Max Wellington

Course: ENZYMOLOGY

Topic: o-DIPHENOLASE AND ITS ROLE IN THE ENZYMATIC BROWNING IN FOODS

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INTRODUCTION

The myriad of biochemical reactions that occur in living systems are nearly all mediated by a series of proteinaceous, biological catalysts known as enzymes. Enzymes differ from ordinary chemical catalysts in several important respects namely:

(i)   Higher reaction rates (10^6 -10^{12} times greater)

(ii)   Milder reaction conditions – (neutral pH; temperature below 100˚C)

(iii)   Greater reaction specificity and

(iv)   Capacity for regulation

Enzymes were first discovered in 1835 (α-amylase) by Jacob Berzelius, but their stereo-chemical and functional elucidation took some time and it was not until 1965 that the X-ray crystallography of an enzyme, Lysozyme, was available. Since then however, nearly some 2000 enzymes have been purified and characterized to at least some extent (Voet and Voet, 1990).

Enzymes display immense specificity for their respective substrates which is achieved through geometrically and physically complimentary interactions which permits enzymes to be absolutely stereo-specific both in binding substrates and catalyzing reactions.

The regulation of enzymatic activity *in vivo* can occur by allosteric alteration of the active site, by substrate binding affinity, by substrate or product feedback inhibition or by gene regulation (Boyer, 1999).

The wide diversity of enzymes and the rapidly growing number of newly discovered enzymes has led the International Union of Biochemistry to adopt a scheme for the systematic functional classification and nomenclature of enzymes. This system is used where possible in conjunction with the informal or trivial name but assumes fundamental

Table 1. Classification of Enzymes

Classification	Type of Reaction Catalyzed
1. Oxidoreductases	Oxidation-reduction reactions
2. Transferases	Transfer of functional groups
3. Hydrolases	Hydrolysis reactions
4. Lyase	Elimination to form double bonds
5. Isomerase	Isomerization
6. Ligases	Bond formation with ATP hydrolysis

importance when ambiguity must be minimized e.g. Peptidyl-L-amino acid hydrolase has classification number EC 3.4.17.1 [where EC signifies "Enzyme Commision"; 3 indicates the enzymes major class, hydrolase(as in Table 1); 4 denotes the subclass, peptide bonds; 17 designates its subclass, carboxypeptidase and the fourth number 1 is the enzymes arbitrarily assigned serial number in its subclass].

Enzymes are proteins and as such their conformation is determined by their amino acid sequence or primary structure which inevitably determines their secondary and tertiary structure. Enzymic structure plays an important functional role as this will determine the steric conformation at the active site of the enzyme and hence its effective activity.

Enzyme activity is affected by any or all of the following: Enzyme Concentration, Substrate Concentration, inhibitors, pH an temperature and as a result the characterization of an enzyme usually involves an assessment of its optimum performance in relation to these criteria as well as in terms of their kinetics with regards to models put forward by Michaelis Menten . According to Michaelis Menten, enzymatic activity can me defined in terms of the Michaelis constant K_m which is equal to the substrate concentration which gives half the maximum velocity (Datta and Ottoway, 1976). The K_m is usually estimated using the Lineweaver-Burke plot, a reciprocal plot of the substrate concentration on the initial enzyme velocity where the negative reciprocal of the x- axis intercept yields the K_m of the enzyme (figure 1).

Figure 1. **Line-Weaver Burke Reciprocal Plot of Substrate Concentration versus Initial Velocity**
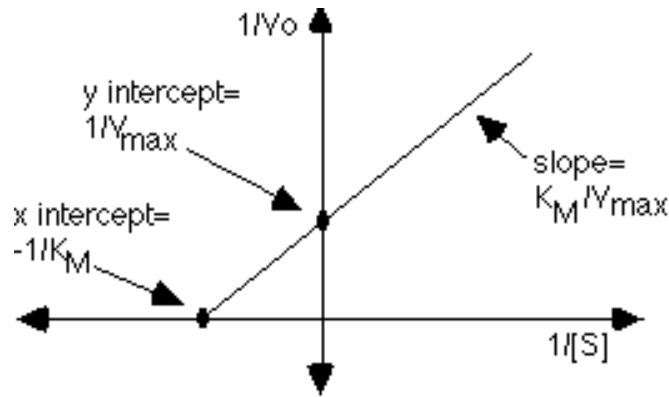
Km could also be estimated from the graph of initial velocity versus Substrate concentration or [S] but the hyperbolic nature of the graph (Figure 2) makes it difficult to estimate the infinite [S] and consequently Vmax (where Km =[S] at ½ Vmax).

