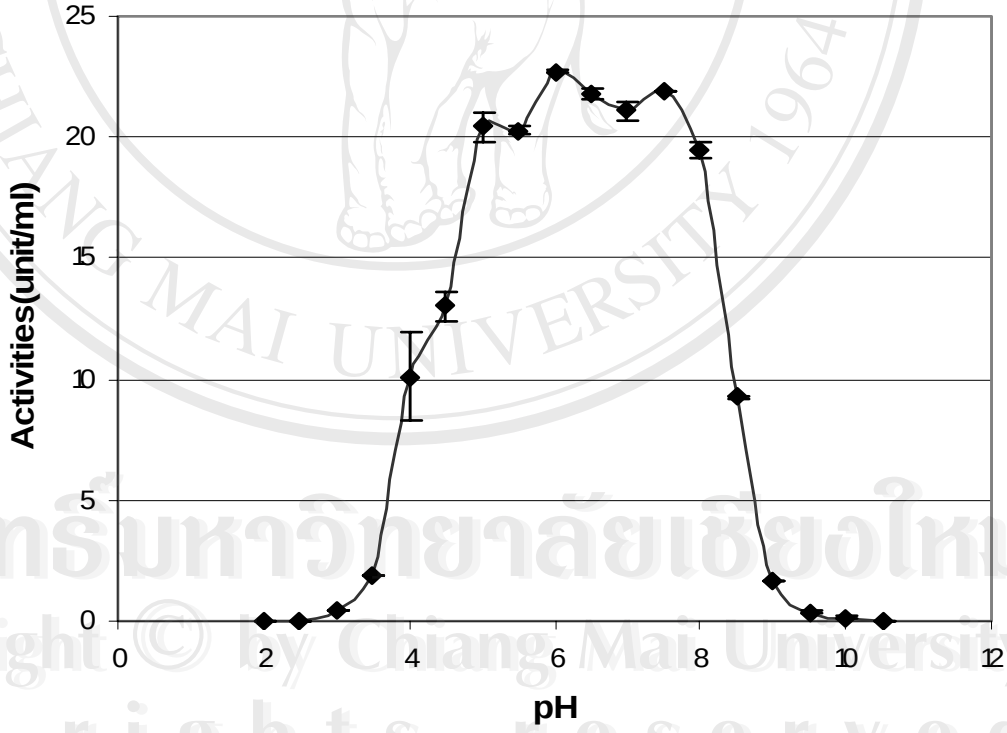


Fig 3.2 The variation of lychee peroxidase activity as a function of pH

3.3.2 pH optima for PPO

For PPO, maximal enzyme activity was at pH 7.0 (Fig 3.3). The pH corresponding to maximal activity is in agreement with the values found for avocado, pear and plum PPO as reported by Weemaes *et al.*(1998c). On raising the pH from 7.0 to 8.0, a sharp drop in enzyme activity was noticed. Moreover, the enzyme showed very low activity at or below pH 4.0. A sharp drop in PPO activity at pH values above 7.0 was also noted by Weemaes *et al.* (1998c) for apple, grape, pear, avocado and plum.

