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Polymerization of phenolic compounds by polyphenol oxidase from bell pepper with increase in their antioxidant capacity

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ABSTRACT

Bell pepper (*Capsicum annuum*) fruits are rich in flavonoids, which have high antioxidant capacity. Polyphenol oxidase (PPO) from bell pepper fruit was partially purified and used to polymerize phenolic compounds. Hydrodynamic diameters of the resulting products were analyzed with dynamic light scattering, and their relation to antioxidant capacity studied. Bell pepper PPO catalyzed polymerization of free phenolic compounds, producing 1500 nm aggregates with antioxidant capacity greater than the corresponding monomers. Polymerization was greatest using epicatechin, forming 5000 nm aggregates with antioxidant capacity higher than the monomer. The aggregates produced from ferulic acid exhibited 15 times more antioxidant capacity than the monomer. Quercetin was the least polymerized, but its aggregates still had antioxidant capacity higher than its monomer.

Polimerización de compuestos fenólicos por Polifenol oxidasa de pimiento morrón con incremento en su capacidad antioxidante

RESUMEN

Los frutos de chile (*Capsicum annuum*) son ricos en flavonoides, los cuales poseen una gran capacidad antioxidante. La polifenoloxidasa (PFO) de *Capsicum annuum* fue parcialmente purificada para polimerizar compuestos fenólicos. Los diámetros hidrodinámicos de los productos resultantes fueron analizados mediante el análisis dispersión dinámica de luz y su relación con la capacidad antioxidante. La PFO catalizó la polimerización de los compuestos fenólicos libres formando agregados de 1500 nm con mayor capacidad antioxidante que sus monómeros. Se identificó una mayor polimerización con epicatequina formando agregados de 5000 nm con una capacidad antioxidante superior al monómero de epicatequina. Los agregados de ácido ferúlico presentaron una capacidad antioxidante 15 veces mayor comparada con su monómero. La quercetina fue la menos polimerizada pero aún con valores de capacidad antioxidante mayores al monómero.

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1. Introduction

Free radicals are generated *in vivo* for specific purposes in metabolism, but can become problematic if imbalances develop between free radical generation and organism's defense mechanisms (Amic, Davidovic-Amic, Beslo, & Trinajstic, 2003). When this occurs, oxidative damage is produced in target cells (specific locations in biomolecules, such as proteins, lipids, and nucleic acids, where pharmaceuticals implement actions) (Einbond, Reynertson, Luo, Basile, & Kennelly, 2004). Antioxidants are the organism's defense against free radicals and the oxidative damage they cause, which has drawn increased interest in the use of natural antioxidants as food additives (Toscano, Colarieti, & Greco, 2003). Flavonoids are an extensive group of phytochemical phenolic compounds with a relatively low molecular weight (MW) and *in vitro* antioxidant properties that depend on the number and position of their hydroxyl groups. Flavones, flavonols (rutin, kaempferol, and quercetin), and anthocyanins belong to this group (Havsteen, 1983). Due to their high antioxidant activity, they play major role in the prevention of

cardiovascular diseases, cancer, and degenerative diseases (Havsteen, 2002; Heim, Tagliaferro, & Bobilya, 2002).

Capsicum annuum L., known as chili, bell pepper, and/or pepper, is a crop grown in Mexico, Central and South America. It is one of the five most consumed species in the world considered as a source of antioxidants such as phenols, flavonoids, and condensed tannins (Obloh, Puntel, & Rocha, 2007). Bell pepper is a good alternative source of phenols obtained from polymerization studies. Polyphenol oxidase (PPO) (E.C. 1.14.18.1) is the main enzyme responsible for browning reactions in fruits and vegetables. However, a possible synergy has been proposed between PPO and peroxidase due to generation of hydrogen peroxide during oxidation of phenolic with PPO (Arslan, Erzenin, Sinan, & Ozensoy, 2004). Reports indicate that in the case of the enzymatic polymerization of simple phenols and some flavonoids, the polymerization affects their radical-scavenging activity (Anthoni et al., 2010, 2008). The enzymatic polymerization of phenolic compounds was the subject of several studies to improve their solubility and/or their stability and provide new properties. These properties are dependent on the molecular mass and the structure of polymers (Mohamed

& Latifa, 2012). Studies have been conducted on phenolic compound polymerization using the enzymes laccase and tyrosinase from the fungus corn smut *Ustilago maydis* and rutin as substrate (quercetin-3-rutinoside) (Desentis-Mendoza et al., 2006). These produced polyrutin, a flavonoid polymer with antioxidant properties superior to those of the rutin monomer (Desentis-Mendoza et al., 2006). Desentis-Mendoza et al. (2006) studied the enzymatic polymerization of phenolic compounds and their effect on the antioxidant activity and the protection effects from oxidative injury of hepatic human cell line WRL-68. The oxidation of (Kurisawa, Chung, Uyama, and Kobayashi, 2003) catechin by horseradish peroxidase (HPR)–hydrogen peroxide and laccase with shuttle oxidant effectively suppresses lipid peroxidation in biological tissues and subcellular fractions such as microsomes and low-density lipoproteins (Hosny & Rosazza, 2002). Anthocyanin and flavonol profiles have been identified as an effective tool in differentiating similar varieties of grapes. Enzymatic polymerization of phenolic compounds (catechol, resorcinol, and hydroquinone) was carried out using laccase. The number-average molecular weights of the polyphenols ranged from 1000 to 1400 Da (corresponding to the degree of polymerization that varied from 10 to 12) with a lower polydispersity value of about 1.10, showing selective polymerization of phenolic compounds catalyzed by laccase (Sun et al., 2013). Kim, An, and Song (2003) showed that the polymerization of cardanol, using soybean peroxidase, produces films with excellent anti-biofouling activity against *Pseudomonas fluorescens*. Figueiredo-González, Cancho-Grande, and Simal-Gándara (2013) reported an incremental decrease in all phenolic compounds throughout the dehydration process. That can be due to the effects of copigmentation reactions of anthocyanins with the flavonols or with the hydroxycinnamic acids, and/or the contact of PPO with highly suitable substrate or with other polyphenols less suitable that can be altered by enzymatically produced quinonas via coupled oxidation reactions. The present study determined the effectiveness of phenolic compound polymerization by partially purified PPO from bell peppers (*Capsicum annuum* L.) and evaluated its relationship to antioxidant activity.

