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PROJECT NO. I-1074-86

Search for Means to Prevent Enzymatic Browning in Fruits and Vegetables

V. Kahn, J.R. Whitaker

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II. Abstract

The research completed during the three years fall within four categories, all of which modulate polyphenol oxidase activity. These areas, all involving specific compounds, are: 1) direct inhibition of polyphenol oxidase; 2) reducing compounds; 3) co-oxidants; and 4) new substrates for polyphenol oxidase.

Direct inhibition of polyphenol oxidase activity can be subdivided into three types. The first is a direct effect on the enzyme (no substrate present) by sulfhydryl compounds, sodium bisulfite, ascorbic acid and by substrate via K_{cat} inhibition. Sulfhydryl compounds and sodium bisulfite inhibit polyphenol oxidase by reducing Cu^{2+} to Cu^{+} , which is more readily lost from the enzyme. This can be reversed by adding Cu^{2+} back. Ascorbic acid, particularly in the presence of $10^{-6} M$ Cu^{2+} , oxidizes the histidine ligands of copper at the active site. The second group of inhibitors probably compete with substrate for binding at the active site. These include benzoic acid, p-hydroxybenzoic acid, p-hydroxymethylbenzoate, p-aminobenzoic acid, vanillic acid, salicylic acid, dihydroxybenzoic acid (2,3-; 2,5-; 3,4-; and 3,5-) gallic acid, methyl gallate and n-propyl gallate and 4-hexylresorcinol. The third type is cyclodextrin which competes with the enzyme for the substrate.

Reducing compounds studied included sodium bisulfite, thiol compounds and ascorbic acid (see above), H_2O_2 , phenylhydrazine, ferrocyanide and gallic acid. While sodium bisulfite and thiol compounds appear to inhibit by reducing Cu^{2+} to Cu^{+} , no such relationship was found for H_2O_2 , phenylhydrazine, ferrocyanide and gallic acid.

4-tert-Butyl catechol, catechol, 4-methyl catechol, protocatechuic acid, benzoic acid, p-hydroxybenzoic acid, p-hydroxymethyl benzoate and 2,3-dihydroxybenzoic acid at low concentrations were co-oxidants with DL-dihydroxyphenylalanine (DOPA), thereby stimulating rate of DOPA oxidation.

Some new substrates identified for polyphenol oxidase include: Tiron, 2,4,5-trihydroxybutyrophenone (an antioxidant), 2,3,4-trihydroxyacetophenone, 2,3- and 2,5-dihydroxybenzoic acids.

IV. Objectives of the Original Research Project

The objectives of this project were to investigate several potentially important methods for the control and prevention of enzymatic browning in plant tissues important for food use in order to minimize the nutritional, economic and sensory losses associated with undesirable browning reactions. These objectives were to be accomplished by investigating the following questions:

1. Is there a direct correlation between the ability of reductants to reduce Cu^{2+} in a chemical system and Cu^{2+} at the active site of tyrosinase and their ability to inactivate the enzyme?
2. Do various copper chelators, including antioxidants, inactivate tyrosinase?
3. What is the mechanism of ascorbic acid and Cu^{2+} in the direct inactivation of tyrosinase?
4. How does sulfite inhibit tyrosinase?
5. Can compounds that show efficient K_{cat} inactivation of tyrosinase be found?
6. Is there an inverse correlation between browning potential and carotenoids content of vegetative tissue? If so, what is the explanation for such a correlation with emphasis to the possibility that carotenoids inhibit browning?

V. Research Report

A. Introduction

The enzyme polyphenol oxidase (E.C.1.14.18.1; diphenol: O_2 oxidoreductase; also called tyrosinase, phenolase and catecholase) (we shall refer to the enzyme as polyphenoloxidase or as tyrosinase) is found in most plant and animal tissues, as

well as in several fungi. In plants, the enzyme is thought to function by forming a "scab" over wounds caused by insect bites and other physical damage. On wounding, polyphenol oxidase interact with certain monohydroxyphenols and o-dihydroxyphenols, together with O_2 , to produce melanin. The insoluble melanin seals off the wound, preventing loss of fluids and microbial infestation.

During the harvesting of fruits and vegetables and during storage there is damage to the plant tissue via bruising and abrasion. Intentional disruption of cellular organization by peeling, slicing, pressing and homogenization provide the ideal conditions for polyphenol oxidase action. Methods for preventing the enzymatic browning have been sought for more than one hundred years. The difficulty of preventing browning, in large part, is because we do not yet understand the complete mechanism of action of the enzyme.

Polyphenol oxidase is known to catalyze two different biochemical reactions:

- 1) monohydroxyphenol + O_2 + AH_2 $\longrightarrow$ o-dihydroxyphenol + H_2O + A
- 2) o-dihydroxyphenol + $1/2 O_2$ $\longrightarrow$ o-quinone + H_2O where AH_2 represents a reductant.

The o-quinone formed is very reactive and polymerizes with itself or with other phenols to form melanins, the insoluble brown pigments responsible for the undesirable effect of the enzyme. The colored pigments are usually brown but may be yellow, red, blue or green when condensation with other phenols occurs. The browning is undesirable because of the off color formed. But the browning reaction also causes change in flavor and decrease in nutritional quality via condensation of the o-quinone with the -amino group of lysyl residues of proteins causing them to become insoluble, as well as modified.

