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Cold plasma effects on enzyme activity in a model food system

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ABSTRACT

Polyphenoloxidase (PPO) and peroxidase (POD) are enzymes that need to be inactivated or inhibited in order to avoid undesirable browning reactions and the loss of sensorial or nutritional quality of fruits and vegetables. As a promising "gentle" alternative to traditional methods, such as pasteurisation or the use of antibrowning compounds, the present study investigates cold plasma's ability to inactivate PPO and POD in a model food system, as well as possible inactivation mechanisms.

The study shows that cold plasma is capable of reducing the activity of both PPO and POD in the model food system. The activity of PPO was reduced by about 90% after a treatment time of 180 s. POD was more stable and was reduced by about 85% after 240 s. Circular dichroism and tryptophan fluorescence measurements indicate that the reason for their loss of activity is based on a plasma-induced modification of their secondary structure. A decrease in the alpha-helix content was accompanied by an increase of the percentage of beta-sheet regions. Reduced and red-shifted tryptophan fluorescence intensities supported these changes.

Industrial relevance: The quality of freshly cut fruits and vegetables greatly depends on the activity of naturally occurring enzymes such as PPO and POD, which catalyse browning reactions at cut surfaces. The presented study shows that cold plasma, as a promising non-thermal pasteurisation technology, is capable of reducing the activity of these enzymes in a model food system. In addition, it describes the impact of different treatment parameters and gives insights into inactivation mechanisms. The results contribute to the understanding of cold plasma effects on enzyme activity and could be a basis for a possible industrial implementation.

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1. Introduction

The enzymes polyphenoloxidase (PPO, also known as tyrosinase), and peroxidase (POD), are to be found particularly in fresh fruits and vegetables. Both are keys to enzymatic browning and thus play a major role in quality losses during post-harvest handling and processing. Only in some cases (such as the production of raisins, cocoa and fermented tea leaves) is the activity of PPO needed in order to produce distinct organoleptic properties (Seo, Sharma, & Sharma, 2003).

PPO and POD belong to a group of plant-based oxidoreductases and are kicked into action through operations such as peeling, slicing or cutting, where they are separated from the membranes and thus come into contact with polyphenolics (Baltes, 2000).

In the case of PPO, monophenolics or o-diphenols are usually dehydrated to instable o-diquinones, depending on the availability of oxygen (Fig. 1). This triggers the formation of melanins and causes the affected areas to become a brownish colour (Boonsiri, Ketsa, & van Doorn, 2007; Valentines, Vilaplana, Torres, Usall, & Larrigaudière, 2005).

Enzymatic browning is also associated with peroxidase, whose primary function is to oxidize phenolic compounds at the expense of H_2O_2, leading to negative flavour changes during storage. Peroxidase is the most heat-stable vegetable enzyme and thus is also used as an indicator for successful blanching (Hendrickx, Ludikhuyze, Van den Broeck, & Weemaes, 1998).

Due to the degradation of phenolic compounds, sensorial losses such as browning and an off-flavour are also associated with losses of nutritional value (Vámos-Vigyázó, 1995). Therefore, the inactivation of PPO and POD is a crucial indicator of quality in the processing of fruits and vegetables.

Several approaches have been established in order to prevent enzymatic browning. Bachem et al. (1995) investigated the modification of PPO- and POD-encoding genes, which led to decreased browning rates and reduced post-harvest inactivation. It has also been shown that PPO and POD levels are influenced by environmental and agricultural factors, such as irrigation (Sannomaru, Katayama, Kashimura, & Kaneko, 1998), fertilisation (Misra, Sukumaran, & Verma, 1991) and salt stress (Sancho, Milrad de Forchetti, Pliego, Valpuesta, & Quesada, 1996), and these factors can therefore be used for control.

In addition, antibrowning compounds, such as reducing agents, chelating agents and inorganic salts, present opportunities in the post-harvest chain. The effects of ascorbic acid, sodium bisulfate and

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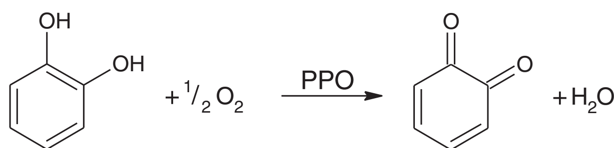

Fig. 1. Conversion of o-diphenol to o-diquinone, catalysed by polyphenoloxidase (PPO).

other reducing agents on PPO have been controversial over the years (Golan-Goldhirsch & Whitaker, 1984), and the use of sulfites has become more and more restricted due to potential health hazards (Taylor & Bush, 1986). Thus, complying with safety and regulatory standards while also maintaining the sensory attributes of the fruit or vegetable is one of the major difficulties when selecting a method to prevent browning (Dorantes-Alvarez & Chiralt, 2000).

The conformational structure of enzymes is also of great importance. Besides the primary structure, which is determined by the amino acid sequence, and the tertiary structure, representing the spatial order of secondary structures, the secondary structure itself is an essential property of enzymes. Owing to the key-lock principle, it is jointly responsible for their degree of activity. By today, the impact of various thermal and non-thermal food processing techniques on the activity of PPO and POD has been studied. All of them are associated with corresponding structural changes.

