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Inhibition of Polyphenol Oxidases Activity by Various Dipeptides

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In an effort to develop natural and nontoxic inhibitors on the activity of mushroom polyphenol oxidase (PPO) the effect of various glycyl-dipeptides (GlyAsp, GlyGly, GlyHis, GlyLeu, GlyLys, GlyPhe, GlyPro, GlyTyr) was investigated. The inhibition study with dihydroxyphenylalanine (DOPA) as substrate is based on separation of the enzymatic reaction components by reversed phase HPLC and the UV detection of the dopachrome formed. The results have evidenced that several of tested dipeptides inhibited PPO activity in the range of 20−40% while GlyPro and GlyLeu had no effect. The study has also permitted the characterization of the following kinetic pattern: a linear-mixed-type mechanism for GlyAsp, GlyGly, GlyLys, and GlyPhe and a hyperbolic-mixed-type for GlyTyr. It was not possible to identify the inhibition mechanism for GlyHis, although it affects PPO activity. In addition the effects of GlyAsp, GlyLys and GlyHis were evaluated for lessening the browning of fresh Golden Delicious apple and Irish White Skinned potato. The effectiveness of such inhibitors was determined by the difference between the colors observed in the dipeptide-treated sample and the controls using the color space CIE-*La*b** system. The % browning inhibition on potato (20−50%) was greater than of apple (20−30%) by the all tested dipeptides. Only GlyLys presented the significant value of 50%.

KEYWORDS: Browning; fruits; tyrosinase; vegetables

INTRODUCTION

Enzymic browning of fruits and vegetables during handling and processing is a main cause of quality loss (*1*, *2*). This is related primarily to the oxidation of phenolic compounds catalyzed by polyphenol oxidases (PPO). Polyphenol oxidases (EC 1.14.18.1), which are copper-containing enzymes, catalyze the hydroxylation of monophenols to *o*-diphenols using molecular oxygen and the oxidation of *o*-diphenols to *o*-quinones. With DOPA as substrate the quinone initially cyclizes to the reddish-orange "dopachrome", than forms 5,6-dihydroxyindole (DHI) derivatives which are the cause of enzymatic blackening in the early stages. After DHI formation, non enzymic polymerizations lead to the formation of brown pigments (*3*).

The chemical control of enzymatic blackening or browning requires the inhibition of PPO activity by adjustment of pH and/or temperature (*4*, *5*) and/or by addition of (a) chelating agents of the copper present in the active site of enzymes (*2*), (b) compounds able to reduce the *o*-quinones to the *o*-hydroxyphenol state (*6*, *7*) (c) chemicals which react with *o*-quinone to give colorless addition products (*8*). The adjustment of pH below the optimum pH range (pH 4−7) can be advantageously employed in browning control as long as acidity can be tolerated tastewise. Heat inactivation of PPO is feasible by applying temperatures of more than 50 °C but may cause undesirable colors and/or flavors. Actually, the best way (*4*, *5*) to inhibit browning is the use of sulfites that act as bleaching, antioxidant, or reducing agents. However their use presents some disadvantages: (i) the corrosion of machinery, (ii) the destruction of nutrients, (iii) the production of tissue softening and off-flavors, and (iv) some adverse health effects so their uses are restricted to a few commodities. Therefore, research and development studies for finding effective substitutes are still ongoing.

Proteins, peptides, and amino acids can affect PPO activities in at least two ways: by reacting with the *o*-quinones and by chelating the essential copper at the active site of PPO (*8*, *9*). It is also reported (*10*) that a small peptide present in a special honey collected from Mediterranean and Agean areas inhibits the mushroom PPO. According to these observations and taking into account the necessity to use nontoxic substances in food chemistry, it appeared of interest to test the effect of some Gly-X dipeptides on the activity of mushroom PPO with DOPA as substrate and on the level of inhibition in the course of the enzymatic reaction. The study was carried out by HPLC method that we have previously described (*11*).

