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Biochemical Properties of Soluble and Bound Peroxidases from Artichoke Heads and Leaves

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Summary

Soluble (SP), ionically bound (IBP) and covalently bound (CBP) peroxidases (POD) from artichoke leaves and heads have been characterized for the main biochemical parameters. The three PODs, in both leaves and heads, showed the major apparent catalytic efficiency ($v_{max,app}/K_{m,app}$) towards ferulic acid, even though, in some cases, they showed higher affinity ($K_{m,app}$) for other substrates. In leaves, SP and IBP showed higher $K_{m,app}$ for ferulic and chlorogenic acids, and CBP for ferulic and caffeic acids. In heads, SP showed higher $K_{m,app}$ for chlorogenic acid, IBP for caffeic and ferulic acids, and CBP for ferulic acid. It was shown that pH optimum for PODs ranged between 5.0 and 6.0 in leaves. In heads, pH optimum for SP and IBP was 5.5, while CBP presented a very low activity in a wide pH range. All PODs showed high thermal stability but different ability to regenerate: the bound forms were more able to regenerate than the soluble one. The results obtained show that (*i*) CBP from heads is able to work under very different cellular conditions, (*ii*) all PODs, in both tissues, have a high apparent catalytic efficiency for ferulic acid, which could explain the effective involvement of POD in lignin biosynthesis, (*iii*) in heads, high $K_{m,app}$ of SP for chlorogenic acid, particularly abundant in artichoke, could justify the possible involvement of PODs in browning mechanism, and (*iv*) in heat-processed artichoke, the ability of PODs to regenerate could contribute to oxidation and loss of product quality.

Key words: artichoke (*Cynara cardunculus*), soluble and bound peroxidases, characterization, thermostability, regeneration, browning

Introduction

Artichoke (*Cynara cardunculus* L. ssp. *scolymus* (L.) Hayek) is one of the most typical vegetables consumed in the Mediterranean countries, well-known for its nutritional properties due to some biochemical constituents. Artichoke is very rich in polyphenols (mono- and dicaffeoylquinic acids and flavonoids such as luteolin and apigenin glycosides), with known antimicrobial and antioxidant properties, and in fructan inulin, which has been reported to have a prebiotic function, stimulating the growth of intestinal bifidobacteria. Metabolic changes of these biochemical constituents during the development of artichoke heads have been investigated (*1*,*2*) since they are important from nutritional as well as technological point of view.

Artichokes, as many fruits and vegetables, are subject to browing during processing and storage, with severe damage of the original quality and loss of acceptability for the consumers (*3–5*). The enzyme principally responsible for browning phenomenon is polyphenol oxidase (EC 1.14.18.1, PPO), but peroxidase (EC 1.11.1.7, POD), as stated by numerous authors (*6–9*), is also involved in enzymatic browning.

Peroxidase oxidizes a wide range of hydrogen donors using peroxides, towards which it is highly specific. The level of H_2O_2 in fruit and vegetables is low and not enough to justify the involvement of POD in brown-

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ing tissue. However, several authors have demonstrated the generation of H_2O_2 during oxidation of some phenolic compounds and the use of generated H_2O_2 as an electron acceptor by POD (*10,11*). Also, superoxide dismutase (SOD), which has been reported to be present in many vegetables, contributes to formation of H_2O_2 from O_2^- produced by the reaction of POD with polyphenols and by autoxidation or light-induced oxidation of brown components (*12*).

The PODs are able to oxidize many polyphenols, among which hydroxycinnamic derivatives, flavonoids (*13*) and flavans (*8,14*), the main phenolic structures implicated in enzymatic browning.

PODs in plants occur in soluble (SP), ionically bound (IBP) and covalently bound (CBP) forms. The SP is located in the cell cytoplasm, whereas the bound forms are generally thought to be associated with particular components such as plant cell walls and some organelles. It is possible to distinguish different classes of POD by comparing their enzymatic affinity for specific substrates: ascorbic acid (class I), veratryl alcohol (class II) and guaiacol (class III).

The distribution of different PODs (SP, IBP and CBP) in both artichoke heads and leaves, their electrophoretic and isoelectrofocusing patterns with related information on molecular mass and pI have recently been investigated (*15*).