Figure 2. The effect of Substrate Concentration on Initial Enzyme Velocity
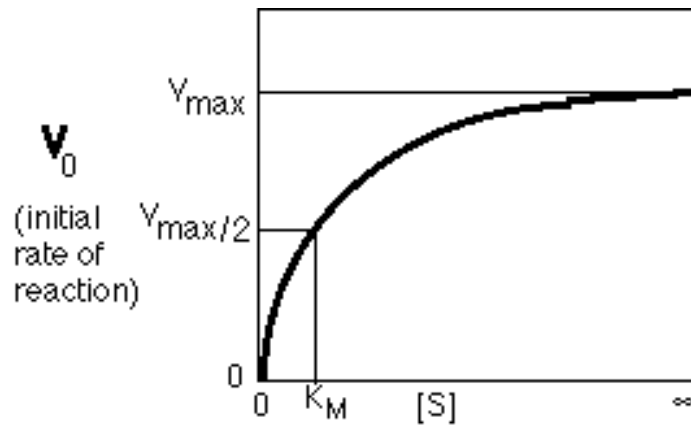
The Km value is also of important in determining whether an inhibitor is competitive (inhibition nullified by increasing substrate concentration) or non-competitive (where there is always inhibition even at high substrate concentrations). For a non-competitive inhibitor the Km value for the enzyme in the presence or absence of the inhibitor remains constant whereas for the competitive Km is less.

Enzymes due to their protein nature can be denatured by extremes of pH and temperature. The optimum pH and temperature of the enzyme is the point where the velocity or activity is maximum at a given substrate and enzyme concentration (Figures 3 and 4)..

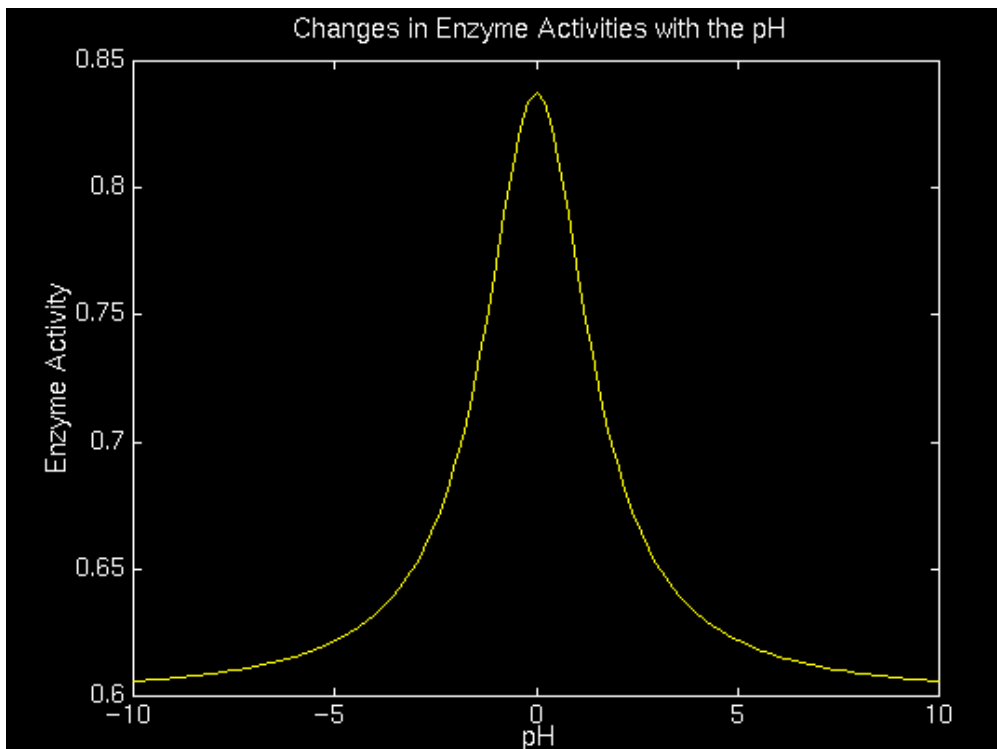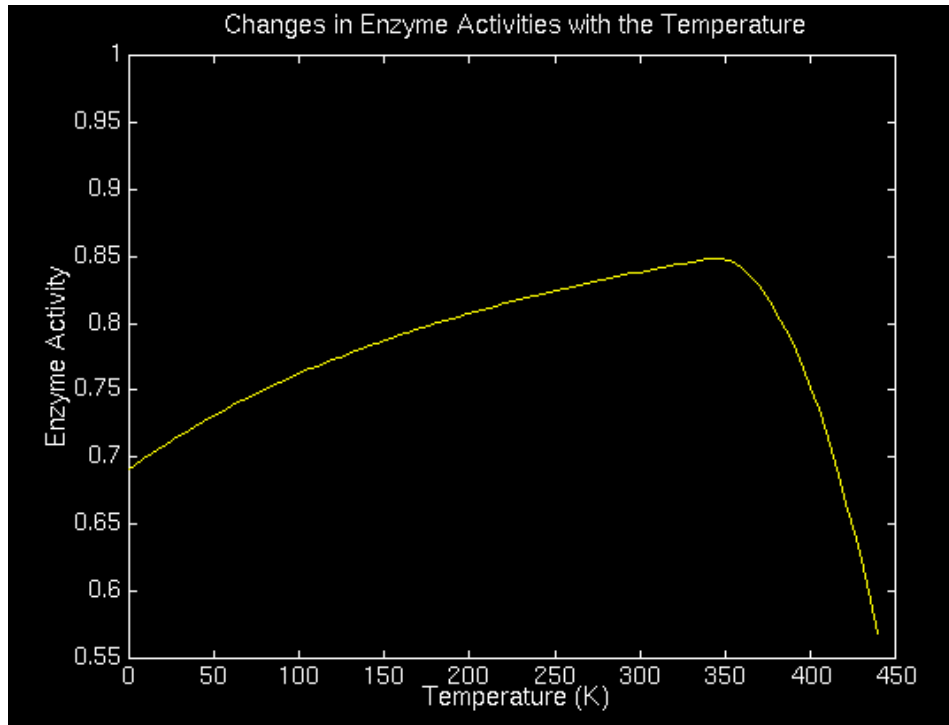Figure 3. Effect of pH Change on Enzyme Activity