Thermal processes are used for the inactivation of enzymes in fruit juices and canned fruits, where heats of up to 80 °C are used to inactivate microorganisms as well as unwanted enzymes. These processes have some negative outcomes, however, such as losses of sensorial and nutritional value and the associated problems concerning marketability (Vámos-Vigyázó, 1995). For these reasons, attention has turned to the development of alternative non-thermal processes. In particular, much attention has been paid to pulsed electric fields (PEF: see Mertens & Knorr, 1992; Knorr & Angersbach, 1998; Barbosa-Cánovas, Góngora-Nieto, & Swanson, 1998). The effect of PEF on the activity of PPO and POD was extensively studied by Ho, Mittal, and Cross (1997), Giner-Segui, Rauret-Ariño, Barbosa-Cánovas, and Martín-Belloso (1997) and Giner, Gimeno, Ortega, Barbosa-Cánovas, and Martín (1999). Another key avenue of research has been high hydrostatic pressure (HHP) processes. For example, Buckow, Weiss, and Knorr (2009) examined the impact of HHP on the stability of PPO in cloudy apple juice.

Less information is available regarding enzyme inactivation by plasma; only the denaturation of proteins by atmospheric pressure glow discharges has been studied (Deng, Shi, Chen, & Kong, 2007). Plasma can be described as the "fourth state of matter", generated by applying energy in the form of heat, voltage or electromagnetic fields to a gas, and leading to such reactions as ionisation, excitation and dissociation. As a consequence, various active components are formed, such as radicals, UV radiation and charged particles. Reactive oxygen species (ROS) play a particularly important role (Laroussi & Leipold, 2004). ROS, like atomic oxygen or OH radicals, attack parts of the cell membrane and start oxidation reactions, leading to unsaturated lipids disintegrating into lipid peroxides. Other targets can be amino acids like tryptophan, which are sensitive to oxidation, as well as the DNA (Mogul et al., 2003). The aromatic amino acids tyrosine, tryptophan and phenylalanine can be found in PPO as well as in POD and particularly tryptophan emits light in the region between 300 and 350 nm after excitation at 280 nm. Changes in tryptophan fluorescence can be used as an indicator of oxidation reactions and (subsequent) changes of the conformation and three-dimensional structure of proteins (Gießauf, Steiner, & Esterbauer, 1995; Vivian & Callis, 2001). It is also suggested that plasma immanent species lead to –C–C– and/or –C–H bond breaking reactions, resulting in the formation of carboxyl and carbonyl groups (Grzegorzewski et al., 2010).

For the generation of cold plasmas, corona discharges, dielectric barrier discharges and atmospheric pressure plasma jets are common setups (Ehlbeck et al., 2010).

Together with their non-thermal operation under atmospheric pressure, cold plasmas could be suitable for the treatment of heat sensitive foods like fruits and vegetables.

This study investigates the effect of an atmospheric pressure plasma treatment on enzyme inactivation in a model food system. The impact of different treatment conditions, such as treatment time and plasma gas composition, has been investigated. In order to gain insights into the enzyme inactivation mechanisms, changes in protein conformation and tryptophan fluorescence were also studied.

2. Material and methods

2.1. Enzymes

PPO (from *Agaricus bisporus*, EC 1.14.18.1, Sigma-Aldrich, Steinbach, Germany) as well as POD (from horseradish, EC 1.11.1.7; Sigma-Aldrich, Steinbach, Germany) were dissolved in a PBS buffer (pH 6.5) to initial concentrations of 2.32 μM and 1.7 μM, respectively. These stock solutions were prepared once for the whole study. There were divided into small portions of 1 ml, put into Eppendorf flasks and stored at −18 °C until usage. The remaining activities were controlled frequently.

The obtained enzyme solutions were then used for CD measurements or for the preparation of poloxamer gel (see Section 2.2).

2.2. Model food system

Shortly before the cold plasma treatment, the enzymes were introduced into the two-layer model system. The lower layer consisted of a gellan gum plate (Gelrite™; Carl Roth, Karlsruhe, Germany), which had a diameter of 12 mm and a height of 1 mm. This type of gel is chemically inert and provides constant quality since it is unaffected by the vagaries of natural conditions. Moreover, it does not contain contaminating substances that could have an influence on plasma processing. Following Fröhling, Baier, Ehlbeck, Knorr, and Schlüter (2012), Gelrite was dissolved in dH_2O under continuous warming and stirring to obtain a 1% solution. After autoclaving, a specified amount of a 10% $CaCl_2$ solution was added to start the gelification. In order to obtain a homogeneous height of 1 mm, 20 ml aliquots of the gel were finally poured into Petri dishes and stored until usage.

Gellan gum plates were cut out and coated with 35 μl of a 30% poloxamer gel (Pluronic F-127®; Sigma-Aldrich, Steinbach, Germany), which was prepared using the appropriate enzyme solution (see Section 2.1) as a solvent. The height of this layer was approximately 310 μm (35 μl spread on an area of around 1.13 cm^2). Pluronic F-127® is a cold-gelling substance, which has solid properties in a temperature range from 20 to 80 °C but liquefies below 15 °C. This characteristic makes it possible to gently introduce the enzymes into it and to extract them very easily after the treatment.

In order to keep the poloxamer gel solid, the sample temperature has been kept in an average temperature range from 20 to 29 °C. Thermal effects, occurring as a result of dehydration due to the gas flow, have been avoided by the property of the lower layer to serve as a kind of water reservoir, which continuously delivers water to the poloxamer. It was therefore possible to consider the reactive plasma species as the only components involved in enzyme inactivation.

2.3. Cold atmospheric pressure plasma jet and experimental set-up

As shown in Fig. 2, cold plasma was generated by a plasma jet (kINPen 09; neoplas tools GmbH, Greifswald Germany), which has already been described and characterized by Weltmann et al. (2009). The device consists of the plasma jet itself (length: 170 mm; diameter:

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