Each POD has a number of isoenzymes, differing in molecular mass, thermal stability, pH optimum, substrate specificity and physiological role, as it has been reported for other species (*14,16*). The soluble and bound PODs differ among themselves in heat stability and regeneration properties, and these characteristics change according to the vegetal species considered (*9,17,18*).

To our knowledge, few studies on biochemical properties of soluble and bound PODs in artichoke have been published (*15,19,20*). The main objective of this paper is the characterization of soluble and bound peroxidase from artichoke leaves and heads. The biochemical parameters obtained could help to understand better the involvement of the three PODs in physiological processes that occur during storage of raw and processed artichokes.

Materials and Methods

The analyses were performed on leaves and heads of artichoke (cv. Violetto di Provenza) grown in an experimental field in Policoro (Matera, Southern Italy). Leaves at different physiological stage were used; very old outer leaves and any damaged tissue were not included in the sample. Heads of about 120 g were deprived of external bracts and only the edible part (heart), representing about 40 % of the total mass, was used for the analysis. Enzyme extraction was performed according to Boucoiran *et al.* (*21*) with minor modifications as described below.

Enzyme extraction

Artichoke leaves and heads (25 g each) were chopped into small pieces and homogenized for 3 min with 100 mL (leaves) or 60 mL (heads) of 50 mM sodium acetate buffer, pH=5.6 and 2 % polyvinylpyrrolidone (PVP), using a Waring blendor (15 000 rpm). The homogenate was centrifuged (17 400×*g*, 30 min, 4 °C), giving the SP as the supernatant. The residue from the centrifugation was washed three times with 50 mM sodium acetate buffer, pH=5.6, then twice more with cold distilled water (4 °C) and after that resuspended with 3 M NaCl and centrifuged (17 400×*g*, 30 min, 4 °C) to give the IBP. To extract the CBP, the pellet was incubated overnight with 0.2 M sodium acetate buffer, pH=5.6, 0.4 g of pectinase and 0.2 g cellulase at 35 °C. The solution was centrifuged and the supernatant separated. The extracts of the three PODs were held at –18 °C until analysis.

Substrate specificity

For the determination of the substrate specificity and classification of the three PODs, the enzyme activity was monitored spectrophotometrically using ascorbic acid, guaiacol and veratryl alcohol. The rate of oxidation of guaiacol was followed at 470 nm (ε_{470nm}=26.6 mM^{-1} cm^{-1}), of ascorbic acid at 290 nm (ε_{290nm}=2.8 mM^{-1} cm^{-1}) and of veratryl alcohol at 310 nm (ε_{310nm}=9.3 mM^{-1} cm^{-1}).

Determination of $K_{m,app}$ and $v_{max,app}$

Specificity of the three PODs from artichoke leaves and heads was determined by calculating the Michaelis-Menten apparent reaction rate constant ($K_{m,app}$) and the apparent maximum velocity ($v_{max,app}$) for a selected monophenol (ferulic acid) and *o*-diphenols (chlorogenic acid and caffeic acid) in a concentration range of 0.01–1 mM. The reaction rate with different substrates was measured as an increase in absorbance at 400 nm of chlorogenic acid and caffeic acid in 0.05 M phosphate/citrate buffer, pH=4.5. However, the oxidation of ferulic acid was measured following the absorbance decrease at 310 nm in 0.1 M potassium phosphate buffer, pH=6.5. The data were plotted according to Lineweaver and Burk (*22*). As different PODs are not purified, the polyphenol oxidase can be present in the extracts. The PPO is oxygen dependent and, differing from POD, it does not require H_2O_2 to work. Therefore, to eliminate the contribution of PPO, the assays were carried out with and without H_2O_2, and the absorbance values obtained without H_2O_2 were subtracted from those obtained in the presence of H_2O_2 (*23*).

POD activity

To evaluate the effect of pH, termostability and the ability to regenerate, POD activity was determined with guaiacol as reducing substrate in a reaction mixture containing 0.1 M potassium phosphate buffer, pH=6, 20 mM guaiacol and 30 mM H_2O_2. The oxidation of guaiacol was assessed by observing the absorbance increase at 470 nm. One enzyme unit is defined as the absorbance change per minute per g of fresh matter (FM) under the above assay conditions (*24*).