It may be possible to remove or reduce the levels of polyphenol oxidase in plants by recombinant DNA techniques sometime in the future. Until that time, we must depend upon normal breeding selection for minimizing the concentration of

this enzyme and via better understanding of its mechanism of action in order to prevent its action. It is in this latter area that we feel most qualified to contribute toward solving one of the major problems of food production, storage and transportation.

B. Materials and Methodology

Because of the numerous projects undertaken during this period, the specific publications, in press items and in preparation items should be consulted for details of the experiments. Some materials and methodologies were general throughout.

1. Materials

Mushroom tyrosinase (polyphenol oxidase; grade III) was from Sigma. All other chemicals were reagent grade.

2. Assays

Most enzymatic assays were measured spectrophotometrically (in a Varian DMS 90 spectrophotometer, a Beckman 35 spectrophotometer or a Shimadzu 160 spectrophotometer with recorders) as a function of time at the wavelength maximum of the intermediates and/or compounds formed. Polarographic determination were also used. pH and temperature were maintained constant as required.

C. Results and Discussion

1. Research results from the Volcani Center

a. Hydrogen peroxide and the lag period of tyrosine hydroxylation by mushroom tyrosinase

Varda Kahn

Oxy-tyrosinase, the active species which interacts with monohydroxyphenols and with o-dihydroxyphenols, is thought to be formed by a direct interaction between tyrosinase and H_2O_2 ,

thereby implying that hydroxylation of monohydroxyphenols by the enzyme would occur anaerobically. However, using the sensitive technique of $^3\text{H}\text{OH}$ release from L-[3,5- ^3H]-tyrosine to measure tyrosine hydroxylation, we could not demonstrate such an anaerobic hydroxylation.

Moreover, catalase, which is known to abolish the oxy-tyrosinase species, did not inhibit the hydroxylation of tyrosine by mushroom tyrosinase.

The above observations, and the data showing that the concentration of H_2O_2 needed to shorten the lag period of tyrosine hydroxylation was in huge excess relative to the concentration of mushroom tyrosinase used, led us to suggest that H_2O_2 shortens the lag period by acting as a reductant (AH_2), analogous to the role played by NH_2OH in the hydroxylation reaction.

b. Ability of various chemicals to reduce copper and to inactive mushroom tyrosinase

Andrawis Andrawis and Varda Kahn

Treatment of mushroom tyrosinase with reducing agents such as hydrogen peroxide, ascorbic acid, phenylhydrazine, gallic acid, ferrocyanide and NH_2OH resulted in inactivation of the enzyme. Under the conditions tested, 50% inactivation of the enzyme was obtained with 4 μM , H_2O_2 , 20 μM ascorbic acid, 40 μM phenylhydrazine, 6 mM gallic acid, 12 mM ferrocyanide and 22 mM NH_2OH .

The ability of the reducing agents to reduce Cu^{2+} in a chemical model system was determined and it was found that gallic acid, phenylhydrazine, ascorbic acid and NH_2OH are relatively good reductants of Cu^{2+} while H_2O_2 and ferrocyanide are relatively poor ones.

The copper content of mushroom tyrosinase before and after inactivation by each of the reducing agents was determined. The copper content of the enzyme inactivated by H_2O_2 , NH_2OH ,

phenylhydrazine, ferrocyanide, gallic acid and ascorbic acid was 100%, 90%, 90%, 85%, 85% and 76% compared to that of the control (enzyme not treated).

It was concluded that the degree of inactivation of mushroom tyrosinase by the reducing agents was not correlated with the decrease in the copper content of the enzyme or with their ability to reduce Cu^{2+} in a chemical model system.

c. Tiron (4,5-dihydroxy-1,3-benzene disulphonic acid) as a substrate for horseradish peroxidase

Varda Kahn

Peroxidase catalyzes reactions in which a variety of hydrogen donors are oxidized in the presence of hydrogen peroxide. Among the known hydrogen donors for this reaction are: guaiacol, benzidine, o-dianisidine, aromatic amines, monohydroxyphenols such as tyrosine and dihydroxyphenols such as caffeic acid, ferulic acid, DOPA and catechol. Peroxidase, in the absence of hydrogen peroxide but in the presence of oxygen, can also catalyze oxidative and hydroxylation reactions.

Tiron is an o-dihydroxyphenol known to be a scavenger of $\text{O}_2^{\cdot -}$, as well as $\text{OH}^{\cdot}$. We have shown recently that Tiron is also a substrate for mushroom tyrosinase. Since o-dihydroxyphenols can serve as substrates for tyrosinase as well as for peroxidase (in the presence of hydrogen peroxide), it was of interest to determine if Tiron could also serve as a substrate for peroxidase.

The data from these experiments provide evidence that Tiron, in the presence of hydrogen peroxide, can serve as a hydrogen donor for horseradish peroxidase.

d. Non-enzymatic of Tiron to a yellow product(s)

Varda Kahn

Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid) was oxidized to a rather stable yellow product(s) at alkaline pH by Ag_2O and by NaIO_4 , when exposed to an $\text{O}_2^{\cdot -}$ -generating system (xanthine-xanthine oxidase system), as well as to an $\text{OH}^{\cdot}$ -generating system (Fe^{2+} -EDTA- H_2O_2 system). In all cases, the yellow product(s) was characterized by a peak at 435 nm.