2. Materials and methods

2.1. Biological material

Bell peppers (*Capsicum annuum*) were purchased from commercial market in Ixtapaluca municipality, State of Mexico, Mexico. Selected fruits were mature (red color) and w1109.28 without signs of rotting or deterioration. They were cleaned and stored at -20°C until use. The PPO activity was evaluated during 16 days of maturation. After that, all the bell peppers were processed.

2.2. Crude extract

Crude extract was prepared following Castro et al. (2008). Briefly, 50 g of bell pepper without seeds was crushed with liquid nitrogen in a porcelain mortar to rupture cell membranes. The cold bell pepper was then homogenized in 75 mL sodium phosphate buffer (0.2 M, pH 6.5) and 4% (v/v) polyvinylpyrrolidone (PVP, Sigma, Sigma Chemical P-6755, Toluca, Mexico). The mixture was shaken for 1 h at 4°C . The resulting homogenate was filtered through layers of

cheesecloth and centrifuged at 15,000 rpm for 20 min at 4°C . The supernatant was recovered as the crude extract and used for enzyme extraction.

2.3. Enzyme extraction

Enzyme extract was obtained by precipitating the crude extract with ammonium sulfate at 90% saturation. The precipitate was collected by centrifuging at 15,000 rpm for 20 min at 4°C . It was then dissolved in a minimum volume of sodium phosphate buffer (20 mM, pH 6.5) and dialyzed for 24 h at 4°C using the same buffer and two volume changes. The dialyzed precipitate was concentrated using ultrafiltration in a nitrogen atmosphere (Amicon[®] Millipore Inc, Beverly, MA, USA) with a 10 kDa molecular membrane.

2.4. Native polyacrylamide gel electrophoresis

Gels were made by electrophoresis under native conditions following Laemmli (1970).

2.5. Gel PPO activity

Post-electrophoresis enzyme activity was identified following the technique described by Casado-Vela, Selles, and Bru (2005). Protein molecular weight (MW) was calculated using molecular weight reference standards ranging from 14.4 to 97 kDa (Low Molecular Weight Calibration Kit, Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA).

2.6. Electroelution

The electroeluted protein was obtained from native electrophoresis (Bio-Rad Electroeluter Model 422, Hercules, CA, USA) following manufacturer's instructions at a constant 60 mA for approximately 8 h. Finally, the PPO activity was measured to electroeluted fraction.

2.7. Protein determination

Protein content was measured by Bradford method (1976) using bovine serum albumin (Sigma) as standard.

2.8. PPO activity

Spectrophotometry was used to measure enzyme activity following Muñoz, Bravo, Zapata, and Londoño (2007) and with catechol (Sigma) as substrate. One PPO activity unit was defined as a 0.001 increase in absorbance per minute at 420 nm and 20°C .

2.9. PPO characterization

2.9.1. Optimum pH

The effect of pH on enzyme activity was measured according to Gasull and Becerra (2006) with some modifications. Different pHs were tested using 10 mM sodium phosphate buffer (pH 4–7), 10 mM HCl-Tris buffer (pH 8–9), and borate buffer (pH 10–11). Enzyme activity was measured as described above using 0.5 M catechol as substrate.

2.10. Optimum temperature

This parameter was determined using the enzyme activity assay described above, and by incubating the mixture at different temperatures (15, 25, 35, 45, and 55°C) using 10 mM sodium phosphate buffer (pH 6.5) in the mixture reaction.

2.11. Substrate specificity

The activity of the PPO activity against catechol, caffeic acid, chlorogenic acid, quercetin, L-DOPA, and protocatechuic acid substrates was determined according to the standard conditions described for the enzyme assay. Each reaction was done in triplicate (Muñoz et al., 2007).

2.12. Free phenolic compounds extraction

According to the method described by Adom and Liu (2002) with some modifications; phenolic compounds were extracted from a mixture of 2 g sample and 8 mL ethanol (80%) for 10 min. The solution was centrifuged at 13,000 rpm for 10 min at 4°C, the supernatant was then removed and the extraction repeated one more time. Extracts were stored at 4°C until use.

2.13. Total phenolic content

Total phenol content was measured following Adom, Sorrells, and Liu (2005). Briefly, 0.75 mL Folin Ciocalteu reagent (Sigma) diluted 10 times with distilled water was added to 0.1 mL extract. This mixture was left to rest for 5 min in darkness and then neutralized with 0.75 mL NaHCO₃ at a 60 g/L. After allowing the mixture to rest for 90 min, absorbance was measured at 725 nm using a spectrophotometer (GBC UV/VIS 918). Total phenol content was expressed as gallic acid (GA) equivalents per gram of sample using GA as standard.

2.14. Radical-scavenging activity

This was measured by adding 10 µL sample to 990 µL ABTS⁺ (2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid), radical adjusted to 0.7 ± 0.02 absorbance. Absorbance measurements were taken at intervals of 1–7 min. Anhydrous ethanol was used as a blank. Antioxidant activity was expressed in millimole Trolox equivalent antioxidant capacity (TEAC) per gram (Adom et al., 2005).

2.15. Dynamic light scattering (DLS) assays

DLS measures Brownian motion and relates this to the size of the particles. Brownian motion is the random movement of particles due to the bombardment by the solvent molecules that surround them (Malvern Instruments, Malvern, UK). Analysis of the hydrodynamic diameter distribution of the partially purified protein (i.e. the enzyme solution) and free phenols was done using the DLS technique with a Malvern Zetasizer (Nano Series S-90, Malvern Instruments) at 25°C in the 'typical protein' option. The samples were filtered with 0.25 µm filters prior to measurement with DLS. Particle diameter measurements are the result of readings taken at each time corresponding to a DV_{0.9} = 6 µm, meaning that 90% of

particle volume measures ≤6 µm. Four combinations (E) of free phenols and enzyme extract were assayed for enzymatic polymerization: (E1) 1.5 mL free phenols (4.5 mg/mL gallic acid (GA)) + 1.5 mL enzyme extract (3.6 U); (E2) 1.5 mL free phenols (2.3 mg/mL GA) in 80% ethanol + 1.5 mL enzyme extract (3.6 U); (E3) 0.4 mL free phenols (4.5 mg/mL GA) + 2.4 mL sodium phosphate buffer (10 mM, pH 6.5) + 0.2 mL enzyme extract (3.6 U); and (E4) 0.4 mL free phenols (2.3 mg/mL GA) in 80% ethanol + 2.4 mL sodium phosphate buffer (10 mM), pH 6.5 + 0.2 mL enzyme extract (3.6 U). Four enzymatic polymerization assays were also done as described above but using pure phenolic compounds (ferulic acid [Sigma]; quercetin [Sigma]; and epicatechin [Sigma]) as substrates at a 0.25 mg/mL initial concentration.