Effect of pH

The effect of pH on the substrate depletion activity of the three PODs was determined using guaiacol following the tetraguaiacol formation at 470 nm. For this purpose four different buffers were used: sodium ace-

tate (pH=3.5–5.0), sodium phosphate (pH=5.0–7.5), Tris-HCl (pH=7.5–9.0), and glycine-NaOH (pH=9.0).

Thermostability

The three PODs were heated for 10 min in a water bath at different temperatures (5, 25, 45, 65 and 85 °C) and immediately cooled on ice. Activity was assayed after 5 min using guaiacol as substrate. The residual activity measured at each temperature was reported as relative percentage activity compared to the activity value at 25 °C.

Regeneration

To study the ability of the three enzymatic forms to regenerate, the extracts were inactivated for the time that allowed the highest reactivation. Thermal inactivation was performed by placing vials in triplicate, filled with 350 μL of enzymatic extract, in a thermostatic water bath, previously equilibrated at 80 °C. After the inactivation time, the vials were immediately cooled in a water-ice mixture to stop the process and assayed for residual POD activity, then held for 1 h at 30 °C before measuring again. The percentage of inactivation and regeneration for all enzymatic extracts was calculated starting from an absorbance value of about 0.300 ΔA/min, utilizing different amounts of enzyme.

Results and Discussion

The SP, IBP and CBP from leaves and heads oxidized guaiacol very quickly, but showed no detectable activity with ascorbic acid or veratryl alcohol, which demonstrates that the enzyme is not an ascorbate nor a lignin POD, but that it is related to the class III POD. This finding is in agreement with the results obtained by other authors on purified POD from artichoke (*19*, *20*).

To evaluate the residual PPO presence in the extract, PODs from artichoke were tested for phenols in the absence of H_2O_2. Some activity was detected only in SP from leaves and heads towards chlorogenic and ferulic acids. To eliminate the contribution of PPO, the absorbance values obtained without H_2O_2 were subtracted from the values obtained in the presence of H_2O_2.

The Lineweaver-Burk (L/B) plots and the linear regression parameters of the data are reported in Fig. 1 for leaves and in Fig. 2 for heads. The R^2 values of L/B plots show that all PODs, if not purified, have approximately Michaelian kinetic behaviour. In fact, the correlation test was significant for all the L/B plots, except in the case of CBP from heads tested for caffeic acid. Besides, half of the plots were significant at p≥99.9 %.

The POD $K_{m,app}$ and $v_{max,app}$ values are presented in Tables 1 and 2 for artichoke leaves and heads, respectively. The three PODs, in leaves and heads, showed the major apparent catalytic efficiency towards ferulic acid, with $v_{max,app}/K_{m,app}$ values higher than towards the other substrates, even if the affinity for the tested substrates was different. In leaves, SP showed higher apparent affinity for ferulic and chlorogenic acids with $K_{m,app}$ values of 0.11 and 0.55 mM, respectively. IBP also showed higher apparent affinity for ferulic and chlorogenic acids with $K_{m,app}$ values of 0.21 and 0.28 mM, respectively. CBP oxidized ferulic and caffeic acids with $K_{m,app}$ of 0.06 and 0.08 mM, respectively. In heads, SP showed high apparent affinity for chlorogenic acid with $K_{m,app}$ value