An intermediate compound, probably a Tiron-o-quinone, with a peak at 365-370 nm, was detected during the initial stage of Tiron's oxidation by NaIO_4 to the yellow product(s) (λ_{max} 435 nm). The conversion of the intermediate compound (λ_{max} 365-370 nm) to the yellow product(s) (λ_{max} 435 nm) was accelerated by H_2O_2 .

It is likely that Tiron-o-quinone is formed when Tiron is acted upon by alkaline pH, by Ag_2O , NaIO_4 , $\text{OH}^{\cdot}$, $\text{O}_2^{\cdot -}$ and that the Tiron-o-quinone polymerizes to low m.w. polymers of Tiron-o-quinone which are the final yellow product(s) (λ_{max} 435 nm). It is also likely that the same products are formed when tyrosinase converts Tiron to oxidized products.

e. 4-tert-Butyl catechol as a substrate for mushroom tyrosinase
Varda Kahn

4-tert-Butyl catechol (t-BC) is an antioxidant and is considered a good scavenger of $\text{OH}^{\cdot}$ radicals (1). t-BC can serve as a substrate for tyrosinase (2,3,4) but relatively little is known about its behavior as a substrate.

In the course of our studies on the effect of various phenols on melanin formation from DL-DOPA we noted that t-BC stimulates the rate of DL-DOPA oxidation by the enzyme. In an attempt to understand the above, and since t-BC is a primary antioxidant approved for use in food, we decided to study in some detail its ability to serve as a substrate for mushroom tyrosinase.

We found that t-BC serves as an efficient substrate for mushroom tyrosinase with a K_m value of 0.5 mM. The product(s)

formed after relatively short incubation periods is yellow, provably t-BC-quinone, characterized by a peak at 400 nm. The molar extinction coefficient (ϵ) of the yellow product(s) at 400 nm was estimated to be $1750 \text{ M}^{-1} \text{ cm}^{-1}$. After relatively long incubation periods, the yellow product(s) is converted to high m.w. beige-orange polymerized t-BC-quinone.

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f. Effect of 4-tert-butyl catechol on the rate of DL-DOPA oxidation by mushroom tyrosinase

Varda Kahn

When DL-DOPA is oxidized by mushroom tyrosinase, dopaquinone is the initial product formed (1,2,3). Dopaquinone is converted very rapidly to dopachrome ($\lambda_{\text{max}} = 475 \text{ nm}$) and melanins, high m.w. red black polymers, are the final product(s) formed (1,2,3).

While studying the effect of various dihydroxy- and trihydroxyphenols on the conversion DL-DOPA to melanin by mushroom tyrosinase, we noted that t-BC, in a wide range of concentrations, stimulated the rate of DL-DOPA oxidation to dopachrome ($\lambda = 475 \text{ nm}$). This was surprising, since we have shown that t-BC is a rather efficient substrate for mushroom tyrosinase with a K_m value of 0.5 mM, similar to the value of mushroom tyrosinase for DL-DOPA (0.48 mM).

Examination of the above phenomenon revealed that t-BC, at concentrations ranging from 0.033 to 16.6 mM, has a pronounced synergistic effect on rate of DL-DOPA oxidation to dopachrome ($\lambda = 475 \text{ nm}$) by mushroom tyrosinase. This phenomenon was found to

be due to the ability of t-BC-quinone (formed by the oxidation of t-BC by mushroom tyrosinase) to oxidize DL-DOPA non-enzymatically to dopaquinone, with the latter undergoing rapid conversion to dopachrome.

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g. 2,3,4- Trihydroxyacetophenone (2,3,4-THAP) as a substrate for mushroom tyrosinase

Varda Kahn

Trihydroxyphenones such as 2,4,5-trihydroxybutyrophenone (2,4,5-THBP) and 2,3,4-trihydroxyacetophenone (2,3,4-THAP) are antioxidants that are used to inhibit oxidation processes in fats, paraffin waxes, mineral oil, carotene extracts, ready-mixes of poultry feeds and cattle fodder (1). In the course of our studies on the oxidation of 2,4,5-THBP by mushroom tyrosinase we tested the ability of related antioxidants to serve as a substrate for enzyme.

We found that 2,3,4-THAP can serve as a substrate for mushroom tyrosinase with a K_m value of 1.2 mM. The product(s) formed is yellow, characterized by a peak at 420-430 nm. A lag period in 2,3,4-THAP oxidation to the yellow product(s) in the presence of ascorbate or phenylhydrazine indicated that the initial product(s) is a quinone of 2,3,4-THAP. An oxime, characterized by a broad peak 510-650 nm, is the likely product formed between 2,3,4-THAP-quinone and NH_2OH .

The molar extinction coefficient (ϵ_M) of the 2,3,4-THAP-quinone was estimated to be $1.7 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ at 430 nm.

During relatively long incubation periods, the peak of the yellow product(s) shifted from 420-430 nm to 430-440 nm; the solution remained yellow and transparent for at least a week and no precipitate was formed. These results indicate that the final yellow product(s) is a low m.w. polymer of THAP-quinone (dimer, tetramer, etc.).