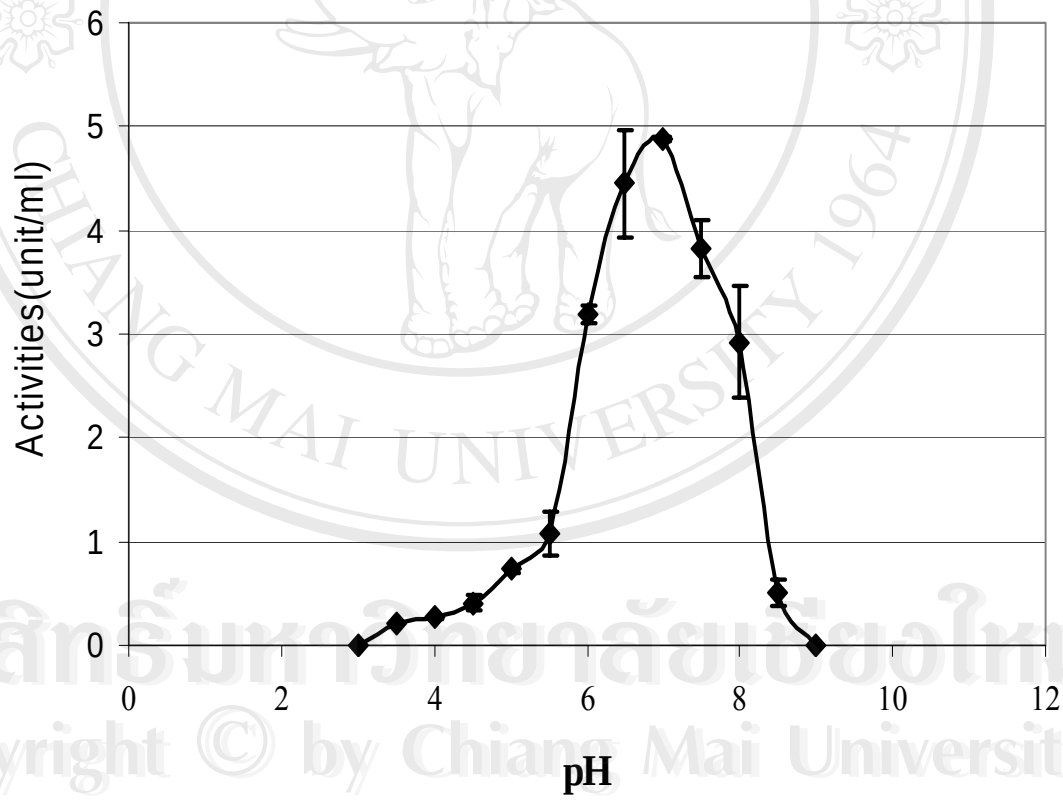


Fig 3.3 The variation of lychee polyphenoloxidase activity as a function of pH

3.3.3 Effect of Combined UHP/Temperature treatment on POD activity

The results of treatment for both 10 and 20 min are summarised in Figs 3.4 and 3.5. It is seen that to a large extent the results obtained for treatment times of 10 and 20 min are similar and perhaps the most marked effect is the apparent increase in activity seen after treatment at 200 MPa in the non-syruped lychees, the effect appearing more marked at 40 °C compared with both 20 and 60°C. This type of effect has also been noted in tomato puree (Hernández and Cano,1998) and for PPO in mushroom (Gomes and Ledward,1996). Because of the activation phenomena, probably related to better availability of the substrate (Gomes and Ledward,1996) the only marked inactivation of POD in lychee was at 600 MPa and 60°C(Figs 3.4 and 3.5). Ogawa *et al*. (1992) observed that peroxidase from citrus juice retained 70 % of its activity and Quaglia *et al*. (1996) found that fresh pea POD retained 50 % of its activity after treatment at 600 MPa and 60°C for 10 min.

When processed in syrup (Figs 3.6 and 3.7) the effects are for less marked and although there is still some evidence of activation at moderate pressure all the differences are small.

These results are undoubtedly due to the baroprotective effect of the syrup as has been found by other workers including Seyderhelm *et al*. (1996) who demonstrated that the inactivation of pectinesterase in orange juice containing 30 % sucrose (60°Brix) was much lower than in orange juice of 11°Brix.

3.3.4 Effect of combined UHP/Temperature treatment on PPO activity

The results are shown in Figs 3.8 and 3.9 and it is seen that although after 10 min treatment the results are non-uniform after 20 min a clear pattern emerges demonstrating that at mild temperatures (20 and 40°C) the degree of inactivation of PPO decreases for with treatment time (10 or 20 min) for the three pressure levels. Increasing temperature brings about significant decreases in activity, with over 90 % loss of activity at 600 MPa and 60°C for both treatment times (10 and 20 min). These results are in good agreement with those of Jolibert *et al*. (1994) on apple PPO, Castellari *et al.* (1997) on grape must and Gomes and Ledward (1996) on potato PPO. These is no evidence of the pressure activation seen by Gomes and Ledward (1996) in mushrooms.