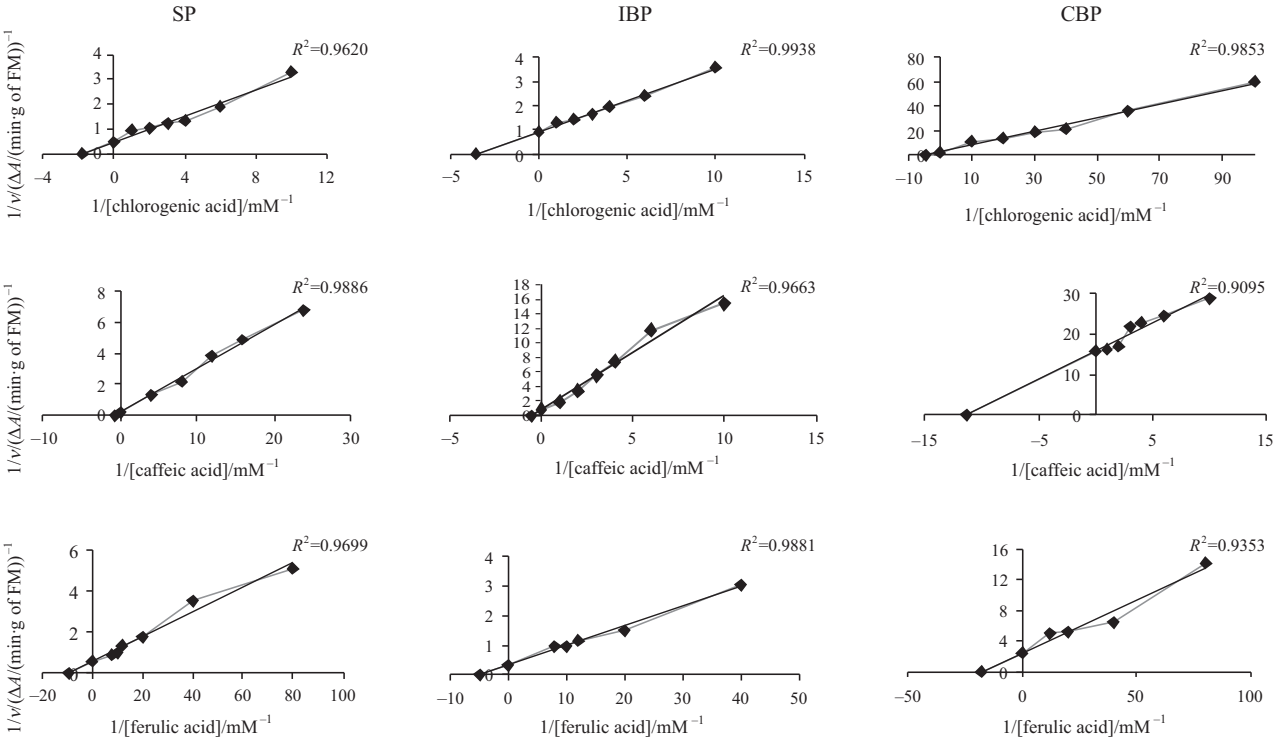

Fig. 1. Lineweaver-Burk plots of SP, IBP and CBP from artichoke leaves in the presence of three different substrates (chlorogenic, caffeic and ferulic acids) at different concentrations