In addition, some measurements have been performed by comparing the relative effectiveness of some dipeptides on the browning inhibition in apples and potatoes. To measure the

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extent of browning and browning prevention, we have used the CIE-*La*b** system, an uniform color space which is produced by plotting the quantities *L*, *a**, *b** in rectangular coordinates, where *L* is the luminosity, *a** is the position on the green(−) to red (+) axis, and *b** is the position on the blue (−) to yellow (+) axis (*12*). The Δ*E** value, considered the most uniform and adequate parameter for the evaluation of total colors difference, is expressed in terms of *L*, *a** and *b** coordinates In this way, luminosity information is separated from color information so that, compared to other-device independent color systems, the advantage of *La*b** is that it takes the human perception into consideration (*13*).

MATERIALS AND METHODS

D,L-DOPA, dipeptides and mushroom PPO were obtained from Sigma (St. Louis, MO). The other chemicals were purchased from Merck (Darmstadt, Germany). Water and acetonitrile used were of HPLC grade. The solutions used for HPLC were also filtered through a 0.45-μm filter (Millipore, Inc. Bedford, MA) and degassed before use.

HPLC Assay for *O*-Dihydroxyphenolase Activity. The *o*-dihydroxyphenolase activity of mushroom PPO was assayed chromatographically at pH 6.5 and at 20 °C using DOPA as substrate. The reaction mixture of 1.54 mL included 9.1 mM DOPA, 0.1 mM sodium phosphate buffer (pH 6.5), and mushroom PPO as reported. At fixed interval times, 20-μL aliquots were injected into the HPLC system. The rate of DOPA oxidation was followed at 217 nm by a Kontron system (Kontron; Milan, Italy), consisting of a model 422 pump and a UV−vis 432 detector, complete with a Rheodyne 7125 injector with a 20 μl sample loop (Rheodyne, Berkeley, CA); a directly connected guard column of LC-18 (5-μm, 75- × 4.6-mm i.d); and a column Alltima LC-18 (5-μm, 250- × 4.6-mm i.d) (Alltech, Deerfield, IL). The mobile phase was NaH_2PO_4 0.1 M/acetonitrile = 99/1 (v/v), the flow rate was 1.2 mL/min. The amount of Dopachrome enzymatically formed was registered by an integrator system constituted by a PC equipped with an INTEL, Pentium III 800 MHz processor CPU and software Agilent ChemStation for LC v. A.08.03(847) (Agilent, Palo Alto, CA) running under MS Windows NT 4.00.31 OS. Rates were linear with time during the first 1200 s of the enzymatic reaction. From these initial linear portions, as described in our previous work (*11*), it was possible to determine PPO activity (U/l) considering that one unit of enzyme produces 1 μmol of Dopachrome per min at 20 °C.

Inhibition Study. Inhibition experiments were performed after a preincubation (5 min) of the enzyme with different amounts of dipeptide, and the reaction was started by adding DOPA as last component of mixture. Successively, at fixed time interval, 20-μL aliquots were injected into the HPLC system. In this way, the reaction rates were determined following the increase of the amount of dopachrome formed, which was identified by its retention time. The concentration was determined by integration of peak areas and comparison with a calibration graph obtained as just described in our preceding work (*11*).

The % of inhibition was determined by the following equation:

$$\%\ \text{inhibition value} = \left(\frac{v_0 - v}{v_0}\right)100$$

where v_0 and v are the reaction rates, expressed as pmol/min dopachrome, in the absence and presence of dipeptides, respectively.

Procedure of Browning and Browning Prevention in Apples and Potatoes. Stock solutions (85 mM) of GlyAsp, GlyLys, GlyHis, and of 9.1 mM DOPA were prepared in 0.1 M sodium phosphate buffer (pH 6.5). A 5-μL portion of dipeptide (inhibitor) or phosphate buffer (control) solutions were deposited at fixed points on a slice of golden apple (*malus domestica*, golden delicious cultivar) and white potato (*solanum tuberosum*, irish white skinned cultivar), then 5 μL of DOPA were immediately stratified on the top. The colors evaluated by CIE*La*b** space system were measured using a digital camera Camedia C100 (Olympus, Milan, Italy) and by Adobe Photoshop v. 7 software