Figure 4. Effect of Temperature Change on enzyme Activity

With advances in biochemistry and molecular biology it has now also become the norm for the molecular structure, gene sequence and molecular catalytic mechanism to be elucidated before the enzyme is deemed fully characterized.

This requires that the enzyme can obtained in pure form (Shi et al, 2001; Ikediobi and Obasuyi, 1982) and usually involves the following sequence of steps. First the tissue from which the enzyme is to be isolated from is grounded at very cold temperature in a stable buffer. Triton X-100 or some other detergent is sometimes added to facilitate the fragmenting of cellular organelles and membranes to release any bound enzyme. The resulting milieu is then centrifuged to remove the particulates after which the proteins in the supernatant are precipitated using ammonium sulfate. The desired enzyme would constitute just one in a mixture of many proteins in the precipitate, however, they usually can be separated quite well based on their molecular weights. The ammonium sulfate can be removed by dialysis and the approximate molecular weight of the enzyme can be established using SDS-Polyacrylamide gel electrophoresis. Testing of the bands using substrate to assay enzyme activity should facilitate identification of the desired enzyme. The molecular weight can then be determined by comparison to known standard.   The

use of column chromatography to extract larger volumes of the enzyme from the supernatant based on the expected molecular weight fraction can be done and once a sufficient quantity is obtained the required biochemical and kinetic data as well as X-ray crystallographic and other structural analytical data can be obtained.

This research paper will examine the distribution of o-diphenolase, its structure, *modus operandi* and economic importance.

ACTUALIZATION

o-Diphenolase and its Role in Enzymic Browning of Foods

o-Diphenolase also referred to as polyphenoloxidase or catechol oxidase which catalyze the oxidation of catechols or ortho-diphenols to orthoquinones has been established to be a copper containing protein (Robb

