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# Tyrosinase Inhibitory Activity of Cucumber Compounds: Enzymes Responsible for Browning in Cucumber

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The inhibition of mushroom tyrosinase by cucumber extracts was evaluated. The inhibitory effect was measured by both polarographic and spectrophotometric methods. The commercial aldehyde, *trans*,*cis*-2,6-nonadienal, described as a major volatile compound of cucumber, was characterized as a noncompetitive inhibitor against 4-*tert*-butylcatechol oxidation by mushroom tyrosinase. The $K_I$ obtained was 3.4 mM. Polyphenol oxidase (PPO) activity was not detected in cucumber skin extracts. However, the presence of PPO was revealed by Western blot; a single band was found with a $M_r$ of 53 kDa. These results support the assumption that the enzyme PPO is present in the cucumber skin, but its activity is inhibited. Peroxidase (PO) was also found in cucumber skin extracts. This enzyme was detected in the soluble fraction but not in the membrane fraction. The kinetic characterization of PO was carried out. Native isoelectric focusing revealed several acidic PO isoenzymes with a p*I* in the range between 5 and 6, a basic isoenzyme, and one principal neutral isoenzyme of p*I* = 7.2.

**KEYWORDS: Tyrosinase inhibitory activity; polyphenol oxidase; peroxidase; noncompetitive inhibition; *Cucumis sativus* L.; *trans*,*cis*-2,6-nonadienal**

## INTRODUCTION

Browning of raw fruits, vegetables, and beverages is a major problem in the food industry and is believed to be one of the main causes of quality loss during postharvest handling and processing. The mechanism of browning in food is well characterized and is mainly enzymatic in origin (*1*). This phenomenon is mediated by endogenous enzymatic activities, such as polyphenol oxidase (PPO) and peroxidase (PO). Enzymatic browning of fruits and vegetables is mostly related to the oxidation of endogenous phenolic compounds. This process ultimately leads to the formation of dark brown polymers of a quinoidal nature (*2*).

Polyphenol oxidase, also known as tyrosinase (monophenol, *o*-diphenol:oxygen oxidoreductase; EC 1.14.18.1), is a copper-containing enzyme that catalyzes two different reactions using molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of the *o*-diphenols to *o*-quinones (diphenolase activity) (*3*). This enzyme is widely distributed in microorganisms, animals, and plants and is responsible not only for browning in plants but also for melanization in animals.

The localization of PPO in the plant cell depends on the species, age, and, in fruits or vegetables, maturity (*4*). In green leaves, a considerable part of PPO activity is localized in the chloroplasts (*5*). Furthermore, PPO has been detected in the soluble fraction in different fruits and vegetables (*6*). In some species, for example, lettuce, broad beans, sugar beet, and grapes, the enzyme was present in a latent form in the membrane fraction (*7*−*10*). However, the membrane fraction from other sources such as potato tubers, tomatoes and corn leaves did not exhibit latency. Thus, latency does not seem to be related to the localization of the enzyme in the cell (*11*). It has been described that during ripening, the concentration of the membrane enzyme decreased with the simultaneous appearance of a soluble fraction (*5*).

Another enzyme involved in browning, peroxidase (donor: $H_2O_2$ oxidoreductase; EC 1.11.1.7), constitutes a group of glycoproteins whose main function is the oxidation of different substrates at the expense of $H_2O_2$. Among these molecules are phenolics and lignins which, in turn, constitute one of the typical structural barriers against infection processes. Robinson (*12*) reviewed the physiological role of POs in postharvest fruits and vegetables and attributed many of the physiological functions to phenol oxidation. Thus, phenol oxidation mediated by PO is believed to be associated with deterioration in the flavor, color, texture, and nutritional qualities of processed foods and their products.

On the other hand, PO is an enzyme that shows strong specific tissue compartmentalization. Thus, in fruits, it is mainly located in the outermost cell layers of the skin and in peripheral vascular strands, a localization that is also valid for leaves and stems.