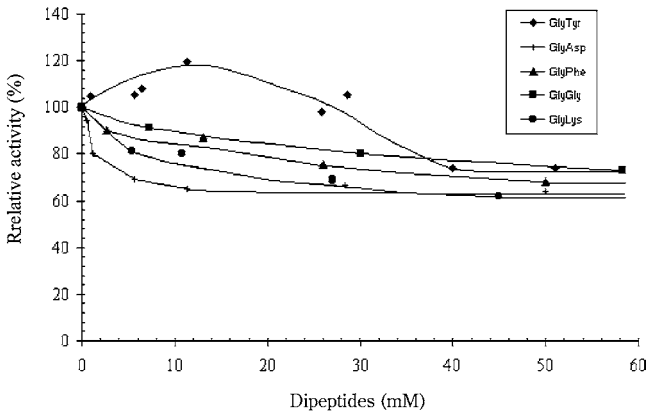


Figure 1. Effect of some dipeptides on the *o*-dihydroxyphenolase activity of mushroom PPO. The reaction mixture included in a total volume of 1.54 mL, 0.1 M phosphate buffer pH 6.5, 89 mU/L mushroom PPO, 9.1 mM DOPA (added last) and dipeptides in buffer as indicated. The activities are expressed relative to those of control (no dipeptides added). Data for GlyLeu and GlyPro not shown for lack of effect.

(Adobe Systems Inc., San Jose, CA) as reading system of *L*, *a**, *b** chromatic coordinates. The readings of each treatment were obtained from the media of three values at different points of the considered areas to get uniform color measurements. The chromatic coordinates measured just after slicing without any treatment, considered as origin fresh color (untreated sample), and those obtained with the dipeptides or phosphate buffer treatment were recorded as time function. By using the color space CIE-*La*b**, the differences between two colors each given in terms of *L*, *a**, *b** is expressed by the measure of total color difference (Δ*E**)

$$\Delta E^* = ((\Delta L)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)^{1/2}$$

The relative dipeptide efficacy as inhibitor was determined as the percent inhibition of browning, expressed as

$$\%\ \text{browning inhibition} = (\Delta E^*_{\text{control}} - \Delta E^*_{\text{inhibitor}}) \times 100/\Delta E^*_{\text{control}}$$

where $\Delta E^*_{\text{control}}$ values were determined considering the different colors between the areas treated with phosphate buffer (control) and the untreated areas of the same apple or potato after a specified time of incubation, and $\Delta E^*_{\text{inhibitor}}$ values were determined considering the different colors between the areas treated with dipeptides and the untreated areas of the same apple or potato after a specified time of incubation.

RESULTS AND DISCUSSION

The effect of various dipeptides on the *o*-dihydroxyphenolase activity of mushroom PPO was tested, but due to solubility limitations, at different maximum concentration value. The reactions were started by the addition of DOPA to the temperature-equilibrated solution containing sodium phosphate buffer-PPO-dipeptide. The initial reaction rates expressed as pmol/min dopachrome formation that were proportional to enzyme activity were chromatographically measured in the absence and in the presence of dipeptides. The obtained results (**Figure 1**) have evidenced that glycylaspartic acid (GlyAsp), glycylphenylalanine (GlyPhe), glycylglycine (GlyGly), glycyllysine (GlyLys), glycyltyrosine (GlyTyr), and glycylhistidine (GlyHis) affected the rates of dopachrome production, whereas glycylproline (GlyPro) (until 55 mM) and glycylleucine (GlyLeu) (until 20 mM) had no effect. The inhibition of DOPA oxidation catalyzed by PPO as a function of dipeptide concentrations (0−50mM) was reported in **Figure 1**. A nonlinear decrease in activity was evidenced for GlyAsp, GlyLys, GlyPhe, and GlyGly reaching