Figure 5. Oxidation of Catechol Catalysed by o-Diphenolase
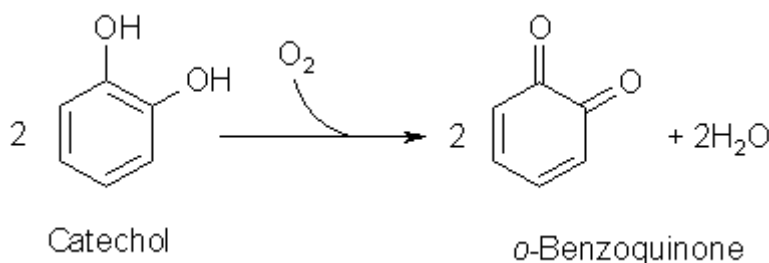
et al, 1965; Kidron et al, 1977, Anosike and Ayaebene, 1982) strongly related to both tyrosinase and haemocyanin, which all have a dinuclear copper complex with histidine ligands at the active site (Siegban, 2004). O-Diphenolases are ubiquitous enzymes capable of mediating or participating in a number of physiological processes. There is a general dubiousness surrounding some of the many functions associated with o-diphenolase, however its role in enzymic browning has been long established. O-Diphenolase has six histidine residues one of which is covalently linked to a cysteine molecule. The distance between the copper atoms has been resolved by X-ray studies to range from about 2.5 to 2.9 Angströms depending on whether it is substrate bound or free. The role which copper assumes involves the binding of oxygen at the active site (Mayer and Harel, 1979; Fennol et al, 2004 and Siegban, 2004). While o-diphenolases from animal issues are relatively specific for tyrosine and dopa (Mason, 1955), the fungal and higher plant enzymes act on a range of mono and diphenols (Mayer and Harel, 1979; Siegban, 2004; Fennol et al, 2004).

Figure 6.   Crystal Structure of a Plant Diphenolase

Extracted from Klabunde et al, 1998

 Marked differences in both the level of o-diphenolase activity and the content of its substrates have been observed between cultivars of fruits (Matthew and Parplia, 1971), vegetables (Ben-Shalom et al, 1978) and yams (Ikediobi and Obasuyi, 1982). o-Diphenolase has been found to be extensively a membrane bound enzyme. Apart from its location in chloroplasts, diphenolases have been reported to be located in mitochondria, peroxisomes and microsomes (Mayer and Harel, 1979). The strength of binding of o-diphenolases to membranes appear to vary depending on the tissues and the stage of development of the plant (Mayer and Harel,1979). In tobacco, washing with buffer suffices to release the enzyme from chloroplast lamellae (Hoffer, 1964). In most cases more drastic conditions are required for the solubilization of membrane bound o-diphenolases such as the use of detergents e.g Triton X-100 (Hrel et.al, 1964; Walker and Hulme, 1966) and sodium dodecyl sulfate (Yamaguchi et al, 1969)

In situ solubilization occurs following exposure to certain stress conditions (Volk et. al., 1977) and also under more natural conditions of ripening of fruits or aging. Thus apple

(Harel et. al, 1966), grape ad banana (Mayer and Harel, 1979) diphenolases become increasingly soluble during fruit ripening

The compartmentalization of phenolic substrates of the enzyme, both in special cells (Mace, 1963) and within cells (Roberts, 1962) have been reported. This results in the separation between the enzyme and the bulk of its phenolic substrates *in situ*.
The rise in diphenolase activity which generally accompanies wounding and stress has been attributed to the *de novo* synthesis of the enzyme (Hyodo and Uritani, 1966). Other researchers have attributed the rise to activation of alredy existing enzyme rather than re-synthesis (Balasbrumani et al, 1971).

Many other roles have been ascribed to diphenolase enzymes due to it's activity response to various stimuli and also by virtue of it's location in the plant cell. Some of these roles are:

(i)   It has been correlated with fruit formation in certain fungi and bacteria (Wilson, 1968; Leonard, 1973).

(ii)   It has been correlated with melanin formation and as such has been deemed to play a role in cellular resistance (Kuo and Alexander, 1967).

(iii) Diphenolases have been suggested to play a role in electron transport (Kabowitz, 1938).

(iv)   Its presence in chloroplast membranous structures have implicated a possible role in photosynthesis (Mayer and Harel, 1979).

(v)   Its involvement in affecting the regulation of plant growth has been implied (Gordon and Paley, 1961; Tomazowski and Thieman, 1966).

(vi)   It has been implicated in rendering seed coats impermeable to water (Marbach and Mayer, 1975).

Enzymic browning is one of the most importantt color reactions that affect foods. It is catalyzed by diphenolase enzymes which facilitate the conversion of phenols to the brown pigment melanin in an oxidation reaction.