3. Results and discussion

3.1. Enzyme activity and protein content

Protein concentration was higher in the crude extract (1.66 g/100 g) than in the enzymatic solution (1.12 g/100 g; Table 1). These contents are higher than the 1 g/100 g reported by Moreiras, Carbajal, Cabrera, and Cuadrado (2007) in bell pepper. Differences in protein concentration can be attributed to fruit-ripening stage at harvest, culture conditions, and region of origin (Rivero et al., 2001). Specific PPO activity in the enzyme extract was 3.6 U/mg protein. This value is higher than 1.92 U/mg from PPO mushroom (Fulya, Ahmet, Arzu, Nagihan, & Ertugrull, 2011). Specific activity values for other plants include 79.83 U/mg protein for potato (Lin et al., 2010) and 1.50 U/mg protein for carnation (Roquesa & Higuera, 2007).

3.2. Native polyacrylamide gel electrophoresis

The enzyme extract had a well-defined, intense band at 54 kDa (Figure 1), which is similar to MWs reported for loquat (58 kDa) (Ding, Chachin, Ueda, & Imahorl, 1998) and olive (55 kDa) (Ortega-García, Blanco, Peinado, & Peragon, 2008). Different MWs have been reported for PPOs from *Boletus erythropus* (40 kDa) (Özel, Colak, Arslan, & Yildirim, 2010), pineapple (104 kDa) (Das, Bhat, & Gowda, 1997), and potato (69 kDa) (Thygesen, Dry, & Robinson, 1994). Arnok, Ruangviriyachai, Mahachai, Techawongstien, and Chanthai (2010) were the first to report the presence of PPO in some hot *C. annuum* L. varieties, but they did not include MW data, and there are no other studies on PPO in *C. annuum*. The reason for such wide variability in MW among PPOs from different origins is still unclear, but has been attributed to one or more polymerization processes (Smith & Montgomery, 1985), glycosylation (Ganesa, Fox, & Flurkey, 1992), proteolysis, and disulfur bonds (Marques, Fleuriet, & Macheix, 1995).

Table 1. Protein concentration and enzymatic activity in bell pepper (*Capsicum annuum*) extracts.

Tabla 1. Concentración de proteínas y actividad enzimática en extractos de pimiento *Capsicum annuum*.

Fraction	Protein concentration (mg/ml)	Protein concentration (g/100 g)	Enzyme activity (U/ml)	Specific enzyme activity (U/mg protein)
Crude extract	7.97	1.66	5.00	0.63
Enzyme extract	7.68	1.12	27.78	3.62

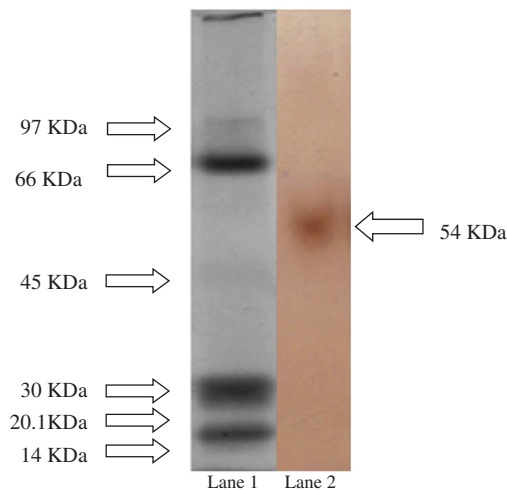


Figure 1. Native polyacrylamide gel electrophoresis of bell pepper (*Capsicum annum*): 1. Low molecular weight marker, Coomassie-stained; 2. Bell pepper enzymatic extract (7.68 mg/ml), stained with 20 mM catechol.

Figura 1. Electroforesis en gel en condiciones de nativas de Chile pimienta *Capsicum annum*: 1. Marcador de bajo peso molecular tinción de Coomassie; 2. Extracto enzimático de pimienta (7,68 mg/ml), teñido con 20 mM de Catecol.

3.3. Partial PPO characterization

Bell pepper PPO exhibited maximum activity (211.30 U/mg) at pH 6.5 (Figure 2(a)), which coincides with values reported for loquat (Dincer, Colak, Aydin, Kadioglu, & Güner, 2002), wheat (Altunkaya & Gokmen, 2011), and litchi (Jiang, 2001). According to previous reports, the optimum pH of PPOs from various plants and vegetables is commonly 4.0–8.0:

pH 7.5 for carnation (Roquesa & Higuera, 2007); pH 6 for eggplant (Concellón, Añón, & Chaves, 2004); pH 6–7 for artichoke (Aydemir, 2004); pH 5 for grape (Rapeanu, Loey, Smout, & Hendrickx, 2006); pH 5.7 for broccoli (Gawlik-Dziki, 2008); and pH 8 for *Cleome gynandra* (Gao, Liu, & Xiao, 2011). The action of PPO enzyme on the substrate depends on the optimum pH for a major enzymatic activity.

Enzyme activity was highest (132.06 U/mg) at 35°C and was absent at 45°C (Figure 2(b)). Maximum PPO activity at 35°C coincides with reports for PPO from loquat (Dincer et al., 2002), butter lettuce (Gawlik-Dziki, 2008), litchi (Jiang, 2001), mamey (Palma-Orozco, Ortiz-Moreno, Dorantes-Álvarez, Sampedro, & Nájera, 2011), and is near that reported for other PPO sources such as mint (30°C) (Kavrayan & Aydemir, 2001) and *Hevea brasiliensis* (35–45°C) (Wititsuwannakul, Chareonthiphakorn, Pace, & Wititsuwannakul, 2002). The bell pepper PPO exhibited preference for a chlorogenic acid substrate, followed by quercetin and caffeic acid (Figure 2(c)). This is similar to a PPO from tomato (Casado-Vela et al., 2005).

