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Inactivation of polyphenol oxidase by ultraviolet irradiation: Protective effect of melanins

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ABSTRACT

Most of the studies about UV irradiation of fruit and vegetable derivatives have been carried out in order to assess its effect on microbial inactivation. Nevertheless, there are few references about UV influence on some enzyme activities that are important in this kind of food, especially polyphenol oxidase, which is responsible for enzymatic browning in fruit and vegetable tissues containing phenolic or polyphenolic compounds. In this work, the effect of UV–Vis irradiation on polyphenol oxidase from *Agaricus bisporus* was investigated. A reduction of 58.7% in enzyme activity was achieved in the first 90 s, and it was completely inactivated after 35 min of treatment. In addition, the protective effect of melanins synthesized by the action of the same polyphenol oxidase was assessed. These pigments absorbed some of the radiant energy and led to a slower inactivation of polyphenol oxidase.

1. Introduction

Polyphenol oxidase (PPO, tyrosinase, E.C. 1.14.18.1) is a copper-containing enzyme that catalyzes two distinct reactions involving molecular oxygen and various phenolic substrates: the *o*-hydroxylation of monophenols to *o*-diphenols (monooxygenase or cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity). Later polymerization of these compounds leads to the formation of an heterogeneous group of melanins (Falguera et al., 2010a). The techniques to control PPO activity should act on one or more of the essential components necessary for the reaction: oxygen, enzyme, copper or substrate. PPO is one of the main enzymes that have to be inactivated during fruit derivatives processing (Falguera et al., 2011).

Food safety is one of the most important issues that food industries and food service companies have to face. The growing demand for a greater variety of prepared food and dishes on a menu makes the risk of contamination increases, especially due to the characteristics of this kind of food that gives a great importance to the quickness of meals. The application of HACCP (Hazard Analysis and Critical Control Points) has increased safety guarantees in production systems, thermal treatments and cold chains, helping to reduce the opportunities for pathogens to gain access to food and/or grow to levels that will pose a risk of infection or toxin production. However, the incidence of foodborne diseases continues to rise in most industrialized countries (Bintsis et al., 2000).

Currently, heat treatment process is the most commonly used hurdle for inactivating microorganisms and enzymes, extending products shelf life. However, this process may have adverse effects on sensory and nutritional quality of food (Braddock, 1999). In this context, non-thermal technologies have received increasing attention in recent years, especially in the preservation of beverages, due to its potential for inactivating spoilage and pathogenic microorganisms (Noci et al., 2008).

Most of the studies about UV irradiation of liquid food have been carried out in order to assess its effect in microbial inactivation. Nevertheless, there are few references about UV influence on some enzyme activities that are important in fruit derivatives, especially tyrosinase, which is still the major practical limitation to fruit handling, storage and processing (Jiang et al., 2004). In addition, fruit juices contain some components that may protect these enzymes to be denatured by the irradiation process (Guerrero-Beltrán and Barbosa-Cánovas, 2004; Koutchma, 2009). Among these compounds, the dark polymers formed by enzymatic browning have a special interest.

Some studies carried out on crystallins have shown that, in those enzymes, UV radiation causes oxidation of SH groups, changes in native conformation of the latter and formation of covalent cross-links between polypeptides (Krivandin et al., 2009). Since enzymatic activity depends on its structure, these modifications lead to enzyme inactivation. Such UV light-induced modifications have been reported to occur via two major routes: direct photo-oxidation arising from the absorption of radiation by the protein structure or bound chromophore and indirect protein oxidation mediated by singlet oxygen generated by energy transfer by either protein bound or other chromophores. In spite of this

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knowledge, the effect of UV light on structure and function of food enzymes is still a matter of speculation (Manzocco et al., 2009).

Thus, this piece of work has had two main aims. The first one has been to assay the inactivation of *Agaricus bisporus* polyphenol oxidase by means of UV–Vis irradiation. The second one to assess the protective effect that melanins synthesized from this enzyme and the two kinds of its substrates (L-tyrosine, a monophenol, and 4-methylcatechol, an *o*-diphenol) have on PPO during the irradiation process.

2. Materials and methods

2.1. Melanin synthesis and recovery

Agaricus bisporus polyphenol oxidase (Sigma Chemical, St. Louis, MO) was diluted in a 50 mM sodium phosphate buffer (pH 6.5) to an enzyme solution activity of 500 U mL^{-1} and distributed in aliquots of 1 mL. L-Tyrosine and 4-methylcatechol (Sigma Chemical, St. Louis, MO) were prepared in sodium phosphate buffer (pH 6.0 and 8.0, respectively, following the optimal pH for each kind of substrate determined in a previous work – Falguera et al., 2010b) in a concentration of 4.0 mM. The final enzyme content in each reaction mixture was 10 U mL^{-1}. After 24 h of reaction, melanins were precipitated adjusting the solution pH to 2.0 with HCl. The mixture was centrifuged in an Avanti J-26XP Centrifuge (Beckman Coulter, USA) for 12 min at 12,000 rpm. The supernatant was discarded; the pellet was recovered with distilled water, lyophilized and rediluted in 50 mL of dimethyl sulfoxide (Sigma Chemical, St. Louis, MO) in different concentrations.