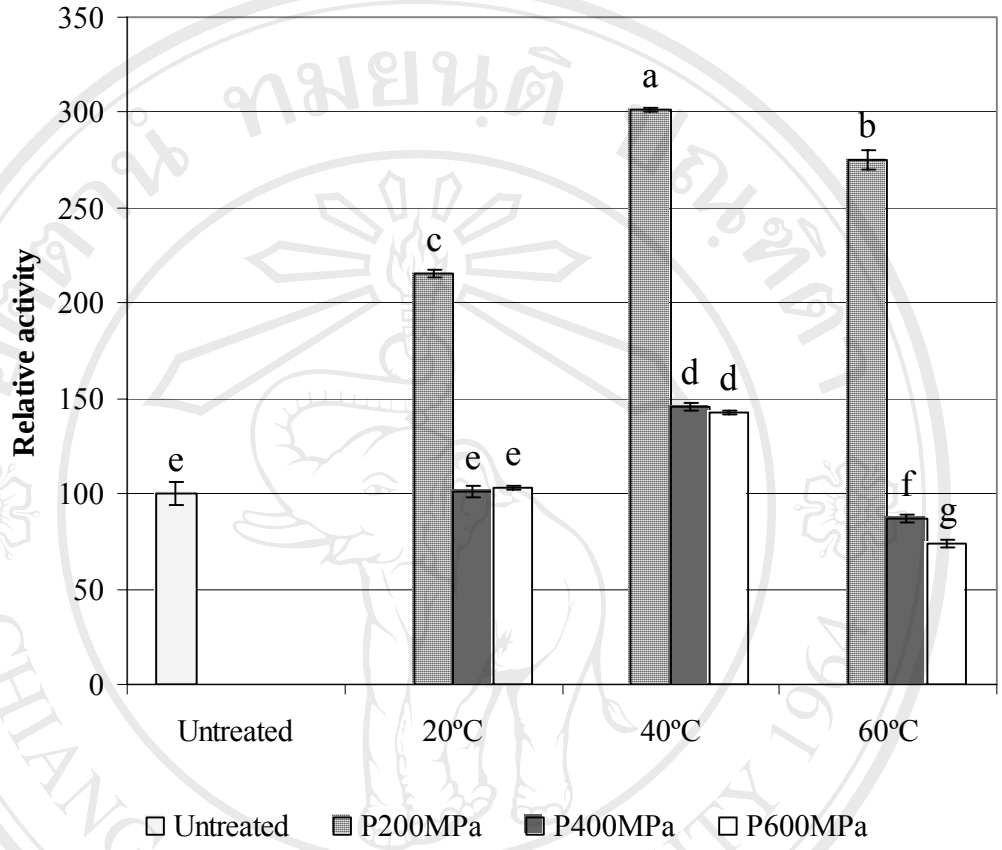


Fig 3.4 Effect of combined UHP/Temperature at 10 min on POD activity of fresh lychee. All values are the means ± S.D. of duplicate determinations on 3 samples. Bars with different superscript were significantly different ($p<0.05$).