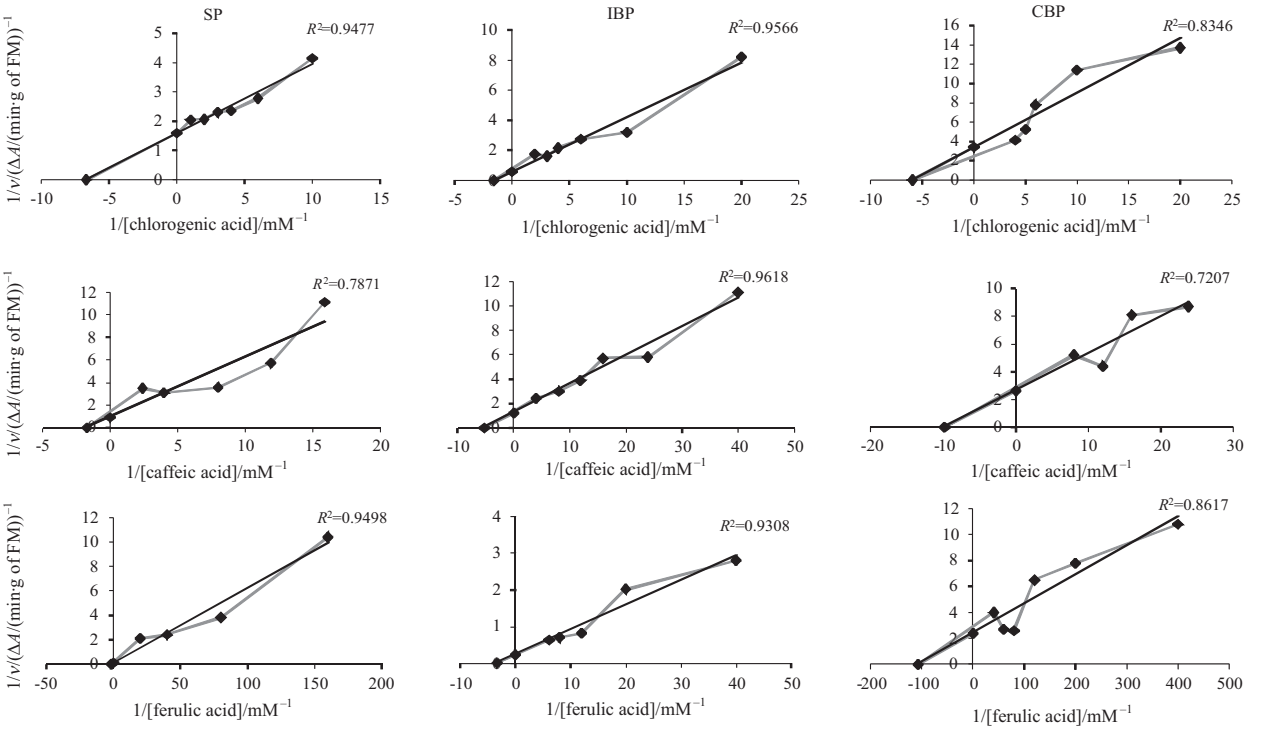

Fig. 2. Lineweaver-Burk plots of SP, IBP and CBP from artichoke heads in the presence of three different substrates (chlorogenic, caffeic and ferulic acids) at different concentrations

Table 1. Michaelis-Menten apparent reaction rate constant ($K_{m,app}$) and relative apparent maximum velocity ($v_{max,app}$) parameters for the three PODs from artichoke leaves

Substrates	SP			IBP			CBP		
	$K_{m,app}$ / mM	$v_{max,app}$ / ΔA/min	$v_{max,app}$ / $K_{m,app}$	$K_{m,app}$ / mM	$v_{max,app}$ / ΔA/min	$v_{max,app}$ / $K_{m,app}$	$K_{m,app}$ / mM	$v_{max,app}$ / ΔA/min	$v_{max,app}$ / $K_{m,app}$
Monophenol:									
Ferulic acid	0.11	1.79	16.70	0.21	3.14	14.90	0.06	0.41	7.32
Diphenols:									
Chlorogenic acid	0.55	2.09	3.80	0.28	1.09	3.90	0.23	0.41	1.77
Caffeic acid	1.59	5.60	3.50	1.90	1.22	0.64	0.08	0.06	0.75

Table 2. Michaelis-Menten apparent reaction rate constant ($K_{m,app}$) and relative apparent maximum velocity ($v_{max,app}$) parameters for the three PODs from artichoke heads

Substrates	SP			IBP			CBP		
	$K_{m,app}$ / mM	$v_{max,app}$ / ΔA/min	$v_{max,app}$ / $K_{m,app}$	$K_{m,app}$ / mM	$v_{max,app}$ / ΔA/min	$v_{max,app}$ / $K_{m,app}$	$K_{m,app}$ / mM	$v_{max,app}$ / ΔA/min	$v_{max,app}$ / $K_{m,app}$
Monophenol:									
Ferulic acid	0.72	11.70	16.25	0.29	4.32	14.90	0.01	0.42	46.70
Diphenols:									
Chlorogenic acid	0.15	0.63	4.20	0.64	1.76	2.75	0.17	0.30	1.77
Caffeic acid	0.59	1.10	1.86	0.19	0.80	4.20	0.10	0.38	3.73

of 0.15 mM; on the other hand, IBP showed high apparent affinity for caffeic and ferulic acids with $K_{m,app}$ values of 0.19 and 0.29 mM, respectively, and CBP oxidized ferulic acid with $K_{m,app}$ value of 0.01 mM.

In heads, the high apparent affinity of SP for chlorogenic acid suggests the possible involvement of the enzyme in the browning mechanism. In fact, the chlorogenic acid, very abundant in artichoke head tissue, is the main substrate for polyphenoloxidase (PPO), principally responsible for browning phenomenon (*25*). Moreover, during oxidation of phenolic compounds, PPO generates H_2O_2 that could be used by POD and so contribute to browning, as already hypothesized for other species (*11*).