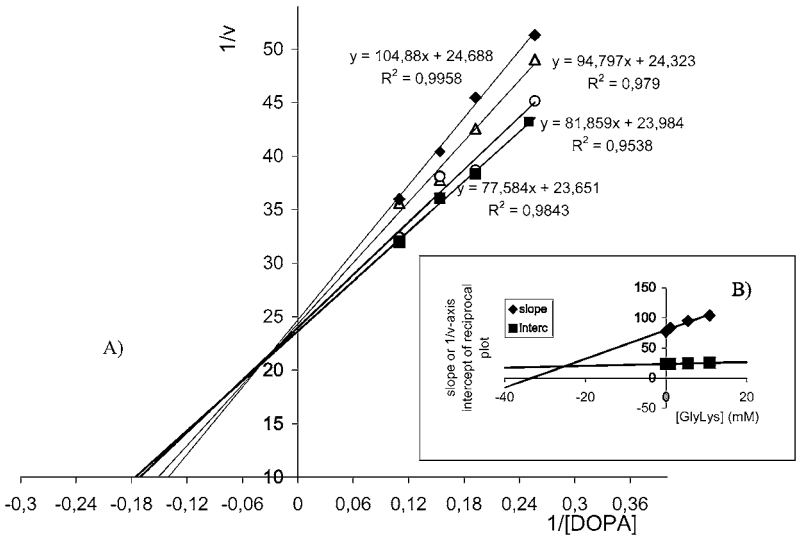
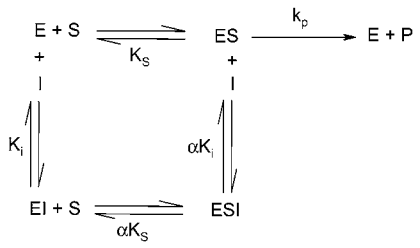


Figure 2. (**A**) Double reciprocal plot of mushroom PPO inhibition by GlyLys with DOPA as substrate. Experimental conditions: PPO 89 mU/L, sodium-phosphate buffer pH 6.5. GlyLys: (■) absent, (○) 1.1 mM, (△) 5.4 mM, (◆) 10.8 mM. (**B**) Secondary plot of slope or 1/V-axis intercept reciprocal versus GlyLys concentration.

Scheme 1



S = DOPA; E = PPO; ES = PPO-DOPA;
I = GlyAsp, .GlyPhe, GlyGly or GlyLys;
EI=PPO-dipeptide; P = product;
ESI=PPO-DOPA-dipeptide;
K_s = dissociation constant of ES complex ;
K_i = dissociation constant of EI complex;
αK_i=dissociation constant of ESI complex when S occupies the enzyme;
αK_s = dissociation constant of ESI complex when I occupies the enzyme;
k_p = rate constant for the breakdown of ES to P.

20−40% of inhibition with concentrations of ≈20, 40, 50, and 60 mM, respectively. On the contrary, GlyTyr initially causes an activity increase up to 120% value and only above ca. 25 mM is present inhibition.

A successive systematic study had been undertaken in order to elucidate the inhibition mechanism. The results obtained for GlyAsp, GlyPhe, GlyGly, and GlyLys are quite similar when the double reciprocal plots (1/v vs 1/[S]) at different concentration of inhibitor were graphed. With increasing [I], the slopes and the *Y*-intercept (1/*V*max) of the reciprocal plots pivoted counterclockwise about the point of intersection with the control curve (**Figure 2***A*) (*14*). In addition, the linear replots of the slopes and the 1/v -axis versus the corresponding inhibitor concentration confirm a "linear mixed" type inhibition (**Figure 2B**).

The scheme of the hypothesized mechanism is displayed in **Scheme 1**.

It is evident that PPO can bind to both substrate (S) or inhibitor (I) to form enzyme−substrate(ES) or enzyme−inhibitor (EI) complexes, and though both substrate and inhibitor are capable of combining with the enzyme at the same time, a ternary complex (ESI) can be formed. Notice that the ESI complex can be produced by two routes, but the final result is the same: an inactive, nonproductive, complex. This means that the inhibitor is *not* out competed by high substrate concentrations and works equally well at low and high concentrations of the substrate. Usually, although the substrate can bind to the enzyme−inhibitor complex, its ability to bind (its affinity) is reduced.