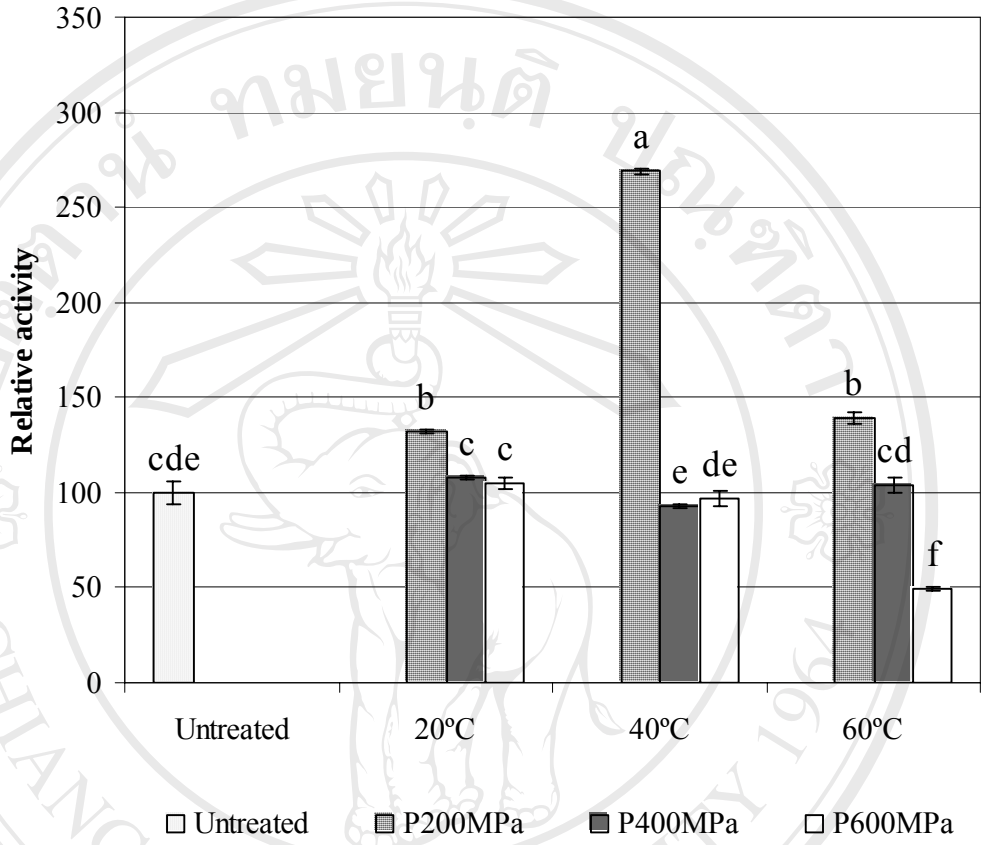


Fig 3.5 Effect of combined UHP/Temperature at 20 min on POD activity of fresh lychee. All values are the means ± S.D. of duplicate determinations on 3 samples. Bars with different superscript were significantly different ($p<0.05$).