3.4. Phenolic compounds and antioxidant activity

Free phenolic compounds extracted from fresh bell pepper have a value of 2042 mg GA/100 g (Table 2). This is higher to the value 296 mg/100 g reported by Kevers et al. (2007) and the 257 mg/100 g reported by Howard, Talcott, Brenes, and

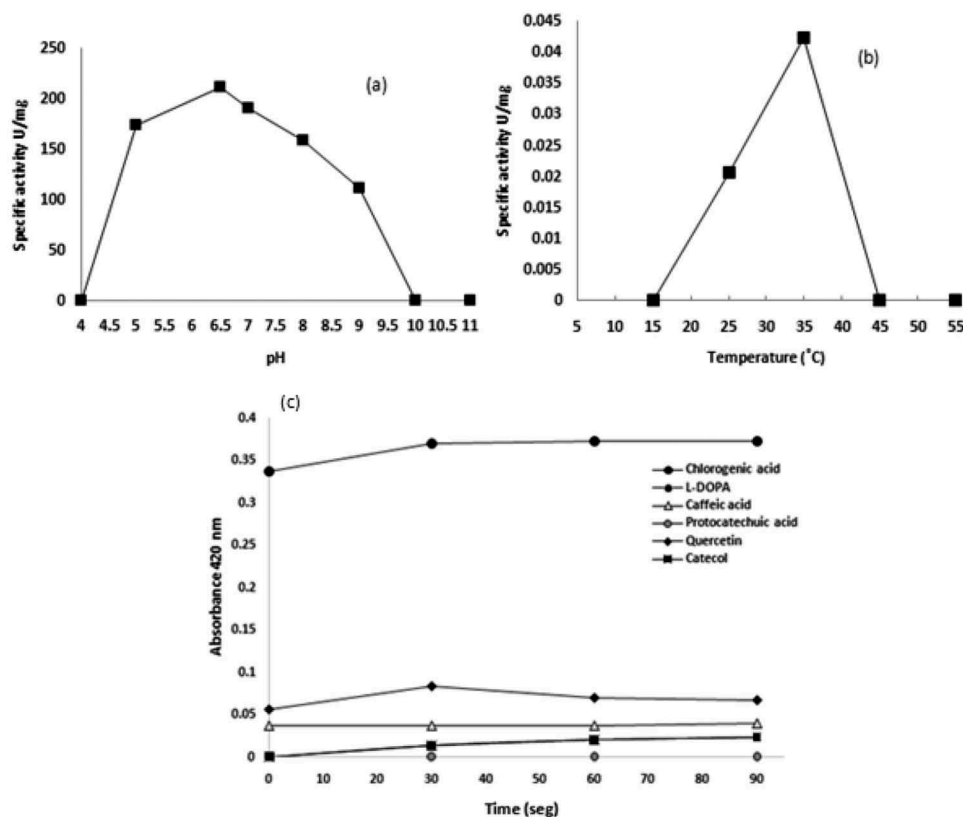


Figure 2. Bell pepper polyphenol oxidase (PPO) characterization: (a) Optimum pH, using 10 mM sodium phosphate buffer (pH 4–7), 10 mM HCl-Tris buffer (pH 8–9), 10 mM borate buffer (10–11). (b) Optimum temperature at 15, 25, 35, 45, and 55°C in sodium phosphate buffer (pH 6.59). (c) Substrate specificity, using 10 mM chlorogenic acid, L-DOPA, caffeic acid, protocatechuic acid, quercetin, and catechol. Each assay was done in triplicate.

Figura 2. Caracterización de polifenol oxidasa de Chile pimienta: (a) pH óptimo, usando regulador de fosfato de sodio a 10 mM (pH 4–7), 10 mM HCl-Tris (pH 8–9), 10 mM borato (10–11). (b) Temperatura óptima a 15, 25, 35, 45 and 55°C en fosfato de sodio (pH 6,59). (c) Especificidad de sustrato, usando 10 mM ácido clorogénico, L-DOPA, ácido caféico, ácido protocatecuico, quercetina y catecol. Cada ensayo fue realizado por triplicado.

Table 2. Concentration and antioxidant capacity of free phenolic compounds from bell pepper (*Capsicum annuum*).**Tabla 2.** Concentración y actividad antioxidante de compuestos fenólicos libres de pimiento *Capsicum annuum*.

Free phenolic compounds	Concentration as gallic acid		Antioxidant capacity	
	(mg/ml)	(mg GAE/100 g bell pepper)	(mmol TEAC/g phenol)	(mmol TEAC/g fresh weight)
	4.55	2042	1109.28	22.66

Villalon (2000), but much higher than the value 69.7 mg/100 g reported by Sun and Tanumihardjo (2007) and the 87.6 mg/100 g reported by Young, Vinson, Su, and Zubik (2006). These differences are attributable to cultivar and culture conditions in different regions. Antioxidant capacity of the phenolic compounds extracted from fresh, ripe bell pepper was 1109 mmol TEAC/g phenol (Table 2), much higher than in serrano or jalapeño chili. However, there has been reports showing antioxidant capacity in bell peppers of 7.6–100 mmol TEAC/g fresh weight (Flores, Jaramillo, Karendash, Hernández, & Dorantes, 2009; Molina-Quijada, 2009; Pellegrini et al., 2003; Sun & Tanumihardjo, 2007). In present study, the antioxidant capacity value was 22.6 mmol TEAC/g, near that reported by Molina-Quijada (2009).