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- h. Effect of dihydroxyphenols (catechol, 4-methyl catechol and protocatechuic acid) and of trihydroxyphenols (gallic acid, 4-methyl gallate and n-propyl gallate) on the rate of DL-DOPA oxidation by mushroom tyrosinase and on melanin formation
Varda Kahn

Melanins (high m.w. pigments) are the final product(s) formed when DOPA is oxidized by tyrosinase (1,2,3). Dopachrome is the initial product formed when DOPA is oxidized by tyrosinase. Dopachrome cyclizes non-enzymatically, rapidly forming leucodopachrome, which is immediately converted to dopachrome. Dopachrome is slowly decarboxylated to 5,6-dihydroxyindole, and the latter is then converted to indole-5,6-quinone (1,2,3). Indole-5,6-quinone polymerizes to melanins, via melanochrome.

Of the various above intermediates, dopachrome is a red product (peak at 475 nm), indole 5,6-quinone is yellow (peak at 300 nm) and melanochrome is purple (peak at 540 nm) while melanin is a dark-red black product(s) with an undefined spectrum (2,3).

In the course of our studies on the effect of various polyhydroxy phenols on melanin formation from DL-DOPA by mushroom

tyrosinase we noted that some prevent melanin formation while others do not (see further below).

In view of the above, we decided to study in some detail the effect of each polyhydroxyphenol on the rate of DL-DOPA oxidation and on melanin formation.

Catechol, 4-methyl catechol and protocatechuic acid are o-dihydroxyphenols that are effective substrates for mushroom tyrosinase - these dihydroxyphenols did not prevent melanin formation from DL-DOPA by the enzyme. On the other hand, gallic acid, 4-methyl gallate and n-propyl gallate are trihydroxyphenols that are less effective as substrates for mushroom tyrosinase, yet these trihydroxyphenols prevented melanin formation from DL-DOPA by the enzyme.

Gallic acid, 4-methyl gallate and n-propyl gallate each inhibited the rate of DL-DOPA oxidation by mushroom tyrosinase so one would theoretically expect that their addition to a reaction mixture containing DL-DOPA, sodium phosphate buffer (pH 6.5) and mushroom tyrosinase would slow down or prevent melanin formation. Protocatechuic acid, in all concentrations tested, inhibited the rate of DL-DOPA oxidation by the enzyme and so did relatively high concentrations of 4-methyl catechol, yet neither of them, even at high concentrations, prevented melanin formation from DL-DOPA by the enzyme.

Wong et al. (4) showed that phloroglucinol, orcinol, resorcinol and d-catechin each complexes (non-enzymatically) with 4-methyl-o-quinone (formed enzymatically or non-enzymatically) to yield isomeric dimers. It is likely that gallic acid, 4-methyl gallate and n-propyl gallate likewise interact (non-enzymatically) with dopaquinone (formed by the action of mushroom tyrosinase or of NaIO_4 on DL-DOPA) to yield various isomeric dimers, trimers and related condensation products of relatively low m.w., thus preventing the eventual conversion of dopaquinone to melanin.

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i. 2,4,5-Trihydroxybutyrophenone (2,4,5-THBP) as a substrate for mushroom tyrosinase

Varda Kahn, David Osuga and John, R. Whitaker

2,4,5-THBP (2,4,5-trihydroxybutyrophenone) is a substrate for mushroom tyrosinase with a K_m value of 1.66 mM. The final product(s) formed is red (λ_{max} 480-490 nm) and it is rather stable with time. An intermediate red product(s), having a maximum at $\lambda=505-515$ nm, was detected and the time course of its conversion to the final red product(s) was studied. The data suggest that the intermediate red product(s) is 2,4,5-THBP-quinone which is converted to colorless polymerized 2,4,5-THBP-OH which is then oxidized to a red polymerized 2,4,5-THBP-quinone(s), which is the final red product(s).

Preliminary data indicate that a dimer is formed from the initial 2,4,5-THBP-quinone, followed by possibly a tetramer.

j. Effect of benzoic acid and its derivatives on the rate of DL-DOPA oxidation by mushroom tyrosinase

Varda Kahn

Preliminary experiments showed that benzoic acid (BA), p-hydroxybenzoic acid (o-OHBA), p-hydroxy methylbenzoate (p-OHBME), p-aminobenzoic acid (p-NH₂BA), vanillic acid and salicylic acid were not substrates for mushroom tyrosinase.

At relatively low concentrations, BA, p-OHBA and p-OHBME individually stimulated DL-DOPA oxidation with stimulation increasing in the order listed. We do not have an explanation for stimulation of DL-DOPA oxidation by relatively low concentrations of BA, p-OHBA and p-OHBME.

Higher concentration of p-OHBME, BA and vanillic acid inhibited the rate of DL-DOPA oxidation by mushroom tyrosinase.

p-NH₂BA and salicylic acid only inhibited the reaction; at relatively low concentrations they did not stimulate the rate of DL-DOPA oxidation by mushroom tyrosinase. p-NH₂BA, BA and p-OHBE, in decreasing order, are very effective inhibitors, salicylic acid moderately so and vanillic acid and p-OHBME, in decreasing order, are very poor inhibitors of DL-DOPA oxidation by mushroom tyrosinase.