It should be emphasized that CBP in both leaves and heads was highly reactive towards ferulic acid, which represents a substrate mainly involved in the stiffening of the cell wall through the formation of diferuloyl bridges between wall polymers (*26*). Moreover, all the three PODs in both tissues have a higher apparent catalytic efficiency ($v_{max,app}/K_{m,app}$) for ferulic acid than for other phenolics. Since the lignin content as well as the POD activity increase during the damage process, it is possible that this enzyme, in the presence of H_2O_2, uses only phenolic compounds as substrates, which are precursors of lignin biosynthesis, such as ferulic acid (*27*).

Many studies have been done to understand if anionic (acidic) or cationic (alkaline) POD isozymes are involved in lignification (*28–30*). Conflicting results have been reported in literature, but some of these studies demonstrated that cationic peroxidases are apparently more efficient than anionic peroxidases in catalyzing NADH oxidation, whereas anionic peroxidases have higher affinity for coniferyl alcohol oxidation than cationic peroxidases. Since H_2O_2 is produced in the first reaction and consumed in the second, it has been speculated that both cationic and anionic PODs are involved in lignification, which requires H_2O_2 for the formation of phenoxy radicals (*31*). In artichoke, several basic and acid isoforms were present in SP and CBP, whereas in IBP only basic components were found (*15*).

At the molecular level, there is no explanation for the different reactivities of PODs towards different phenols containing 1 or 2 hydroxyl groups. One possible explanation could be that the electrostatic field produced by the charged residues of the enzymes would affect the reduction potential of the active site (*32*).

To evaluate the effect of pH, termostability and the ability to regenerate, POD activity was determined with guaiacol as reducing substrate. When guaiacol was used as a substrate, no activity was detected for PPO.

The pH optimum of the PODs in leaves ranged between 5.0 and 6.0 (Fig. 3). In heads, SP and IBP showed the same pH optimum (pH=5.5), while CBP presented a very low, but constant activity in the pH range from 3.5 to 9.0 (Fig. 3). This result shows that CBP is able to work under different cellular conditions. Generally, the active site of the enzymes is often composed of ionized groups (prototropic groups) that must be in the proper ionic form in order to maintain the conformation of the active site, the enzyme-substrate binding and the reaction catalysis (*33*). It must be difficult for the H^+ ion to reach the active site of the CBP form due to covalent immobilization of the enzyme in a heterogenous matrix, then the enzyme works at any pH with the same activity.

The results of termostability analysis show that different forms of the enzyme have high stability between 5 and 65 °C, while after a drastic heat treatment, such as 10 min at 85 °C, the enzymes were inactivated (Fig. 4). The termostability properties of PODs suggest that the contribution of this enzyme to browning could increase at elevated temperatures, because of the lower heat stability of PPO (*34*).

All the three enzymatic forms from artichoke leaves and heads showed ability to regenerate. The inactivation percentage allowing greater reactivation ranged between 30 and 70 %: less than 50 % for SP, about 50 % for CBP and up to 70 % for IBP. After an incubation period of 1 h at 30 °C, the three forms regenerated at different degrees. The SP in leaves as well as in heads regained less

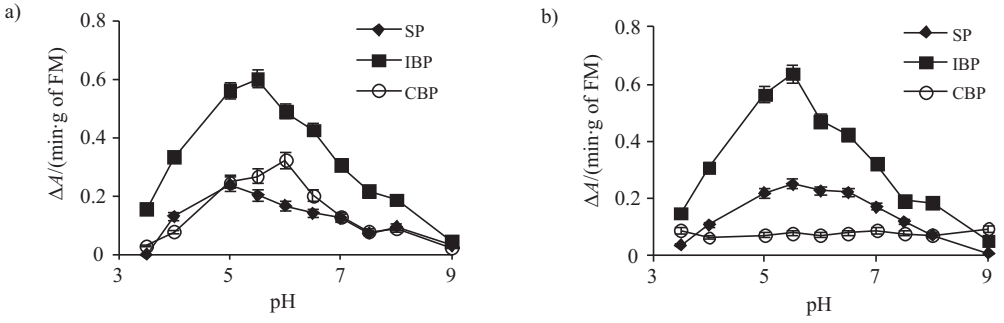

Fig. 3. POD activity at different pH in SP, IBP and CBP from (a) artichoke leaves, and (b) heads (mean of three samples±SD)