Figure 7. Formation of melanin (Browning) from tyrosine. (From Lerner, 1953).
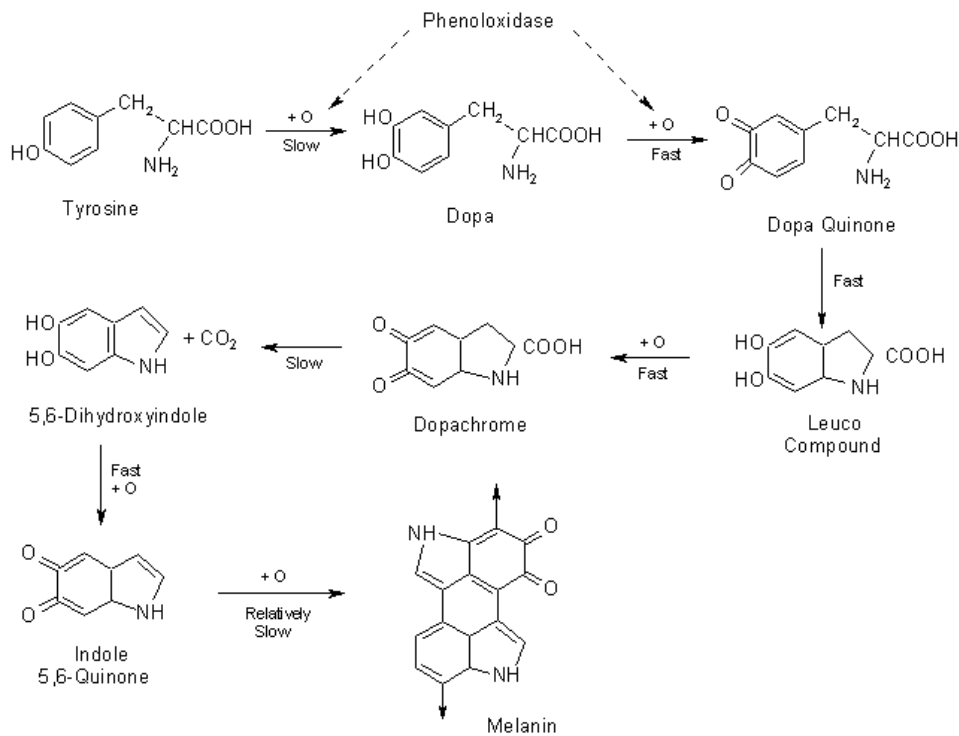
Ikediobi and Obasuyi (1982) purified the enzyme from yam and found the molecular weight to be $107{,}000 \pm 5400$ with temperature and pH optima of 25°C and 6.8 respectively. Activity was illustrated on catechol, chloregenic acid, dopamine and pyrogallol. The enzyme was found to be inhibited strongly by dithiothreitol, diethyldithiocarbamate, potassium cyanide, sodium metabisulfite, 2-mercaptoethanol and L-cysteine. The rate for catechol conversion in sweet potatoes has been measured to be $2.3 \times 10^3 S^{-1}$ (Baruah and Swain, 1959) corresponding to a rate-limiting free energy barrier of around 13 kcal/mol.

Figure 8. Comparison of reactions catalysed by o-Diphenolase and *p*-Diphenolase. (From Walker, 1995).
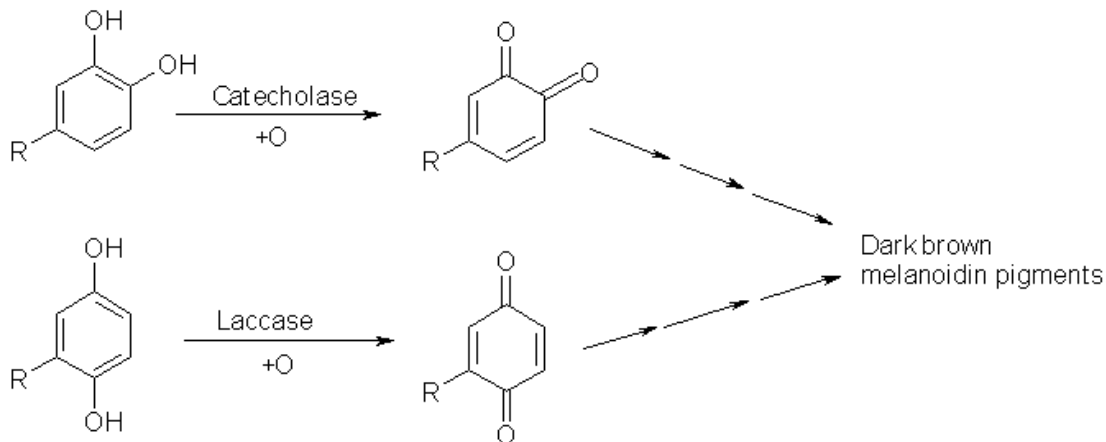
Studies done on ripe banana o-diphenolase show that dopamine is the only significant substrate in the browning reaction of banana. The first reaction results in the orthohydroxylation of phenol and the second, oxidation of the diphenol to orthoquinone. The remaining portion of the reaction sequence involve non-enzymic oxidations and ultimate polymerization of indole 5,6-quinone to brown pigments (Melanins) – as schematized in figures 7 and 8.