2.2. UV–Vis irradiation process

UV–Vis irradiation was carried out in a dark chamber containing the sample and the lamp. Five hundred milliliters of the sample was placed in a methacrylate tank of 22 × 15 × 10 cm. Each sample contained 12.5 U mL^{-1} of PPO in sodium phosphate buffer (pH 6.5) and different melanin concentrations, from 0.00 to 0.20 mg mL^{-1}. A refrigeration system consisting in a metallic coil fed with cold water was used to control temperature and avoid heating. Temperature was maintained at 25 ± 1 °C in all experiments. A magnetic stirrer was used during irradiation to ensure that the entire sample was subjected to the same UV dose. UV radiation was produced with a Philips HPM-12 high-pressure mercury lamp of 400 W of nominal power that emits in a range between 250 and 740 nm (Philips, Eindhoven, The Netherlands), used in previous pieces of work (Ibarz et al., 2009; Falguera et al., 2011). Experimental series were carried out by duplicate.

2.3. PPO activity determination

PPO activity was assayed measuring the increase in absorbance at 420 nm using 4-methylcatechol as substrate, prepared in a phosphate buffer solution with pH 6.5. The reaction was carried out in a 1 cm light path optical glass cell. One unit of PPO was defined as the amount of enzyme that caused the increase of one unit of absorbance at 420 nm in one min (Ülker-Yerlitürk et al., 2008). PPO activity was assayed by duplicate.

2.4. Kinetic models

In some cases using different techniques, enzyme inactivation has been reported to follow a first order kinetic mechanism (Giner et al., 2001):

$$N \xrightarrow{k} D$$

where N and D are the native and the inactivated form of the enzyme, respectively.

This scheme leads to the following kinetic equation:

$$RA = RA_0 \cdot e^{(-k \cdot t)} \quad (1)$$

where RA is enzyme residual activity, RA_0 is the intercept of the curve, k is the first order kinetic constant and t is the time of treatment. In other cases, enzyme inactivation is supposed to occur in two consecutive irreversible first order steps with the presence of intermediate active forms of the enzyme (I), being the first one faster than the second one (Giner-Seguí et al., 2006):

$$N \xrightarrow{k_1} I \xrightarrow{k_2} D$$

and leading to the equation:

$$RA = e^{-k_1 \cdot t} - \frac{k_1 \cdot \Lambda}{(k_1 - k_2)}(e^{-k_1 \cdot t} - e^{-k_2 \cdot t}) \quad (2)$$

being k_1 and k_2 are the kinetic constants for the first and the second stages, respectively, and Λ *is* the ratio between the activities of the intermediate (partially inactivated, I) and the native (N) forms of the enzyme.

Experimental data were fitted to the kinetic expressions by non-linear regression procedures using Statgraphics Plus 5.1 statistical data processing software (STSC Inc., Rockville, MD, USA). The fittings and the estimates were calculated at a 95% significance level.

3. Results and discussion

3.1. Inactivation of polyphenol oxidase in a model solution without any pigmentation

The irradiation of the sample containing 12 U mL^{-1} of *A. bisporus* polyphenol oxidase led to the complete inactivation of the enzyme after 35 min of treatment. However, in the first 90 s a big decrease in PPO activity was observed, being reduced to nearly the 70% of its original value. After 4 min of irradiation, residual activity was only 30% of the initial activity. It is also important to state that PPO activity was not recovered after 24 h in any taken sample, so the achieved inactivation was irreversible. In a previous piece of work performed with the same equipment, 20 min of irradiation were required to completely inactivate bovine carboxypeptidase-A, and 12 min were necessary to denature porcine trypsin (Ibarz et al., 2009).

3.2. Inactivation of polyphenol oxidase in a model solution containing melanins from L-tyrosine

Fig. 1 shows the evolution of polyphenol oxidase residual activity with irradiation time depending on melanin concentration in the medium. The enzyme was completely inactivated in all experimental series; the time that was necessary to accomplish this denaturalization was higher as the pigment concentration increased, showing the protective effect of these polymers. Thus, as it has been stated in the previous section, with no pigments in the medium polyphenol oxidase was inactivated after 35 min of irradiation. Meanwhile, with 0.200 mg mL^{-1} of melanin from L-tyrosine 70 min were required. The bigger decrease in enzyme activity in the first 90 s of treatment was especially remarkable in the samples with low melanin content. Another time, PPO activity was not recovered after 24 h in any sample, so the achieved inactivation was also irreversible.

In a previous piece of work carried out with the same equipment, 100 min were required to inactivate polyphenol oxidase in apple juices from four different varieties (Falguera et al., 2011). These differences show that the other compounds that juices contain have also a protective effect against the enzyme denaturaliza-