論文の内容の要旨

応用生命化学専攻

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論文題目

A Study on the Inhibition Mechanism of Halide and Disulfide
on the Activity of Polyphenol Oxidase (PPO)
(ハロゲン化物ならびにジスルフィド物による
ポリフェノールオキシダーゼの阻害機構)

Introduction

Polyphenol oxidase (PPO) is a ubiquitous enzyme widely distributed in living organisms. It acts as an oxygenase, catalyzing the hydroxylation at the ortho position of monophenols. PPO is unique as it can further act on the ortho-diphenols, catalyzing its oxidation to the corresponding quinones.

PPO contains two copper (Cu) ions on its active site, classifying it as a type-3 copper metalloenzyme. Crystal structures reveal that each Cu is bound to the N-ε of three histidine residues. Although the two Cu ions, termed CuA and CuB, both have histidines as coordinating ligands, they are not identical. Further, which of the dual actions of PPO will occur depends on which Cu is involved in the reaction. Although PPO is found in many living organisms, alignment of PPO sequences from different biological sources show little homology, except for the amino acid sequences of the copper-binding domain. The conservation of this moiety across all PPOs asserts that the binuclear copper center plays a fundamental role in the activity and function of the enzyme. The Cu in the active site is redox active and exists in at least two oxidation states in biological systems. The catalytic activity of PPO is carried out alongside the redox transformations of this binuclear Cu center.

It is known that PPO is widespread in nature. However, its biological functions vary according to the source. In fruits and vegetables, it is the main culprit behind the undesirable discoloration normally equated to food senescence. Because of the detrimental effects of PPO to aesthetics and food security, many efforts are being undertaken to control its reaction. Numerous methods and chemical inhibitors have been proposed. However, if all the determinants of food quality are to be considered, no one perfect method to prevent undesirable browning can be pointed. Thus, it remains a challenge for food scientists to find the most effective method to curb the unsatisfactory products of PPO oxidation without sacrificing nutrition and food quality.

In this study, the binding of inorganic ligands with inhibitory activity to PPO is studied, using mushroom PPO. This enzyme is commercially available, at a lower cost, and with relatively higher purity as compared to proteins extracted from plants. Further, mushroom PPO has been well-characterized and is assumed to maintain the same active site structure as those found in plants.

Kinetic Analysis of PPO Activity and Inhibition

A steady-state kinetic analysis was provided by monitoring the PPO-catalyzed oxidation of catechol in the presence and absence of the inhibitors. In the absence of any inhibitor, substrate inhibition was observed. When greater than 1 mM catechol was added to the enzyme solution, quinone formation slowed down. On the other hand, the halide inhibitors decreased PPO activity in a dose-dependent manner. However, the degree to which the halide inhibited the reaction was dependent on the substrate concentration. These data show that, at pH 5.0, both substrate and halide inhibition occurs for PPO. Moreover, it seems that excess substrate and halide compete with each other for a common binding site (Lim, *et al.*, 2012). At low catechol concentration where no substrate inhibition occurs, it was demonstrated that the halides chloride and bromide inhibit PPO via a non-competitive mechanism, able to bind to both free and substrate-bound enzyme. On the other hand, iodide exhibits uncompetitive inhibition behavior, with affinity only to the enzyme-substrate complex. Fluoride showed very strong inhibition, and a small concentration resulted in an almost total elimination of PPO activity. Disulfide-containing compounds such as cystine and 2-hydroxyethyl disulfide (HEDS) were also tested for their inhibitory effect on PPO. These ligands decreased the rate of quinone formation, but in a time-dependent manner that is observable in room temperature.

Electrochemical analysis of halide binding to PPO

Since PPO activity occurs concomitant with the redox transformations of the Cu in the enzyme, it is plausible that the oxidation state of the Cu center exerts some influence on ligand binding. Cyclic voltammetry (CV) experiments were performed to make known the probable mechanism of the bioelectrocatalytic effect of PPO, without the use of substrates. When iodide was titrated into the protein solution, the potential shifted to a more negative value. This is the result of the altered electron cloud and distortion of coordination environment around Cu after iodide binding. On the other hand, titration of PPO with fluoride caused the redox peaks of the enzyme to disappear. The strong binding of fluoride may have caused conformation changes within the vicinity of Cu that affected the electron movement between PPO and the electrode. Both CV experiments on fluoride and iodide showed pH dependence. The alteration of electron cloud resulting in a shifted potential or disappearance of signal occurred only in pH 5.0 but not in pH 7.0. This behavior is in agreement with the kinetic data.