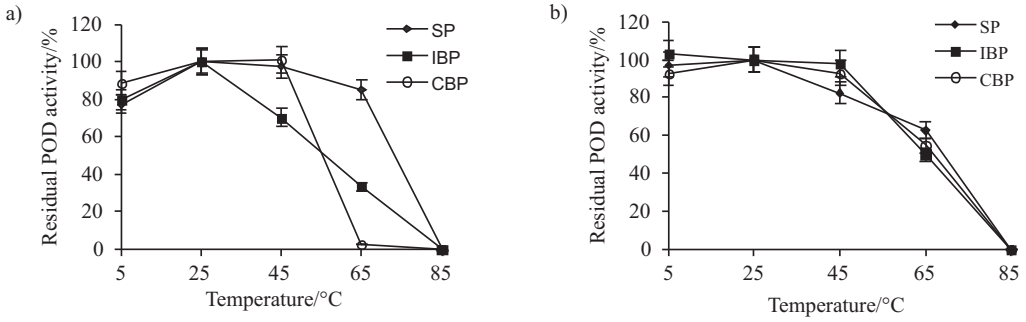

Fig. 4. Residual POD activity of SP, IBP and CBP from (a) artichoke leaves, and (b) heads after heat treatments for 10 min at different temperatures (mean of three samples±SD)

than 10 % of the original activity, reactivation of IBP ranged between 15 % in heads and 30 % in leaves, while CBP regenerated for 50 % in both leaves and heads (Fig. 5). The results show that the bound forms regenerated more than the soluble ones.

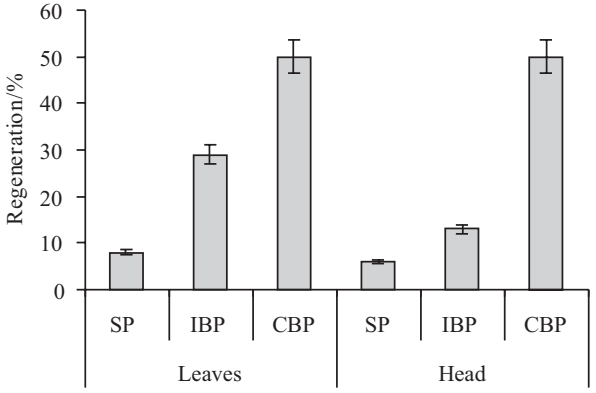

Fig. 5. Regeneration ability of SP, IBP and CBP from artichoke leaves and heads (mean of three samples±SD)

It could be useful to underline that the reported results were obtained on the unpurified enzymes. It is possible that the regeneration capacity of purified forms is very strong, as demonstrated for SP purified from artichoke leaves that, after complete inactivation, regained its activity almost completely (*20*).

Conclusions

The soluble and bound PODs from artichoke leaves and heads have been characterized assessing the main biochemical parameters. While SP, IBP and CBP from artichoke leaves and SP and IBP from heads show a narrow pH optimum range, CBP from heads shows a particular behaviour since its activity does not change in response to pH changes. It must be difficult for the H^+ ion to reach the active site of the CBP form due to covalent immobilization of the enzyme in a heterogenous matrix.

All the three PODs in both tissues have a higher apparent catalytic efficiency for ferulic acid than for other phenolics. It is possible that this enzyme, in the presence of H_2O_2, can use ferulic acid as a substrate for lignin synthesis. Thus, the reddish brown coloration of lignins or their intermediaries could contribute to the browning of fresh-cut products. In heads, the high apparent affinity of SP for chlorogenic acid, among the most abundant phenols in artichoke, could explain the possible involvement of the enzyme in browning mechanism of artichoke.

The termostability properties of PODs suggest a greater contribution of this enzyme to browning at elevated temperatures, because of the lower heat stability of PPO. All the PODs showed ability to regenerate: in particular the bound forms regenerated more than the soluble ones. This information could have a great importance from the biochemical as well as technological point of view. In heat-processed artichokes, which frequently contain residual POD activity, the regeneration of POD could contribute to oxidation phenomenon that might damage the product quality.

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