The mathematical treatment of data has been allowed to determine for GlyAsp, GlyLys, GlyGly, and GlyPhe the α factors, the inhibition constants (K_i) and K_i' (= α K_i) values (**Table 1**). The α factor is the value parameter by which K_s changes when I occupies the enzyme. The values of K_i and K_i' were determined by calculating the intercepts of the slopes or intercept replot on the [I]-axis, respectively. By comparison of inhibition constants values it appeared that GlyAsp is the dipeptide with the highest inhibition power, probably due to its ability to interact with the enzyme through the additional carboxylate group present in the side chain.

Table 1. Inhibition Constants (K_i), α, and α K_i Values of Glycilaspartic Acid (GlyAsp), Glycilphenylalanine (GlyPhe), Glycillysine (GlyLys), and Glycilglycine (GlyGly)

dipeptide	K_i (mM)	α	$K_i' = \alpha K_i$
GlyAsp	0.66	21.8	14.4
GlyPhe	32.42	4.9	158.0
GlyLys	33.34	5.76	191.1
GlyGly	33.20	12.9	428.3

When the kinetic data of the reaction in the presence of increasing concentration of GlyTyr were graphed using the double reciprocal plot, a different pattern was found. The results, reported in **Figure 3A**, show that the slopes of the family curves of the added GlyTyr are less than the control curve and the reciprocal plot pivots clockwise about the point of intersection with the control curve.

This result can be related to an hyperbolic mixed-type inhibition[14] expressed by **Scheme 2**.

In this case, the complex ESI is catalytically active, and its action is quantitatively expressed by β, the kinetic factor by which k_p changes (see **Scheme 2**). By mathematical elaboration

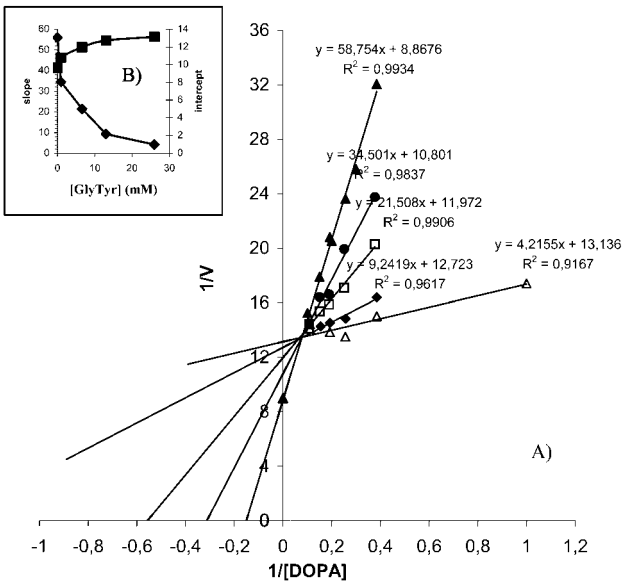


Figure 3. (**A**) Double reciprocal plot of mushroom PPO inhibition by GlyTyr with DOPA as substrate. Experimental conditions: PPO 89 mU/L, phosphate buffer 0.1 M pH 6.5. GlyTyr: (▲) absent, (●) 0.9 mM;, (□) 6.6 mM, (◆) 13 mM, (△) 26 mM. (**B**) Secondary plot of slope (◆) or 1/V-axis intercept (■) reciprocal versus GlyTyr concentration.

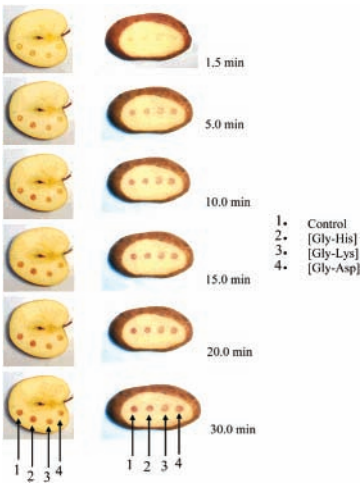


Figure 4. Photographs at different times of apple and potato slices treated with DOPA and the inhibitor solutions listed above.

of data, the values of 0.04 and 0.66 for α and β were obtained, respectively, in agreement with the hyperbolic mixed type inhibition characterized by $\alpha < 1$ and $0 < \beta < 1$ with $\alpha < \beta$ as reported by Segel (*14*). These α and β values justify the behavior previously evidenced in **Figure 1** for GlyTyr. Indeed, in the presence of this dipeptide, there will be a higher concentration of ESI than of ES (because EI has a higher affinity than E for S). The greater concentration of ESI more than compensates for the fact that ESI is not as effective as ES in forming product. However, as [GlyTyr] increases, this advantage (activation effect) is lost when values higher than 25 mM are reached.