3.5. Polymerization of free phenolic compounds by enzymatic oxidation and their antioxidant capacity

The activity of the enzyme and the solubility of the substrate are the two important factors for high polymerization

reaction performances. The natural environment of the enzyme is water, while the phenolic substrates are more soluble in organic solvents (Mohamed & Latifa, 2012). In the first treatment E1 (E1: 1.5 mL free phenols (4.5 mg/mL GA) + 1.5 mL enzyme extract (3.6 U)), the hydrodynamic diameters were similar or lower than the free phenols blank (1500 nm) and enzyme solution blank (1000 nm) (Figure 3(a)). This could indicate a low interaction between the PPO and free phenolic compounds. The low or absent reactivity of some phenols might be explained by their molecular structure and their higher redox potential (Mohamed & Latifa, 2012).

In the reaction of the enzyme solution (3.6 U) with a 2.3 mg/mL free phenolic compound concentration (E2: 1.5 mL free phenols (2.3 mg/mL GA) in 80% ethanol + 1.5 mL enzyme extract (3.6 U)), molecular diameters were larger than for the free phenols, and enzymatic solution blanks were at the 40, 50, 90, and 130 min reaction times. This shows that 800 nm dimers and 1000 nm trimers were formed as a result of enzymatic reaction with the substrates. Dordick, Marletta, and Klibanov (1987) observed changes in the average polymer weight of poly(phenol) from 1000 to over 26,000 g.mol⁻¹ upon varying the 1,4-dioxane content in the polymerization reaction. Mita, Tawaki, Uyama, and Kobayashi (2002) have investigated various polar solvents and their influence on the chemoselectivity of the peroxidase-initiated polymerization of phenols. In E2, the polymerization profile was not significant with respect to the antioxidant capacity (297 mmol TEAC/g phenol), this behavior is due to interactions between the enzyme, the solvent, the monomer, and the polymer, particularly to the decrease of the enzyme affinity to the substrate. In organic solvent,

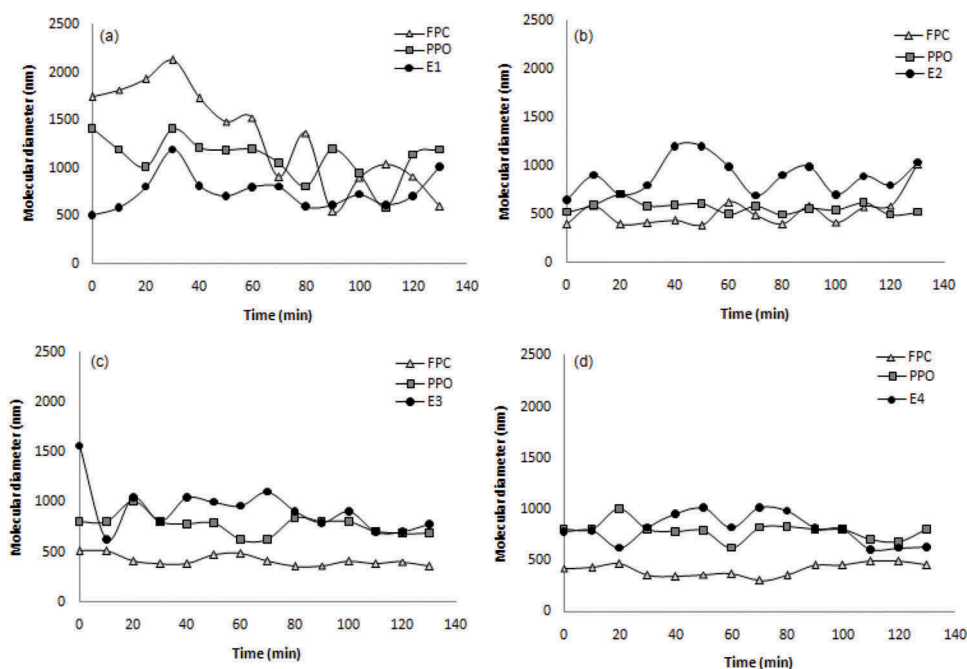


Figure 3. Dynamic light scattering (DLS) analysis of hydrodynamic diameter distributions of free phenolic compounds (FPC) and polyphenol oxidase (PPO) from bell pepper, and polymerized compounds (E1–E4). (a) (E1) 1.5 ml free phenols (4.55 mg/ml GA) + 1.5 ml enzyme extract (3.62 U); (b) (E2) 1.5 ml free phenols (2.275 mg/ml AG) in 80% ethanol + 1.5 ml enzyme extract (3.62 U); (c) (E3) 0.4 ml free phenols (4.55 mg/ml GA) + 2.4 ml 10 mM sodium phosphate regulator, pH 6.5 + 0.2 ml enzyme extract (3.62 U); and (d) (E4) 0.4 ml free phenols (2.275 mg/ml GA) in 80% ethanol + 2.4 ml 10 mM sodium phosphate regulator, pH 6.5 + 0.2 ml enzyme extract (3.62 U).

Figura 3. Análisis de la distribución de diámetros hidrodinámicos de compuestos fenólicos libres (CFL), polifenoloxidasas (PFO) de pimiento y compuestos polimerizados (E1–E4) mediante dispersión dinámica de luz (DLS). (a) (E1) 1,5 ml fenoles libres (4,55 mg/ml AG) + 1,5 ml extracto enzimático (3,62 U); (b) (E2) 1,5 ml fenoles libres (2,275 mg/ml AG) en 80% etanol + 1,5 ml extracto enzimático (3,62 U); (c) (E3) 0,4 ml fenoles libres (4,55 mg/ml AG) + 2,4 ml 10 mM regulador de fosfato de sodio, pH 6,5 + 0,2 ml extracto enzimático (3,62 U); y (d) (E4) 0,4 ml fenoles libres (2,275 mg/ml AG) en 80% etanol + 2,4 ml 10 mM regulador de fosfato de sodio, pH 6,5 + 0,2 ml extracto enzimático (3,62 U).

the activity of oxidoreductases is reduced due to their denaturation in such environment (Rodakiewicz-Nowak, 2000). For that, the use of cosolvent was a good alternative to aqueous or organic solvents.

The polymerization profile for E3 (0.4 mL free phenols (4.5 mg/mL GA) + 2.4 mL sodium phosphate buffer (10 mM, pH 6.5) + 0.2 mL enzyme extract (3.6 U)) began with formation of a 1550 nm trimer, followed by formation of dimers at 20, 40, 50, and 70 min (Figure 3(c)). The polymerization was stable until 70 min as result of oxidative polymerization of phenols via recombination process (Mohamed & Latifa, 2012).