Table 3. lists a number of phenols found in fruits and vegetables. Relatively few of these serve as sustrate for diphenolase. The most important are catechin, 3,4 dihydroxyphenylalanine (DOPA) and tyrosine and the substrate specificity varies depending on the source of the enzyme.

Table 2. Phenolic substrates of Diphenolase in fruits, vegetables, and seafoods.

Source	Phenolic substrates
Apple	chlorogenic acid (flesh), catechol, catechin (peel), caffeic acid, 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxy benzoic acid, *p*-cresol, 4-methyl catechol, leucocyanidin, *p*-coumaric acid, flavonol glycosides

Apricot	isochlorogenic acid, caffeic acid, 4-methyl catechol, chlorogenic acid, catechin, epicatechin, pyrogallol, catechol, flavonols, *p*-coumaric acid derivatives
Avocado	4-methyl catechol, dopamine, pyrogallol, catechol, chlorogenic acid, caffeic acid, DOPA
Banana	3,4-dihydroxyphenylethylamine (Dopamine), leucodelphinidin, leucocyanidin
Cacao	catechins, leucoanthocyanidins, anthocyanins, complex tannins
Coffee beans	chlorogenic acid, caffeic acid
Eggplant	chlorogenic acid, caffeic acid, coumaric acid, cinnamic acid derivatives
Grape	catechin, chlorogenic acid, catechol, caffeic acid, DOPA, tannins, flavonols, protocatechuic acid, resorcinol, hydroquinone, phenol
Lettuce	tyrosine, caffeic acid, chlorogenic acid derivatives
Lobster	tyrosine
Mango	dopamine-HCl, 4-methyl catechol, caffeic acid, catechol, catechin, chlorogenic acid, tyrosine, DOPA, *p*-cresol
Mushroom	tyrosine, catechol, DOPA, dopamine, adrenaline, noradrenaline
Peach	chlorogenic acid, pyrogallol, 4-methyl catechol, catechol, caffeic acid, gallic acid, catechin, Dopamine
Pear	chlorogenic acid, catechol, catechin, caffeic acid, DOPA, 3,4-dihydroxy benzoic acid, *p*-cresol
Plum	chlorogenic acid, catechin, caffeic acid, catechol, DOPA
Potato	chlorogenic acid, caffeic acid, catechol, DOPA, *p*-cresol, *p*-hydroxyphenyl propionic acid, *p*-hydroxyphenyl pyruvic acid, *m*-cresol
Shrimp	tyrosine
Sweet potato	chlorogenic acid, caffeic acid, caffeylamide

Tea	flavanols, catechins, tannins, cinnamic acid derivatives

Extracted from Marshall et al, 2002 (Structures of the listed Phenolics can be found in the Appendix)

Browning is desirable in some foods e.g tea and coffee and in most plants it has been associated with pest and bacterial resistance and wound healing .Some have even ascribed anticancer and antioxidant properties to the melanins produced during the browning reaction (Marshall et al, 2000).

Projected increases in the fruit and vegetable market for the future will however not occur if enzymatic browning is not understood and controlled (Marshall et al, 2000). It is estimated that over 50 percent losses in fruit occur as a result of enzymatic browning (Whitaker and Lee, 1995) and this has increased interest in understanding and controlling diphenolase enzymes in foods. Browning or melanosis has also been observed during the storage of some high value crustaceans such as shrimp and lobster connoting spoilage (Otwell et al, 1992) and losses and browning has been shown to adversely affect flavor and nutritional value of foods (Marshall et al, 2002)..

Figure 9. Examples of enzymatic browning in banana

Extracted from Marshall et al, 2000

Figure 10. Examples of enzymatic browning in potato
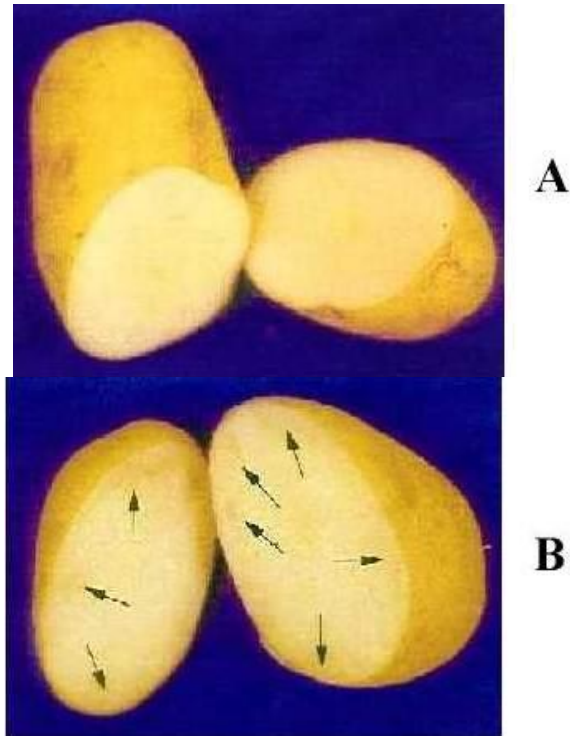
Extracted from Marshall et al, 2000