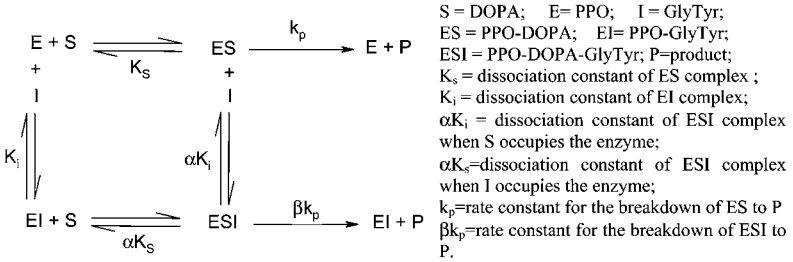
An anomalous effect was observed when GlyHis is added to the reaction mixture. The time course of the Dopachrome accumulation, chromatographically monitored, was not always linear for 1200 s, the time required for three successive injections and indispensable to have a good accuracy of the rate measurements. This pattern is dependent on the ratio DOPA/GlyHis; when this value is > 1, a linear increase of Dopachrome within the 1200 s with the same slope of the control was observed. On the contrary, when the value DOPA/GlyHis was <1, a not linear increase of dopachrome formation was evidenced preventing us to effect a systematic study for the determination of inhibition mechanism type. However, the behaviors of a 50mM GlyHis solution preincubated for 5 min with PPO, to which was added 9.1 mM DOPA as starting agent, and of the corresponding control solution were monitored for 6 days. It appeared that the color of the control solution became light gray in 4 h and black in 7 h, and the solution became turbid with a black precipitate in 5 days. Meanwhile, the solution containing GlyHis remained uncolored until it reached a straw-colored yellow in 20 h and a deep cherry red in 5 days. Both solutions were chromatographated, and a great difference in the retention times of the products was derived by the spontaneous rearrangement of Dopachrome. Indeed, in the case of the control, they appeared at retention times longer than that of Dopachrome (t_r = 6.8 min), while in the presence of GlyHis they appeared in the range of 2.5−5.2 min. This confirms the different nature of these products. We have hypothesized that GlyHis may interact with dopaquinone through the imidazole group, forming copolymers that are more polar than DOPA and that have a less dark color than the oxidation products of DOPA alone. This pathway is supported by some reported results for other heterocyclic nitrogen compounds (*15*). In this view, GlyHis may also be considered an inhibitor of mushroom PPO.

Browning Inhibition in Apples and Potatoes. The degree of browning of the apple and potato was monitored by photographs taken immediately after the treatment with dipeptides-DOPA or buffer-DOPA (**Figure 4**).

GlyAsp and GlyLys were chosen because they were the most efficient inhibitors of DOPA oxidation by mushroom PPO. In this study, GlyHis was also added. Because of its peculiar behavior, a close study by HPLC was impossible to realize, even if an influence on dopachrome formation was shown.

The results expressed as percent browning inhibition as a function of time, shown in **Figure 5**, were obtained by the measure of total color difference ΔE^* in according to the CIE-La^*b^* system (see Experimental Section) of potato and apple portrayed in **Figure 4**. The reaching of a constant value is always evidenced, in **Figure 5**, after just 10 min. Moreover, a

Scheme 2



S = DOPA; E= PPO; I = GlyTyr;
ES = PPO-DOPA; EI= PPO-GlyTyr;
ESI = PPO-DOPA-GlyTyr; P=product;
K_s = dissociation constant of ES complex ;
K_i = dissociation constant of EI complex;
αK_i = dissociation constant of ESI complex when S occupies the enzyme;
αK_s=dissociation constant of ESI complex when I occupies the enzyme;
k_p=rate constant for the breakdown of ES to P
βk_p=rate constant for the breakdown of ESI to P.