In the E4 (0.4 mL free phenols (2.3 mg/mL GA) in 80% ethanol + 2.4 mL sodium phosphate buffer (10 mM), pH 6.5 + 0.2 mL enzyme extract (3.6 U)) profile (Figure 3(d)), phenol dimers and trimers were formed at 40, 50, and 70 min, with a clear tendency to increase from 20 to 70 min. After 70 min, the polymers underwent disaggregation, and the profile began to decline and then again began to form dimers beginning at 120 min.

The tendency to maintain polymerization was clear in all four treatments, and, although no polymers appeared, a number of molecular aggregates appeared. Conditions in E4 were most favorable to PPO activity in formation of phenolic polymers. It resulted in production of species from 900 to 1500 nm molecular diameter, corresponding to phenol trimers and tetramers. This result can be due to the presence of cosolvent with organic solvent in E4. The ratio of organic solvent/buffer affects drastically the yield, the solubility, and the average molecular weight of obtained polymers (Anthoni et al., 2008; Mita et al., 2002; Mohamed & Latifa, 2012). For more complex phenols like flavonoids, there are few works that discussed the effect of the nature and proportion of solvent (Anthoni et al., 2008; Tonam, 2003). López et al. (2013) identified an enzymatically synthesized polymer soluble in water, and it possesses active pendant groups, which might increase its range of applications. The effect of solvent on selectivity could be attributed to different interactions of the solvent molecules with the radical intermediates during their coupling reactions.

E4 showed the highest antioxidant capacity (2196 mmol TEAC/g phenol), followed by E3 (1152 mmol TEAC/g phenol) (Figure 4). The free phenolic group had an antioxidant capacity value of 1109 mmol TEAC/g phenol, higher than E1 (457 mmol TEAC /g phenol) and E2 (297 mmol TEAC/g phenol). The nearly 100% increase (+97.98%) in antioxidant capacity in the E4 treatment compared with the pre-reaction phenolic compounds extract was favored by the conditions in E4 and was probably the result of formation of molecular aggregates with diameters from 900 to 1000 nm.

This result indicated that pH is other important factor that plays a vital role in the enzymatic reaction, besides the ratio of organic solvent/buffer factor. The effect of the pH on the polymerization reaction can be related to the nature of the substrate. For substrates having a redox potential independent of pH (ABTS, $K_4Fe(CN)_6$), laccase activity decreases with the increase of pH (Mohamed & Latifa, 2012). Few data are available on the effect of pH on the structure of the synthesized polymers. Tonam (2003) indicated that the buffer pH also affected the polymerization behaviors, the yield was the highest in buffer pH 7 in oxidative polymerization of phenolic compounds catalyzed by peroxidase. López et al. (2013) indicated that pH affects the type of linkage between

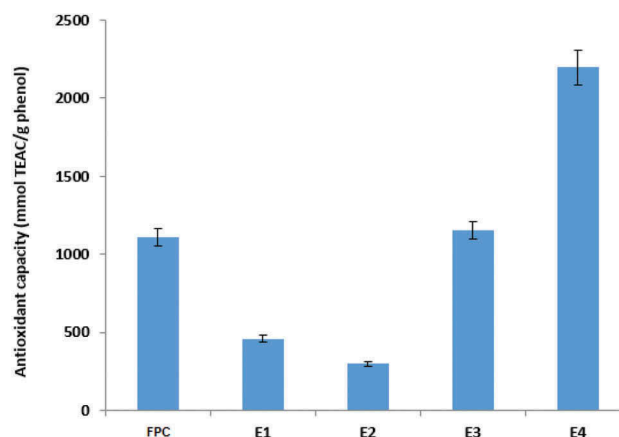


Figure 4. ABTS⁺ antioxidant capacity assay of bell pepper free phenol (FP) compounds and bell pepper polyphenol oxidase (PPO) polymerized extracts: E1: 1.5 ml free phenols (4.55 mg/ml GA) + 1.5 ml enzyme extract (3.62 U); E2: 1.5 ml free phenols (2.275 mg/ml GA) in 80% ethanol + 1.5 ml enzyme extract (3.62 U); E3: 0.4 ml free phenols (4.55 mg/ml GA) + 2.4 ml 10 mM sodium phosphate regulator, pH 6.5 + 0.2 ml enzyme extract (3.62 U); and E4: 0.4 ml free phenols (2.275 mg/ml AG) in 80% ethanol + 2.4 ml 10 mM sodium phosphate regulator, pH 6.5 + 0.2 ml enzyme extract (3.62 U).

Figura 4. Capacidad antioxidante de compuestos fenólicos libres de pimiento mediante ABTS⁺ (FL) y de extractos polimerizados con polifenoloxidasas de pimiento: E1: 1,5 ml fenoles libres (4,55 mg/ml AG) + 1,5 ml extracto enzimático (3,62 U); E2: 1,5 ml fenoles libres (2,275 mg/ml AG) en 80% etanol + 1,5 ml extracto enzimático (3,62 U); E3: 0,4 ml fenoles libres (4,55 mg/ml AG) + 2,4 ml 10 mM regulador de fosfato de sodio, pH 6,5 + 0,2 ml extracto enzimático (3,62 U); y E4: 0,4 ml fenoles libres (2,275 mg/ml AG) en 80% etanol + 2,4 ml 10 mM regulador de fosfato de sodio, pH 6,5 + 0,2 ml extracto enzimático (3,62 U).

monomeric units in *Trametes versicolor* laccase oxidation of GA. An acidic pH promotes the C–C linkage, while C–O linkage is abundant at basic pH. The antioxidant activity may be dependent on the molecular weight of the synthesized polymers and the type and the position of the linkages (Mohamed & Latifa, 2012).

The reaction conditions could be controlled by the polydispersity, the molecular weight of polymers synthesized, and the type of linkages between the monomers (Kobayashi & Higashimura, 2003; Mohamed & Latifa, 2012).