CONTROL OF BROWNING

Browning does not occur in intact plant cells due to vacuolar separation of the phenolic

substrates from the enzyme which is present in the cytoplasm. Cutting or damage to the

tissue brings the enzyme and substrate together resulting in the observed brown

pigmentation which impacts both the organoleptic and biochemical characteristics of

fruits and vegetables (Marshall et al, 2000).

The role of browning has been shown to be mediated by several factors, namely:

    (i)   Tissue Diphenolase level [E]

    (ii)   Tissue Phenolic content [S]

    (iii)   pH

(iv)   Temperature and

(v)   Oxygen Availability

The control of browning consequently can be effected through the manipulation of these factors (Marshall et al, 2002). Some resulting methods of control include:

(i)   The elimination of oxygen by vacuum packing, immersion in liquid, treatment with reducing agents e.g. ascorbic acid and antioxidants e.g butylated hydroanisole (BHA).

(ii)   Inactivation of the enzyme by: (a) Chelating the copper prosthetic group of the active site using EDTA, Sorbic Acid or (b) denaturing with steam treatment, blanching, solar drying or freezing or (c) Inhibition with cysteine, honey, heylresocinol etc.

(iii)   Removal of the enzyme e.g. from juices by precipitation and ultrafiltration and

(iv)   Reducing Enzyme activity by acidifying or lowering the pH e.g Citric Acid (Most diphenolases exhibit optimal activity at pH 6.8).

Other non-conventional methods of reducing enzymic browning of foods include the use of antienzymes or enzymes which destroy some cofactor necessary for the reaction e.g some cleavage oxygenases (Kelly and Prinkle, 1969); Catechol Transferase (Prinkle and Nelson, 1963) and Protease (Labuza et al, 1992).

With the advent of recombinant DNA technology, numerous amino acid sequences of diphenolase isozymes have been deciphered using cDNA sequencing techniques (Marshall et al, 2000).

The inactivation of genes coding for these enzymes using anti-sense RNA specific for diphenolase should lower the browning reaction as it effectively reduces diphenolase gene expression and hence the concentration of the enzyme in situ. Anti-sense RNAs were recently observed to selectively block the gene expression of other plant enzymes such as polygalacturonase and peroxidase in tomatoes (Marshall et al, 2002).

Bachem et al (1994) determined that the expression of diphenolase in potatoes was decreased through the use of anti-sense cDNA.

It is hoped that though the use of this technology that browning resistant varieties maybe developed to prevent or significantly curtail the production of Diphenolase.

CONCLUSION

In closing Diphenolases have been shown to be ubiquitous enzymes which can have significant impact on the shelf life and indeed the quality of fruits, vegetables an certain shell fish due to their facilitation of enzymic browning. Enzymic browning was shown to be responsible for about 50% of spoilage of fruits and vegetables and therefore its control can be of significant economic impact to the global food supply. Several method for its control during the processing and handling of foods were explored bearing in mind that treatments administered should not affect product flavor, texture and color.

The use of enzyme inhibitors, reducing agents, anti-oxidizers, heating, refrigerating, anti-enzyme and anti-sense RNA technology were all examined.

It is hoped that in the very near future the use of anti-sense RNA technology will be able to see the production of food varieties with a much reduced propensities for enzymic browning.

RECCOMENDATIONS:

1.   Study to quantify the economic value of global food losses due to enzymic browning.
2.   Elucidation of the genes for diphenolase enzymes for all major food crops impacted by enzymic browning to facilitate the use of anti-sense RNA technology to reduce browning.
3.   Further explore the potential of melanin in fighting cancer.
4.   Intensify research in food technology to find novel yet economical ways to reduce enzymic browning in foods accompanied by an education drive to enlighten the masses and other stakeholders.