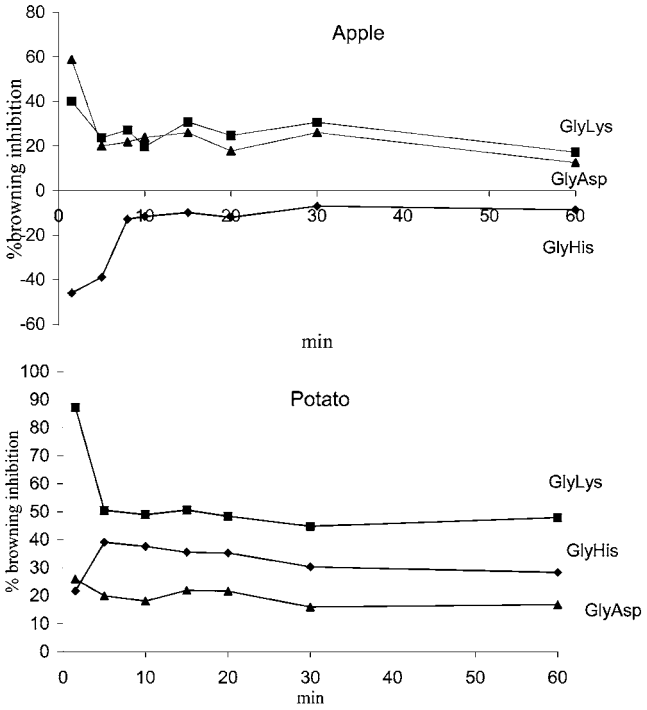


Figure 5. Effect of some dipeptides on browning of apple and potato versus time. Conditions as reported in the Experimental Section.

value of 20% inhibition for GlyAsp was found in both the foods, while for GlyLys and GlyHis a greater browning inhibition in potato was found. In addition, it is interesting to note that GlyLys appeared to be the most effective inhibitor reaching the value of 50% in potato, while in apple appeared only slightly more effective than GlyAsp.

GlyHis presented an anomalous behavior. Indeed, it appeared that (i) the % browning inhibition value of GlyHis increased in both foods, instead of decreasing, as a function of time, and (ii) the % values are always negative in apple. These anomalous results, taking into account the given definition of % browning inhibition, are obviously due to the more intense "total color difference" of the GlyHis treated samples ($\Delta E^*_{\text{inhibitor}}$) than the controls ($\Delta E^*_{\text{control}}$). In this case, the formation of an adduct quinone−GlyHis may be due to quinones enzymatically formed by DOPA and other natural phenolic compounds present in apple. An example is chlorogenic acid, present in plentiful amount in fruit, which is known (*16*) to form an *o*-quinone, which combines very well with imidazole group (like that present in hystidine), giving compounds more intensely colored than the simple polymers.

Conclusions. The obtained results show that dipeptides varied widely in their inhibition effect on mushroom PPO activity. In the major cases (GlyAsp, GlyGly, GlyTyr, GlyPhe, GlyLys) prevention of melanin formation is due to the direct inhibition of the enzyme catalyzed reaction. In the case of GlyHis, the inhibition is indirect, due to blocking subsequent melanin-forming reactions of the enzymatically formed dopaquinone. In the remaining cases such as GlyPro and GlyLeu, no inhibition effect is evidenced.

In addition, it is demonstrated that some dipeptides influence the browning of apple and potato with an appreciable degree of variation depending on the nature of both dipeptides and matrix. This is an important point when considering the control of enzymatic browning, and universal methods are unlikely to be appreciable. Consequently, specific control measurements may have to be developed for individual systems. It is also evidenced that although only GlyLys produces a significant effect on potato browning prevention, it may be suggested to survey other dipeptides with the aim of finding more effective nontoxic browning inhibitors.

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