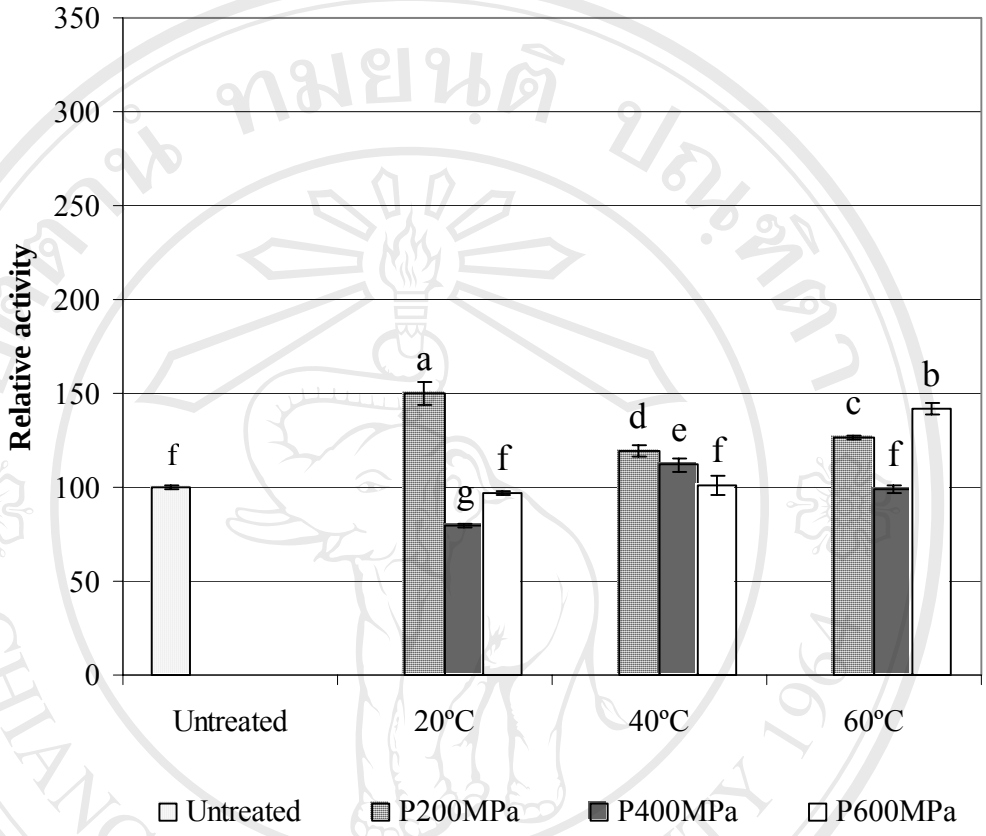


Fig 3.6 Effect of combined UHP/Temperature at 10 min on POD activity of syrup lychee. All values are the means ± S.D. of duplicate determinations on 3 samples. Bars with different superscript were significantly different ($p<0.05$).

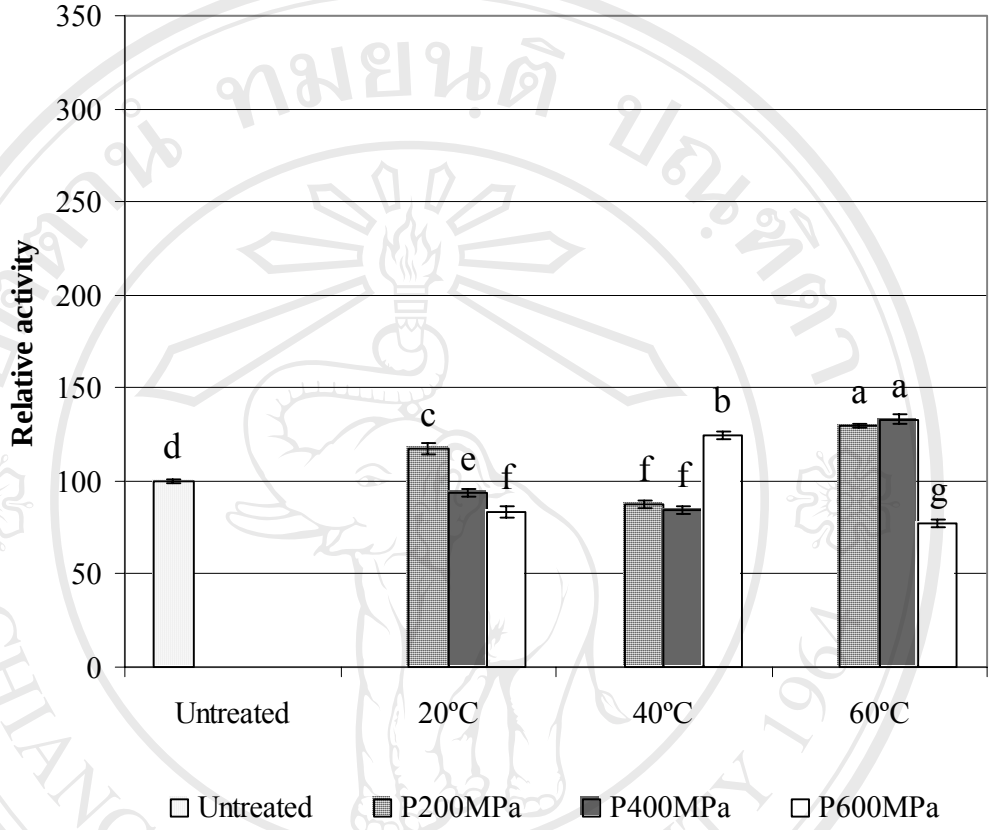


Fig 3.7 Effect of combined UHP/Temperature at 20 min on POD activity of syrup lychee. All values are the means ± S.D. of duplicate determinations on 3 samples. Bars with different superscript were significantly different ($p<0.05$).