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Regarding the subcellular localization of PO, different isoenzymes have been found both associated with the soluble fraction and bound to the membrane and cell-wall fractions in the same tissue (*13*). Due to this high polymorphism, the functionality of each isoenzyme depends on its nature (acidic or basic) and its subcellular localization (*14*).

Because of the undesirable effects of enzymatic browning on fruits and vegetables, a considerable number of inhibitors of PPO are known. Chelating agents such as EDTA, are reported to be used as inhibitors for PPO activity (*1*). On the other hand, a low $M_r$ cooper-chelating peptide from *Dactylium dentroides* (*15*) and a cooper-metallothionein from *Aspergillus niger* (*16*) were reported to inhibit mushroom tyrosinase.

Compounds structurally analogous to phenolic substrates, such as tropolone (*17*), kojic acid (*18*), 4-substituted resorcinols (*19*), and 4-substituted benzaldehydes (*20*), constitute another important group of browning inhibitors. In addition, tyrosinase inhibitors have also become increasingly important in medical and cosmetic products (*21*) in regard to hyperpigmentation. Flavonols isolated from flower petals have been studied for their tyrosinase inhibitory activity (*22, 23*).

A widely used method in the food and beverage industries to control browning is the addition of reducing agents (sulfites, ascorbic acid, etc.), which chemically reduce the *o*-quinones to the less reactive colorless diphenols. However, these compounds can have adverse health effects and can also react with other components in the food system, resulting in unwanted effects (*1*). The need for a safe and effective PPO inhibitor has focused the research in the finding of natural inhibitors for PPO (*23*−*25*). Owing to the importance of finding alternative PPO inhibitors, this study was designed for the search of natural browning control agents, because PPO inhibitors isolated from regularly consumed foods may be safer compared to non-natural products. Therefore, one of the aims of the present paper is to carry out a study about the inhibition of PPO activity by cucumber extracts.

PPO activity in cucumber fruit was reported by Miller et al. (*26*). In the same paper, anodic PO isoenzymes were detected mainly in the cucumber skin. Both enzymes are involved in phenolic metabolism, so this study was also designed to investigate their presence in cucumber, to understand their possible function in this fruit.

## MATERIALS AND METHODS

**Plant Material.** Cucumbers (*Cucumis sativus* L. cv. Español) were grown from plants in an ecological plantation without adding any pesticide. The cucumbers were harvested, and cucumber skin was separated from the fruit; both were frozen in liquid nitrogen and stored at −80 °C until use.

**Chemicals.** Mushroom tyrosinase, dopamine, and 4-*tert*-butylcatechol (4tBC) were obtained from Sigma (Madrid, Spain); *trans,cis*-2,6-nonadienal was purchased from Aldrich (Madrid, Spain). All other chemicals were of analytical grade.

**Preparation of Cucumber Extract.** One hundred and twenty grams of cucumber fruit was homogenized in a model 230 Omnimixer with 240 mL of 50 mM sodium phosphate buffer (pH 7), at 4 °C. The homogenate was filtered through two layers of cheesecloth and centrifuged at 120000*g* for 40 min. The sediment was discarded, and the supernatant was ultrafiltered through a YM-10 membrane from Amicon (Madrid, Spain) to remove proteins. The filtered material was used as a source of PPO inhibitors.

**Enzyme Extraction.** Soluble and membrane fractions were obtained from 30.6 g of cucumber skin, which was homogenized twice at maximum speed for 10 s in a model 230 Omnimixer with 100 mL of 0.1 M sodium phosphate buffer (pH 7) containing 0.33 M sorbitol, 10 mM ascorbic acid, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM PMSF, and 1 mM benzamidine. All procedures were carried out at 4 °C. The homogenate was filtered through two layers of cheesecloth and centrifuged at 1000*g* for 10 min. The supernatant was centrifuged at 120000*g* for 40 min, and the resultant supernatant was considered to be the soluble fraction. The pellet containing the membrane fraction was resuspended with 5 mL of 1.5% (w/v) TX-114 in extraction buffer and digested for 1 h. After high-speed centrifugation (120000*g* for 40 min) at 4 °C, the supernatant was subjected to temperature phase partitioning by adding TX-114 so that the final detergent concentration was 8% (w/v). The mixture was kept at 4 °C for 15 min and then warmed to 37 °C. After 15 min, the solution became spontaneously turbid due to the formation, aggregation, and precipitation of large micelles composed of detergent, hydrophobic proteins, and the remaining chlorophylls and phenolic compounds. This solution was centrifuged at 5000*g* for 15 min at 25 °C. The aqueous phase was used as a membrane-bound enzyme (*9*).