The effect of benzoic acid and its derivatives on melanin formation from DL-DOPA initiated by mushroom tyrosinase was examined. BA, p-OHBA, p-OHBME, p-NH₂BA, vanillic acid and salicylic acid (0.33-33.3 mM) did not prevent melanin formation after 24 h incubation (in a reaction mixture that included 6.7 mM DL-DOPA, 47 mM sodium phosphate buffer (pH 6.5) and 100 ug mushroom tyrosinase (in a total volume of 4 ml). Apparently, although BA and its derivatives inhibited the rate of DL-DOPA oxidation by mushroom tyrosinase, dopaquinone was slowly formed and converted non-enzymatically to melanin within the 24 h incubation period.

k. 2,3-Dihydroxybenzoic acid (2,3-DBA) as a substrate for mushroom tyrosinase

Fernando Schved and Varda Kahn

When 2,3-dihydroxybenzoic acid (2,3-DBA) is acted upon by mushroom tyrosinase, a yellow intermediate, 2,3-DBA-o-quinone, characterized by a peak at 415 nm, is the first product detected. 2,3-DBA-o-quinone is an intermediate which gives rise to a "blue

polymer" (λ = 230 nm, 410 nm, 620 nm) and to "soluble oxidation product(s)" (λ_{max} = 275-280 nm, 350-360 nm).

Kinetic data (determined spectrophotometrically and polarographically) obtained when different concentrations of 2,3-DBA were oxidized by a fixed amount of mushroom tyrosinase deviated from the classic Michaelis-Menten kinetic model.

Reduction of the "blue polymer" with ascorbate resulted in loss of the blue chromophore at 620 nm and concomitant appearance of a "yellowish reduced polymer". The "yellowish reduced polymer" could be reoxidized with either mushroom tyrosinase or with NaIO_4 to the "blue polymer", indicating that the latter has carbonylic quinonoid groups in ortho position to each other.

1. The effect of different isomers of dihydroxybenzoic acids (DBA) on the rate of DL-DOPA oxidation by mushroom tyrosinase
Fernando Schved and Varda Kahn

The effect of dihydroxybenzoic acid isomers (2,3-DBA, 3,4-DBA, 2,5-DBA, 2,4-DBA, 3,5-DBA) on the rate of DL-DOPA oxidation by mushroom tyrosinase to dopachrome (via dopaquinone) was studied. 2,3-DBA and 2,5-DBA, each, at relatively low concentrations, stimulated rate of dopachrome formation but at relatively high concentrations they inhibited the reaction. 2,4-DBA, 3,4-DBA and 3,5-DBA, at all concentrations tested, inhibited the rate of DL-DOPA oxidation by mushroom tyrosinase to dopachrome. 2,3-DBA, 2,5-DBA, 3,4-DBA and 2,4-DBA each served as a substrate for tyrosinase while 3,5-DBA did not.

The stimulation exerted by relatively low concentrations of 2,3-DBA on DL-DOPA oxidation to dopachrome was due to the ability of 2,3-DBA-o-quinone (formed by the interaction of 2,3-DBA with tyrosinase in the reaction mixture) to oxidize DL-DOPA non-enzymatically to dopaquinone with the latter being immediately converted non-enzymatically to dopachrome.

The inhibition exerted by relatively high concentrations of 2,3-DBA on DL-DOPA oxidation by mushroom tyrosinase was due to a

competition between 2,3-DBA and DL-DOPA for the active site of the enzyme.

It is likely that the explanation for the stimulation effect of relatively low concentrations of 2,5-DBA and the inhibitory effect of relatively high concentrations of 2,5-DBA on the rate of DL-DOPA oxidation by mushroom tyrosinase is the same as that for 2,3-DBA, but it was difficult to establish since the rate of 2,5-DBA oxidation by the enzyme is slow and the absorbancy of 2,5-DBA-o-quinone is low.

m. Synergism exerted by 4-methyl catechol, catechol and their respective quinone on the rate of DL-DOPA oxidation by mushroom tyrosinase

Fernando Schved and Varda Kahn

4-Methyl catechol and catechol, at concentrations ranging from 0.03 to 9 mM and 0.066 to 20 mM, respectively, each has a synergistic effect on the rate of DL-DOPA oxidation by mushroom tyrosinase to material absorbing at 475 nm. The synergism results from the ability of 4-methyl catechol-o-quinone (4-methyl-o-benzoquinone) and of catechol-o-quinone (o-benzoquinone) to oxidize DL-DOPA non-enzymatically to dopaquinone, with the latter being immediately converted to dopachrome ($\lambda = 475 \text{ nm}$).

2. Research from the University of California, Davis

a. Cu^{2+} -Affinity chromatography purification of commercial mushroom polyphenol oxidase

David Osuga and John R. Whitaker

Commercially available mushroom polyphenol oxidase is widely used to study the properties and control of polyphenol oxidase and has been used in research in past BARD projects by Kahn and Whitaker. The product is brown, indicating chemical reaction of the proteins with benzoquinones formed by the enzyme from phenols

during purification. However, the specific activity is high, although several protein bands are obtained by gel electrophoresis. Previous attempts of this laboratory to further purify the commercial preparation by antibody affinity chromatography, by pseudo substrate affinity chromatography and by more classical methods have not been very successful (Golan - Goldhirsh and Whitaker, Israel J. Bot. 35, 203 (1986)).