Binding analysis of Halides to PPO

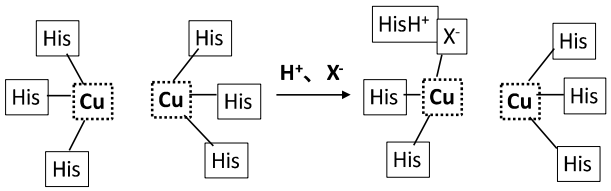
Nuclear magnetic resonance (NMR) spectroscopy was used to further characterize the binding of ligands to the macromolecule. This method is convenient especially since the ligands have magnetic nuclei and its binding to the protein causes a change in an observable NMR parameter such as the chemical shift, linewidth, or relaxation rate.

The ^{127}I NMR spectra show that iodide preferentially binds to the reduced form of PPO. The initial addition of PPO caused the linewidth of iodide to broaden. However, titrating stoichiometric amounts of ligand to the enzyme resulted in peak sharpening, evidence of an interaction between the two molecules. A decreasing linewidth was also observed as temperature was increased, characteristic of a fast exchange between free and protein-bound iodide in reduced PPO.

To observe the interaction of PPO with cystine, the ^{1}H NMR spectra of the enzyme was observed under increasing concentrations of the disulfide ligand. The peak at 4.1 ppm that slowly appeared as cystine was titrated is assigned to the methine proton of cystine that is in direct interaction with PPO. An inversion recovery fourier transform experiment following selective irradiation of this methine proton showed that T_1 was hastened in the presence of PPO.

Discussion

In summary, at pH 5.0 where inhibition was most apparent, the following model may be imagined for the binding of inhibitor ligands to PPO.



Because of the acidic conditions, one of the copper-bound histidine residues becomes protonated and cleaves from the active site Cu. The site vacated by histidine provides a binding site for the inhibitor ligands. Considering this, it can be thought that ligand binding to PPO is strongly dictated by its interaction with Cu. The proposed model, alongside the known properties of Cu, offers the following mechanisms to account for the observed behaviors of the inhibitor ligands tested in this study:

1. Weak hydrophobic interactions exhibited by chloride and bromide.
   Since chloride and bromide are inhibitors that do not have considerable redox character, it is understood that its binding to PPO is influenced only by its ionic size. These halides showed indiscriminate binding to PPO and the addition of chloride did not affect the spectra of PPO in NMR.
2. Influence of PPO redox state on protein-ligand interaction as observed in iodide binding.
   The kinetic behavior of iodide showed that iodide preferential binds to the reduced form of the enzyme. The halide showed uncompetitive inhibition, able to bind only when the substrate is bound to PPO. At this stage, Cu is thought to be in the cuprous state. The

irreversibility of the CV reaction further give light to this, where only a reduction peak, and no oxidation peak was observed. With iodide bound to the reduced Cu, no oxidizable Cu(I) species remain in solution. Lastly, the different NMR spectra for the reduced and the oxidized PPO clearly present that iodide has stronger affinity for the reduced enzyme. This preferential binding of iodide to the reduced Cu shows that the redox state of the active site Cu influences ligand binding predictable by the principles of hard and soft acids and bases (HSAB).

3. Strong ionic interactions such as occurring for fluoride.
   The almost total elimination of activity by fluoride in the kinetic experiments suggest a strong binding of fluoride to PPO. This is further understood from CV data, where the addition of the inhibitor severely disrupted the electron cloud around Cu, causing the redox peaks to disappear. Binding is likewise assumed for both the oxidized and reduced form of PPO. This behavior suggests that fluoride attachment occurs via a strong ionic interaction with the active site Cu or with other ionizable groups on the protein surface. The pH dependence of the CV results also provides support to this supposition since ionic groups that could be involved in binding are not as many under neutral conditions.
4. Covalent overlap of the empty d orbitals of Cu with nucleophiles such as disulfides.
   The ^{1}H NMR data as well as the enhanced T_1 value of cystine in the presence of PPO gives light on the interaction between the two compounds. However, the kinetic data shows that disulfide binding to PPO requires incubation and can only be achieved in room temperature. Although disulfides are also capable of redox reactions, the overriding mechanism that dictates its attachment to PPO is the covalent overlap of the empty orbitals of Cu with the valence electrons of disulfide.

The gathered information proves the versatility of PPO in terms of reactivity with various halides. This is hopeful for a food scientist as it opens many ways of controlling the activity of this enzyme. At the same time, it can also prove taxing as a clear-cut mechanism applicable to all PPOs and inhibitor ligand is yet to be provided. Albeit only but a surface, this study has provided additional knowledge that can be tapped in the development of PPO inhibitors. Optimum conditions that can influence PPO-ligand binding likewise have been mentioned.