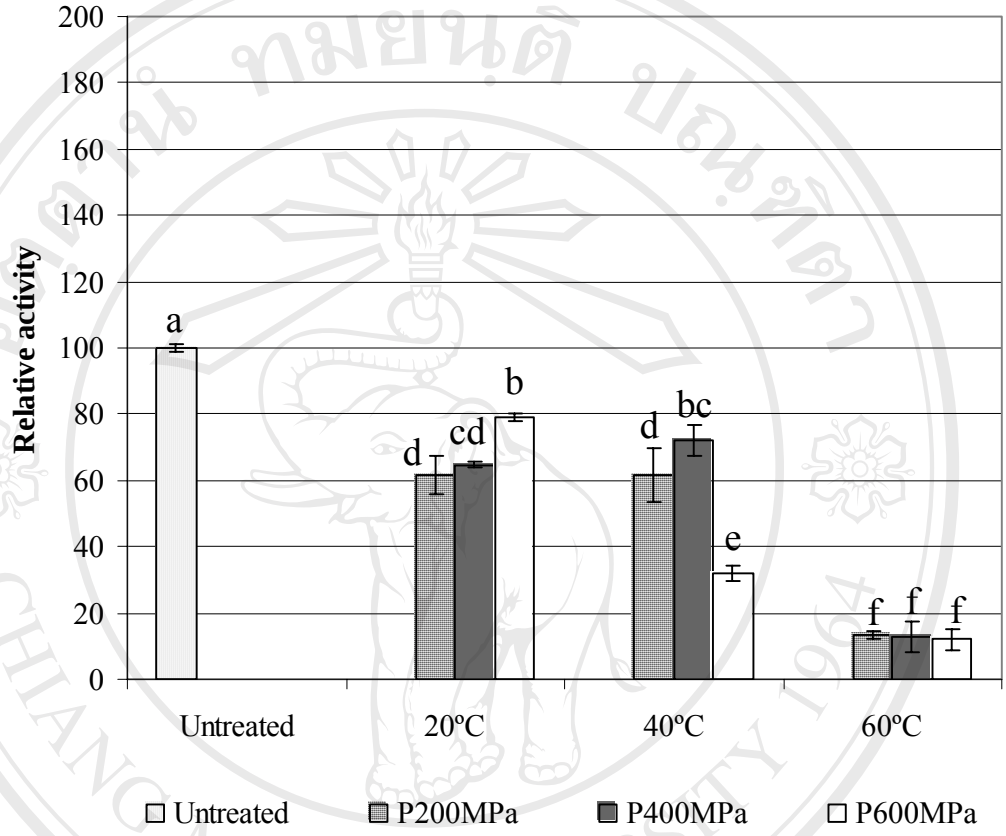


Fig 3.8 Effect of combined UHP/Temperature at 10 min on PPO activity of fresh lychee. All values are the means ± S.D. of duplicate determinations on 3 samples. Bars with different superscript were significantly different ($p<0.05$).