3.6. Polymerization of pure phenolic compounds by PPO

Of the three substrates used in the PPO reactions, the most effective, or specific, was epicatechin (0.25 mg/mL) (Figure 5 (a)). In all four tests it formed dimers, trimers, tetramers, pentamers, and even larger polymers, which were present from the beginning of the assay. The 5000 nm order polymer was most frequent in the E3 treatment. In E3, it appeared at 10 and 80 min with a molecular diameter of 5560 nm, at 90 min with a diameter of 5408 nm, and at 140 min with one of 5532 (Figure 5(a)). According to Mohamed and Latifa (2012) for the same enzyme, the type of substrate modifies the molecular weight and yield of obtained polymers. This may be due to the difference in the polymer solubility in the solvent.

Ferulic acid followed epicatechin in effectiveness, forming dimers and large polymers in all four treatments (Figure 5 (b)). It is a useful substrate for enzymatic polymerization, and treatments E3 and E4 produced the best results due to pH medium. With the three tested substrates, E3 and E4 were most effective for molecular aggregate formation; indeed, they produced 15 times more than their monomers. The

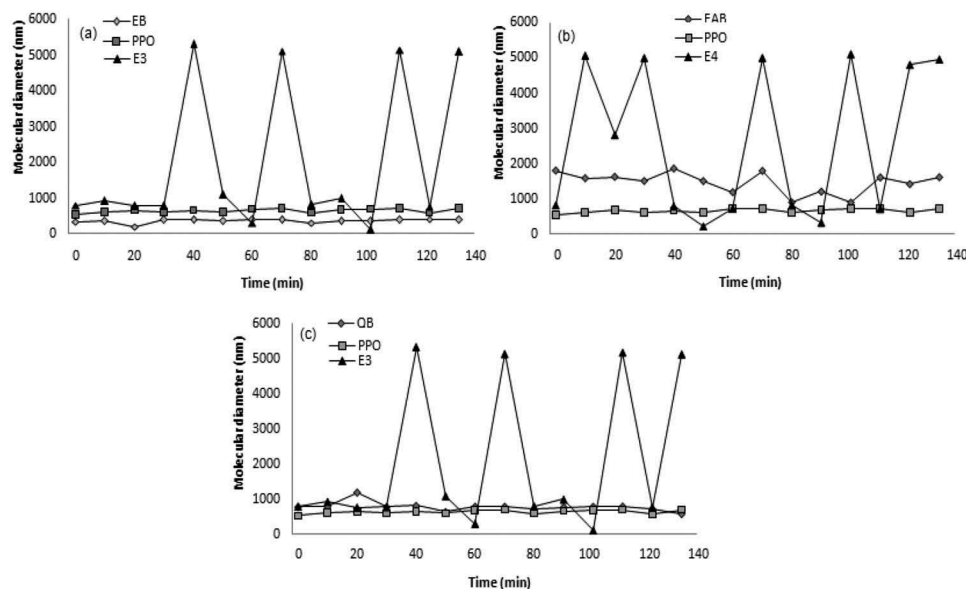


Figure 5. Dynamic light scattering (DLS) analysis of hydrodynamic diameter distributions for free phenolic compounds polymerized by bell pepper polyphenol oxidase (PPO): (a) Epicatechin (0.25 mg/ml), PPO (3.62 U), E3 [0.4 ml epicatechin (0.25 mg/ml) + 2.4 ml 10 mM sodium phosphate regulator, pH 6.5 + 0.2 ml enzyme extract (1.34 U)]. (b) Ferulic acid (0.25 mg/ml), PPO (3.62 U), E4 [0.4 ml ferulic acid (0.125 mg/ml) in absolute alcohol + 2.4 ml 10 mM sodium phosphate regulator, pH 6.5 + 0.2 ml enzyme extract (1.34 U)]. (c) Quercetin (0.25 mg/ml), PPO (3.62 U), E3 [0.4 ml quercetin (0.25 mg/ml) + 2.4 ml 10 mM sodium phosphate regulator, pH 6.5 + 0.2 ml enzyme extract (1.34 U)].

Figura 5. Análisis de la distribución de diámetros hidrodinámicos mediante dispersión dinámica de luz (DLS) de compuestos fenólicos libres (CFL) polymerizados por la polifenol oxidase (PFO) de pimienta: (a) Epicatequina (0,25 mg/ml), PFO (3,62 U), E3 [0,4 ml epicatequina (0,25 mg/ml) + 2,4 ml 10 mM regulador de fosfato de sodio, pH 6,5 + 0,2 ml extracto enzimático (1,34 U)]. (b) Ácido ferúlico (0,25 mg/ml), PFO (3,62 U), E4 [0,4 ml ácido ferúlico (0,125 mg/ml) en alcohol absolute + 2,4 ml 10 mM regulador de fosfato de sodio, pH 6,5 + 0,2 ml extracto enzimático (1,34 U)]; (c) Quercetina (0,25 mg/ml); PFO (3,62 U), E3: [0,4 ml quercetina (0,25 mg/ml) + 2,4 ml 10 mM regulador de fosfato de sodio, pH 6,5 + 0,2 ml extracto enzimático (1,34 U)].

largest polymers were detected in the presence of 10 mM sodium phosphate buffer at pH 6.5 (Figure 5(b)), which favored the enzymatic activity because its pH is optimum for PPO (Arnnok et al., 2010). pH influences the conversion rate and the molecular weight of obtained oligomers (Ikeda, Sugihara, Uyama, & Kobayashi, 1998). Few data are available on the effect of pH on the structure of the synthesized polymers. Acidic pH will promote C–C linkages, while C–O linkages are favored at basic pH (Kobayashi & Higashimura, 2003). The nature of solvent affects also both the position (Intra, Nicotra, Riva, & Danieli, 2005) and the type of the linkage (Mita, Tawaki, Uyama, & Kobayashi, 2003; Ncanana, Baratto, Roncaglia, Riva, & Burton, 2007).

The least effective substrate for polymerization of PPO was quercetin because it mostly formed dimers in all four treatments (Figure 5(c)). A 5000 nm order polymer was detected in E1 and E2. However, in E3 only dimers, and no polymers, were formed (Figure 5(c)), which was also the case in E4 (data not shown). Use of the sodium phosphate buffer at pH 6.5 (particularly adequate for PPO) was expected to produce a more stable enzymatic reaction and favor polymerization. However, this did not occur since the quercetin had low affinity for PPO, even though it had produced a small number of polymers in preliminary tests. The efficiency of the enzymatic transformation is strictly dependent on the chemical features of the phenolic substrate such as the number of OH groups, the nature, and the molecular weight of the substituents and their position on the aromatic ring (*ortho* or *para*) (Mohamed & Latifa, 2012).