Another possible method of further purifying the enzyme is by Cu^{2+} -affinity column chromatography. We prepared the Cu^{2+} -affinity column material by the method of Sundberg and Porath (J. Chromat. 90, 87 (1974)) and Porath et al. (Nature 258, 598 (1975)). The apo-polyphenol oxidase (removal of Cu^{2+}) was prepared by the method of Kertesz et al. (Biochem. Biophys. Res. Commun. 49, 1209 (1972)) using dialysis against 0.1 M NaCN at pH 7.0. The apo-enzyme had 0.26% of the original activity. Chromatography on the Cu^{2+} -affinity column gave a brown enzyme with a specific activity of 17.9 units/mg of protein (when Cu^{2+} was added back) vs a starting specific activity of 25.5 units/mg of protein. We are led to the conclusion that little further purification of the commercial enzyme is possible and that we should purify the enzyme from mushrooms under better conditions.

b. Inhibition of mushroom polyphenol oxidase by phytic acid

David Osuga and John R. Whitaker

In 1987, Graf et al. (J. Biol. Chem. 262, 11647 (1987)) reported that 10 and 50 mM phytic acid (myo-inositol hexaphosphoric acid; a naturally occurring compound) inhibited polyphenol oxidase activity when measured using the substrate dihydroxyphenylalanine (DOPA) in 0.1 M phosphate buffer, pH 6.5, at 30°C. We obtained the same results reported by Graf et al. when we reproduced their conditions. However, phytic acid or myo-inositol had no effect on polyphenol oxidase activity when catechol (1,2-dihydroxybenzene) was used as substrate under the same conditions. Phytic acid or myo-inositol did not protect

polyphenol oxidase from inactivation by ascorbic acid or ascorbic acid plus Cu^{2+} .

We believe the effect observed by Graf et al. is due to electrostatic interaction between the highly negatively charged phytic acid and the positively charged DOPA at pH 7. We are testing this hypothesis further using other substrates with different charges and by determining the effect of ionic strength on the Graf et al. system.

c. Direct inactivation of polyphenol oxidase by dithiothreitol (and other thiol compounds).

David Osuga and John R. Whitaker

Golan-Goldhirsh and Whitaker reported that thiol compounds directly inactivated mushroom polyphenol oxidase (J. Agric. Food Chem. 32, 1003 (1984)). However, the mechanism of inactivation was not determined. Thiol compounds can reduce disulfide bonds in proteins, as well as chelate metal ions, such as Cu^{2+} . We verified the results of Golan-Goldhirsh and Whitaker. To determine the mechanism of inactivation, we incubated mushroom polyphenol oxidase (1.11 mg/ml) with dithiothreitol (2 mM) at pH 6.5 (phosphate buffer) in the presence of 1 mM EDTA. After 155 min at 23°C, the activity decreased to 20% of the starting value. The treated enzyme was separated from other reactants by Sephadex gel filtration equilibrated with pH 6.5 phosphate buffer containing 1 mM EDTA. There was no increase in -SH groups on the enzyme, as determined by the dithionitrobenzoic acid method of Ellman. However, incubation of the purified treated enzyme with Cu^{2+} increased its activity. We have now determined that dithiothreitol inactivates polyphenol oxidase by removal of Cu^{2+} . Our experiments included restoration of activity by added Cu^{2+} , and measurement of Cu^{2+} -content of treated enzyme by atomic absorption spectroscopy.

d. Direct inactivation of polyphenol oxidase by bisulfite/sulfite

David Osuga and John R. Whitaker

Sulfites have been used for many years to prevent the enzymatic browning of fruits and fruit juice. In 1984, Golan - Goldhirsh and Whitaker (J. Agric. Food Chem. 32, 1003 (1984)) showed that these compounds directly inactivate polyphenol oxidase, in addition to their efforts on reduction of benzoquinones produced by the enzyme back to substrates (thereby preventing browning).

We postulated that sulfites might inactivate polyphenol oxidase by sulfitolysis of essential disulfide bonds. Lysozyme (4 disulfide bonds per mol) and polyphenol oxidase (two disulfide bonds per 32,000 MW subunits) were separately incubated with 0.1 M NaHSO₃ at pH 9.5 in 0.2 M Tris - 3 mM EDTA buffer. Sulfitolysis was measured by the method of Damodara (Anal. Biochem. 145, 200 (1985)). No sulfitolysis was measurable with either protein unless incubation was performed in the presence of 2 M guanidinium thiocyanate (Gu-HSCN), a denaturing agent. Under these conditions, 75% of the disulfide bonds of lysozyme and 65% of those of polyphenol oxidase were sulfitolyzed. We conclude from these experiments that sulfites do not inactivate polyphenol oxidase by sulfitolysis of disulfide bonds.

e. Mechanism of inactivation of polyphenol oxidase by ascorbic acid and ascorbic acid/Cu²⁺

Avi Golan-Goldhirsh, David Osuga, Andi Chen, Patricia Velasco and John R. Whitaker