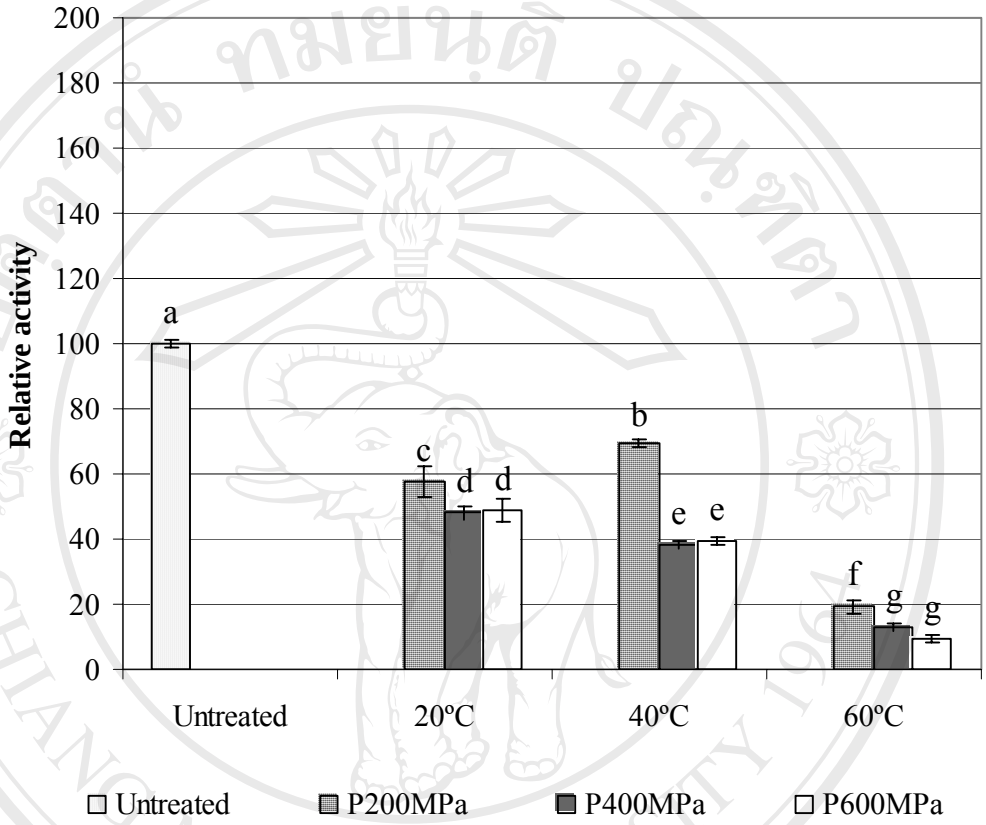


Fig 3.9 Effect of combined UHP/Temperature at 20 min on PPO activity of fresh lychee. All values are the means ± S.D. of duplicate determinations on 3 samples. Bars with different superscript were significantly different ($p<0.05$).

When treated in syrup the baroprotective effect of the mixture is again seen although there is still significant inactivation under the more extreme conditions (Figs 3.10 and 3.11)

In conclusion this initial work has demonstrated that the use of high pressures and moderate temperatures may be an effective means of extending the shelf life of lychees with minimal effect in the quality although the high residual activity of peroxidase may lead to problems.