3.7. Antioxidant activity in polymers obtained

Of the three tested pure commercial substrates, quercetin had the highest antioxidant capacity, closely followed by

epicatechin and finally ferulic acid (Table 3). Phenolic compound antioxidant capacity is attributed to the number of hydroxyl groups bonded to the molecule (Muñoz et al., 2007; Oliveras & López, 2005). This agrees with the present results, since ferulic acid has the lowest number of hydroxyl groups of the three tested phenolic compounds and exhibited the lowest antioxidant capacity. Quercetin and epicatechin have a similar number of hydroxyl groups and these molecules performed similarly. However, the carbonyl group in ring position four of the pyrane group provides quercetin greater antioxidant capacity (Cai, Sun, Xing, Luo, & Corke, 2006), and explains its higher value compared with epicatechin (Table 3).

The highest antioxidant activity in polymerization of epicatechin with PPO was observed in treatment E2 (135,539 mmol TEAC/g phenol), followed by E4, E1, and E3. Decamers and octamers were detected in E2 and E4, and polymers of the order of 5000 nm were present in E4. The polymers formed using epicatechin exhibited dual properties, like those formed from ferulic acid and quercetin.

With ferulic acid, each of the four treatments exhibited different antioxidant capacities, the highest being in the pure phenol. The identified dimers, trimers, and polymers had different properties depending on the product formed in the enzymatic reaction. Antioxidant capacity was highest in E4 (91,256 mmol TEAC/g phenol), followed by E1, E3, and E2. Throughout the kinetic in E4, 14 readings were made of large polymers greater than 5000 nm in order, which occurred in larger quantities than in the other treatments. However, this treatment also had fewer readings of dimers and trimers compared with the other treatments (Table 3).

Polymerization of quercetin by bell pepper PPO produced dimers and trimers that contributed to increasing its antioxidant capacity above that of the pure phenol. Treatment E2

Table 3. ABTS⁺ Antioxidant capacity of pure phenolic compounds after polymerization with bell pepper (*Capsicum annuum*) polyphenol oxidase (PPO).**Tabla 3.** ABTS⁺ Capacidad antioxidante de compuestos fenólicos puros después de la polimerización con polifenol oxidasa de pimiento *Capsicum annuum*.

Phenolic compound	ABTS ⁺ antioxidant capacity(mmol TE/g phenol)				
	Pure phenol	E1	E2	E3	E4
Ferulic acid	5831	64,753 ± 0.02	16,089 ± 0.01	44,466 ± 0.01	91,256 ± 0.01
Quercetin	14,718	47,311 ± 0.01	78,360 ± 0.01	47,695 ± 0.02	64,647 ± 0.01
Epicatechin	14,476	49,075 ± 0.01	135,539 ± 0.02	43,650 ± 0.01	106,938 ± 0.02

E1: 1.5 ml phenol (0.25 mg/ml) + 1.5 ml enzyme extract (1.34 U).

E2: 1.5 ml phenol (0.125 mg/ml in absolute alcohol) + 1.5 ml enzyme extract (1.34 U).

E3: 0.4 ml phenol (0.25 mg/ml) + 2.4 ml 10 mM sodium phosphate regulator, pH 6.5 + 0.2 ml enzyme extract (1.34 U).

E4: 0.4 ml phenol (0.125 mg/ml in absolute alcohol) + 2.4 ml 10 mM sodium phosphate regulator, pH 6.5 + 0.2 ml enzyme extract (1.34 U).

All experiments were done in triplicate and repeated at least three times.

E1: 1,5 ml fenol (0,25 mg/ml) + 1,5 ml extracto enzimático (1,34 U).

E2: 1,5 ml fenol (0,125 mg/ml en alcohol absoluto) + 1,5 ml extracto enzimático (1,34 U).

E3: 0,4 ml fenol (0,25 mg/ml) + 2,4 ml 10 mM fosfato de sodio, pH 6,5 + 0,2 ml extracto enzimático (1,34 U).

E4: 0,4 ml fenol (0,125 mg/ml en alcohol absoluto) + 2,4 ml 10 mM fosfato de sodio, pH 6,5 + 0,2 ml extracto enzimático (1,34 U).

Todos los experimentos fueron realizados por triplicado y repetidos al menos tres veces.

exhibited the highest antioxidant capacity (78,360 mmol TEAC/g phenol), followed by E4, E3, and E1 (Table 3). Larger polymers with a diameter of 5362 nm were observed in 6 readings in E2, and dimers were detected in 10 readings. The antioxidant capacity of E4 confirms the role of dimers in this capacity since it had the largest number of dimers and the second highest antioxidant capacity. The polymers formed from quercetin apparently had a dual nature, increasing antioxidant capacity in E2 and decreasing it in E1. Perhaps this difference is due to release of hydroxyl groups during polymer formation, which would favor antioxidant capacity. Desentis-Mendoza et al. (2006) proposed a possible reaction mechanism for enzymatic polymerization of polyphenols in which products with variable hydroxyl group counts are formed. They reported high antioxidant activity values beginning in low-molecular-weight quercetin molecular aggregates.

Each substrate used in enzymatic polymerization with bell pepper PPO exhibited molecular aggregates with different characteristics and antioxidant capacities. These differences were caused by medium pH, amount of added substrate, and the reaction medium; in addition, each substrate's specificity is different. The magnitude of these effects depends on the degree of polymerization or the synthesized oligomers (Anthoni et al., 2008, 2010).

4. Conclusion

PPO from bell pepper is generally promising for polymerization of phenols and results in some products with high antioxidant activity. However, extensive further research is needed to fully understand the process and its products, and to develop possible uses for them. The solvents used during the polymerization reaction also affect the structure of polymers and the selectivity of the reaction. This behavior is variable according to the nature of substrates. The oxidative grafting of flavonoids to polymers is enjoying increased attention especially aiming at functionalized textiles or food applications.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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