As reported previously in BARD reports, there is no doubt that the ascorbic acid/Cu²⁺ system directly inactivates polyphenol oxidase by destruction of the histidine residues which ligand the Cu²⁺ in the active site. The products formed, as well as the essentiality of O₂, indicate the reaction is via a free

radical mechanism, with the free radicals formed in proximity of the active site by a specific complex of the Cu^{2+} /ascorbic acid/ O_2 with the imidazole ring of histidine. However, it has been impossible to determine whether destruction of the imidazole ring is the direct result of $\text{OH}^\bullet$, $\text{OH}_2^\bullet$, $\text{O}_2^\bullet$, H_2O_2 or the dehydroascorbic acid semiquinone. We are continuing to work on this system by ESR, by studying effect of added scavengers of free radicals, changes in experimental conditions, etc. We feel this information is essential to the design of good Kcat inhibitors of the enzyme and possible commercial control of enzymatic browning.

f. Effect of ascorbic acid and copper on proteins

Avi Golan-Goldhirsh, David T. Osuga, Andi Chen and John R. Whitaker

Ascorbic acid and copper are both essential for good nutrition. They function together in at least two monooxygenases and separately in a number of other oxidoreductase systems. The exact role of ascorbic acid is still unclear. In in vitro systems, ascorbate and cupric ions together with O_2 form a strong oxidizing system that has been shown to fragment carbohydrate and DNA chains and to damage proteins, including enzymes. Our research has shown that histidine residues of polyphenol oxidase, bovine serum albumin, ovalbumin and Kunitz soybean trypsin inhibitor are readily destroyed, with smaller losses of the methionine residues. Aspartic acid, glutamic acid, glycine and alanine are formed. The proteins retained their original size by size exclusion measurements but had marked changes in electrophoretic properties as a result of the increase in aspartic acid and glutamic acid residues. It is suggested that histidine residues are selectively destroyed as a result of formation of a specific quaternary complex involving histidine, Cu^{2+} , ascorbate, O_2 . The reactive species could be a histidine, Cu^{2+} , semidehydroascorbate, O_2 complex or

semidehydroascorbate radical, superoxide radical ($O_2^{\cdot -}$), peroxy radical ($HO_2^{\cdot}$ or hydroxyl radical ($OH^{\cdot}$) formed in proximity to the imidazole ring of histidine of the protein.

g. Direct effect of sodium bisulfite on polyphenol oxidase inactivation

Davis Osuga, Cheng-Jian Liu, Patricia Velasco and John R. Whitaker

Micromolar concentrations of sodium bisulfite, when incubated with polyphenol oxidase in the absence of substrate, rapidly inactivates the enzyme. There are several possibilities to explain this direct effect. These include: 1) breakage of essential disulfide bonds by sulfitolysis; 2) reduction of Cu^{2+} to Cu^+ in the active site, permitting the more loosely bound Cu^+ to dissociate; and 3) some other effect on the protein. We have shown that sulfitolysis of disulfide bonds does not occur in the native enzyme. Only when the enzyme is denatured does sulfitolysis occur. Copper is lost from the enzyme on incubation with sodium bisulfite. This has been shown by atomic absorbance measurements on the enzyme before and after sodium bisulfite treatment. Enzyme that had been inactivated to 10 to 20% remaining activity had less bound copper as shown by atomic absorption analysis after passage through a Sephadex G-25 column to remove sodium bisulfite. Up to 80% of the lost activity could be restored by incubation with added $CuSO_4$. We want to verify these experiments with a homogeneous enzyme.

We have also determined that one or two of the polyphenol oxidase isozymes are more sensitive to sodium bisulfite treatment. We will attempt to correlate this enhanced sensitivity with the K_d for dissociation of Cu^+ from the isozymes.

h. Purification of mushroom polyphenol oxidase

Patricia Velasco and John R. Whitaker

Much of our research has been done on commercially available mushroom polyphenol oxidase. We have tried repeatedly to purify the commercial enzyme, including the use of Cu^{2+} -affinity chromatography (reported last year). Not being successful in purifying the commercial enzyme, we have devoted considerable time to purifying the enzyme from mushrooms. We have solved the problem of browning due to large concentrations of phenolic compounds in mushrooms and have at least one of the isozymes pure. We consider this to be critical, as we need to confirm the results of the direct effect of sodium bisulfite and dithiothreitol in inactivation of polyphenol oxidase.

i. Kcat inactivation of polyphenol oxidase

David Osuga and John R. Whitaker

We are looking at the potential of reaction inactivation of polyphenol oxidase (PPO) as a control method. It is well known that PPO inactivates itself during the course of conversion of substrate to product. The enzyme, completely stable in the absence of substrate, can lose all activity within 2-4 min in presence of substrate. There is an increase in unbound copper, indicating loss of one or more histidine ligands to the copper. Little research is available on the effect of oxygen, enzyme and substrate concentrations, pH, temperature and substrate structure on rate of inactivation. We are systematically examining these factors, using 4-methyl catechol and catechol as substrates.

j. Improved inhibitors of polyphenol oxidase

David Osuga and John R. Whitaker

A number of phenolic compounds are competitive inhibitors of PPO activity. One of the most effective compounds is 4-hexylresorcinol with a K_i of $\sim 1 \times 10^{-7}$ M. This compound is widely used as a topical antiseptic (Sucrets) and anthelmintic. We are determining the effect of substrate structure, pH, storage

in complex systems (such as grape juice and wine) on the effectiveness of this inhibitor.

k. Cyclodextrin inhibition of polyphenol oxidase activity

Andre van der Schaaf and John R. Whitaker

Cyclodextrins are known to form occlusion complexes with phenols, slowing down the rate of browning of juices and fruit slices. The most effective use of cyclodextrins to prevent browning is with fruit juices where the phenols are removed from the juices. We are determining the effect of phenol structure and concentration, type of cyclodextrin (α , β or γ) and pH on complex formation. We are also determining the kinetic and thermodynamic consequences of removing some of the substrate from contact with the enzyme.