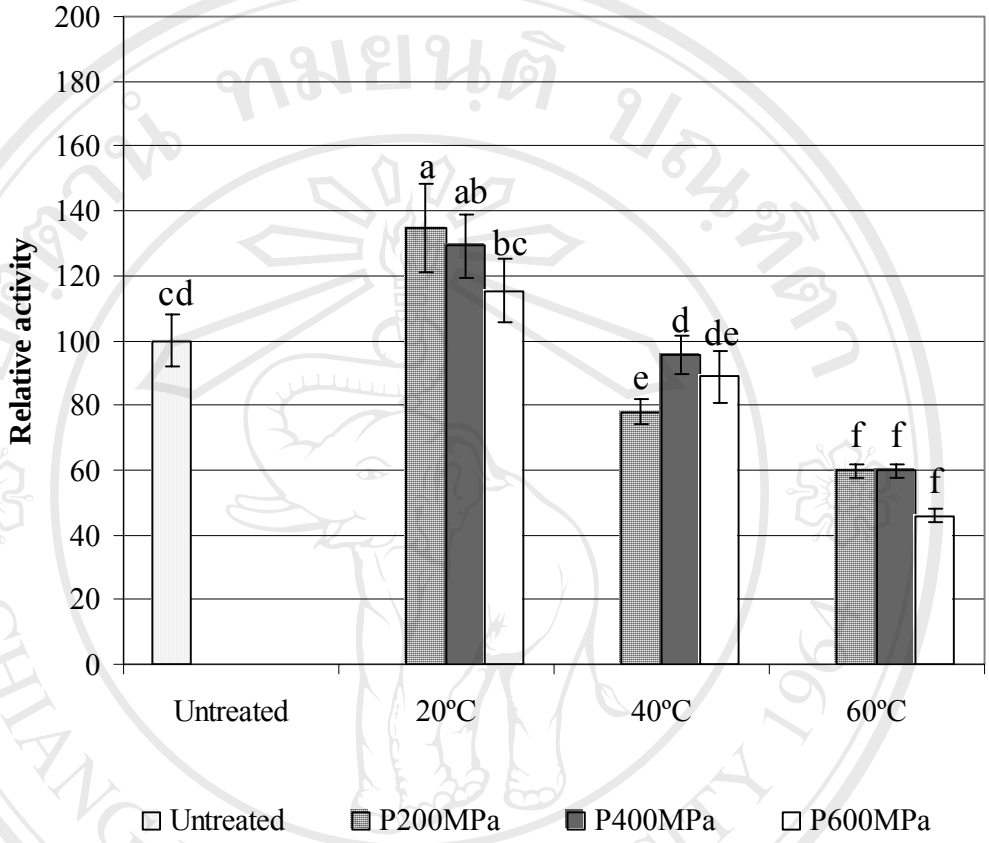


Fig 3.10 Effect of combined UHP/Temperature at 10 min on PPO activity of syrup lychee. All values are the means ± S.D. of duplicate determinations on 3 samples. Bars with different superscript were significantly different ($p<0.05$).

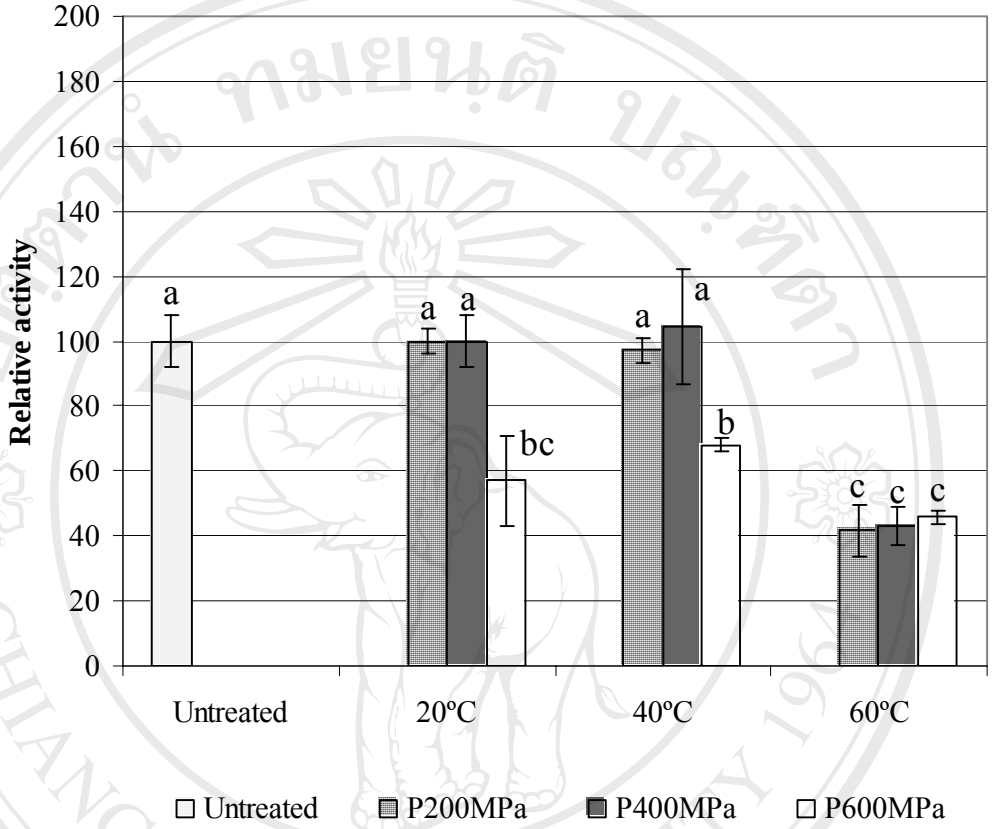


Fig 3.11 Effect of combined UHP/Temperature at 20 min on PPO activity of syrup lychee. All values are the means ± S.D. of duplicate determinations on 3 samples. Bars with different superscript were significantly different ($p<0.05$).