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APPENDIX 1

Structures of common phenolic compounds.
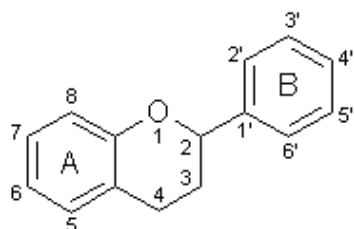
Flavonoid structure
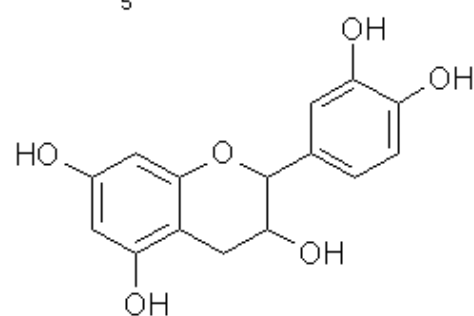
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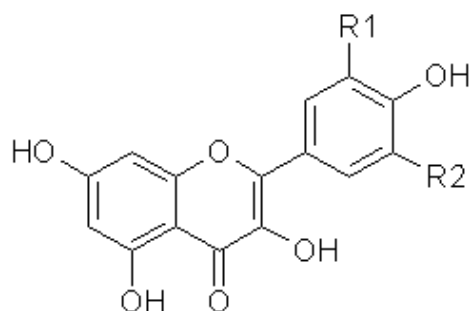
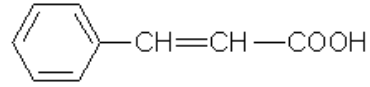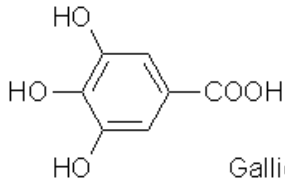Quercetin (R1=OH, R2=H)

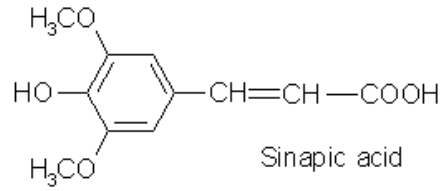Myricetin (R1=R2=OH)

Kaempferol (R1=R2=H)
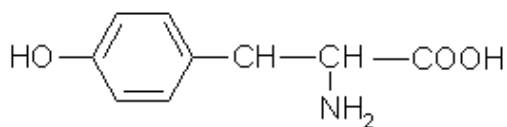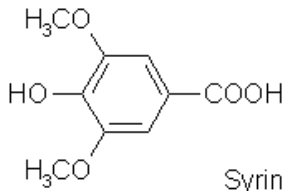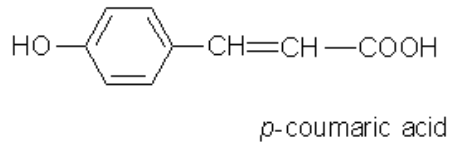
Tyrosine
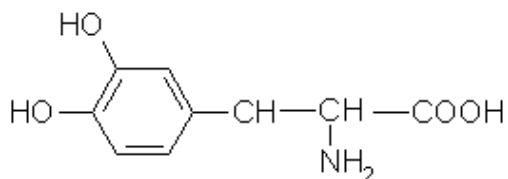
3,4-dihydroxyphenylalanine (DOPA)
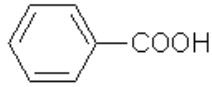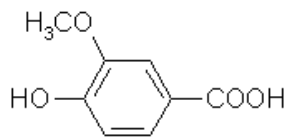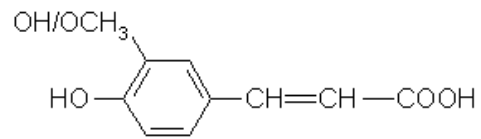
Benzoic acid

Cinnamic acid

Gallic acid

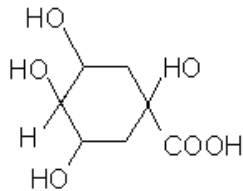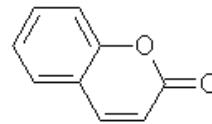Sinapic acid
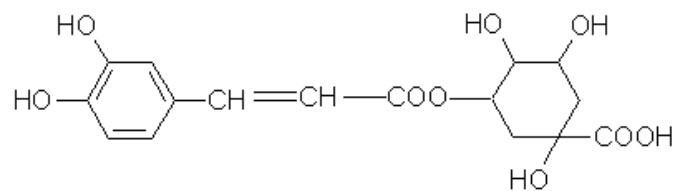
Syringic acid

p-coumaric acid

Vannilic acid

Caffeic acid/Ferulic acid

Quinic acid

Coumarin

Chlorogenic acid