VI. Description of Cooperation

Dr. Kahn spent several weeks in Davis in December 1987 and December 1989 working directly with Dr. Whitaker in analyzing accumulated data, writing manuscripts and planning future research. She plans to come to Davis again in March or April 1991 for collaboration in manuscript writing and data analyses. They exchanged ideas and manuscripts regularly. They collaborated experimentally on one of the research areas and have a joint publication (see list of publications).

VII. Evaluation of research achievements with respect to the aims of the original research proposal and emphasizing benefit to agriculture

A. Is there a direct correlation between the ability of reductants to reduce Cu^{2+} in a chemical model system and Cu^{2+} at the active site of tyrosinase and their ability to inactivate the enzyme?

The data clearly showed this not to be the case. Thiol compounds and sodium bisulfite appear to reduce Cu^{2+} to Cu^{+} , ascorbic acid/ Cu^{2+} degrades the imidazole ring of histidines liganding the $\text{Cu}^{2+}/\text{Cu}^{+}$ and the mechanism of other reductants is not yet known.

B. Do various copper chelators, including antioxidants, inactivate tyrosinase?

We were unable to find a correlation between copper chelating ability and inactivation of tyrosinase at pHs near the optimum pH of the enzyme (pH 6.5), indicating that the $\text{Cu}^{2+}/\text{Cu}^{+}$ is very tightly liganded.

C. What is the mechanism of ascorbic acid and Cu^{2+} in the direct inactivation of tyrosinase?

Ascorbic acid/ Cu^{2+} inactivate tyrosinase by a free radical scission of the imidazole ring of histidine at the active site of the enzyme. Similar scissions occurred in bovine serum albumin, ovalbumin and Kunitz trypsin inhibitor. Specificity resides with the Cu^{2+} /ascorbate system whereby the Cu^{2+} ligands to the imidazole group of histidine, at the same time binding with ascorbic acid and O_2 .

D. How does sulfite inhibit tyrosinase?

Sodium bisulfite appears to inhibit tyrosinase by reducing the active site Cu^{2+} to Cu^{+} , which then is lost from the enzyme.

E. Can compounds that show efficient K_{cat} inactivation of tyrosinase be found?

We do not yet have an answer to this question. Only recently have we developed the methodology to fully explore this issue. This is because of the complexity of the system, involving changes in substrate concentration, O_2 concentration, non-specific modification of PPO by benzoquinone and variable

effects depending on enzyme concentration. We will continue to work on this problem in a systematic way.

F. Is there an inverse correlation between browning potential and carotenoids content of vegetative tissue? If so, what is the explanation for such a correlation with emphasis to the possibility that carotenoids inhibit browning?

We did not work on this question directly.

We believe all the work reported here will be important in controlling the browning caused by polyphenol oxidase. Some has already been implemented by the food industry, especially in avocado products.

VIII. List of Publications

A. Publications of Dr. Varda Kahn

- Kahn, Varda. (1989). Tiron as a substrate for horseradish peroxidase. Phytochemistry 14, 41-44.
- Andrawis, A. and Kahn, Varda. (1990). Ability of various chemicals to reduce copper and to inactivate mushroom tyrosinase. J. Food Biochem. 14, 103-115.
- Kahn, Varda. (1990). Synergism exerted by 4-tert-butyl catechol and tert-butyl catechol-quinone on the rate of DL-DOPA oxidation by mushroom tyrosinase. J. Food Biochem. 14, 177-188.
- Kahn, Varda. (1990). 2,3,4-Trihydroxyacetophenone (2,3,4-THAP) as a substrate for mushroom tyrosinase. J. Food Biochem. 14, 189-198.
- Kahn, Varda, Osuga, D.T. and Whitaker, J.R. (1991). 2,4,5-Trihydroxybutyrophenone (2,4,5-THBP) as a substrate for mushroom tyrosinase. J. Food Biochem. (in press).
- Schved, F. and Kahn, Varda. (1991). 2,3-Dihydroxybenzoic acid as a substrate for mushroom tyrosinase (in preparation).

Schved, F. and Kahn, Varda. (1991). Synergism exerted by 4-methyl catechol, catechol and their respective quinones on the rate of DL-DOPA oxidation by mushroom tyrosinase (in preparation).

B. Publications by Dr. John Whitaker

Kahn, Varda, Osuga, D.T. and Whitaker, J.R. (1991). 2,4,5-Trihydroxybutyrophenone (2,4,5-THBP) as a substrate for mushroom tyrosinase. J. Food Biochem. (in press).

Golan-Goldhirsh, A., Osuga, D.T., Chen, A.O. and Whitaker, J.R. (1991). Effect of ascorbic acid and copper on proteins. In The Bender Legacy, CRC Press (in press).