The soluble fraction was brought up to 35−85% $(NH_4)_2SO_4$. The salt content was removed by dialysis against 10 mM sodium phosphate buffer, pH 7.0.

**Protein concentration** was determined according to the Bradford assay (*27*).

**Enzyme Assays.** *PPO Activity.* The diphenolase activity was determined using the substrate 4tBC, which was especially used for the stability of the 4-(*tert*-butyl)benzo-1,2-quinone obtained as a product of the enzymatic activity (*28*). The enzyme activity was determined at 25 °C by spectrophotometrically monitoring the appearance of the 4-(*tert*-butyl)benzo-1,2-quinone product of the reaction at 390 nm ($\epsilon = 1150\ M^{-1}\ cm^{-1}$) (*9*). In this work, the international unit of enzyme activity (IU) was defined as the quantity of enzyme that produces 1 $\mu$mol of 4-(*tert*-butyl)benzo-1,2-quinone per minute at pH 6.5 and 25 °C.

Unless otherwise stated, the reaction medium (1.0 mL) contained 50 mM sodium phosphate buffer (pH 6.5) and 6 mM 4tBC. The inhibitors tested were first dissolved in ethanol. The final concentration of ethanol in the reaction medium was 20%. Controls, without inhibitor, containing ethanol at that concentration were routinely carried out.

The steady-state rate was defined as the slope of the linear zone of the product accumulation curve. The lag period was estimated by extrapolation of the linear zone of the product accumulation curve to the abscissa axis.

*PO Activity.* Peroxidase activity was determined by using 4-methoxy-α-naphthol (4MN) and $H_2O_2$ as substrates (*29*). The enzymatic activity was determined spectrophotometrically by measuring the appearance of the dye product at 593 nm ($\epsilon_{593} = 21\ 000\ M^{-1}\ cm^{-1}$). In this work, IU was defined as the quantity of enzyme that produces 1 $\mu$mol of the dye product per minute at pH 5.0 and 25 °C.

Unless otherwise stated, the reaction medium (1.0 mL) contained 50 mM sodium acetate buffer (pH 5.0), 0.45 mM $H_2O_2$, and 1 mM 4MN.

Spectrophotometric measurements were performed in a Kontron Uvikon 940 spectrophotometer.

**Oxymetric Assays.** Oxygen consumption was followed by a Hansatech D. W. oxymeter based on the Clark electrode covered with a Teflon membrane, equipped with an Amel 863 digital X/Y recorder. The electrode calibration was carried out according to the 4tBC/PPO method (*30*). The assay mixture was under continuous stirring at 25 °C controlled with a circulating bath.

**Denaturing SDS-PAGE.** Electrophoresis was carried out using the method of Laemmli (*31*). Samples were applied to 10% polyacrylamide gels. The slab gels of 1.5 mm thickness were run in a Miniprotean II cell (Bio-Rad) at a constant current of 175 mV. Proteins were fixed and stained by incubation with a Coomassie Brilliant Blue solution (0.1% in 40% ethanol, 10% acetic acid) and destained with 7% acetic acid and 5% ethanol.

**Partially Denaturating SDS-PAGE.** The SDS-PAGE was performed as described above but in the absence of $\beta$-mercaptoethanol and without heating in order to preserve enzymatic activity.

**Isoelectric Focusing (IEF).** IEF was performed on 5% (w/v) polyacrylamide gels in 3.5−10.0 pH gradients using a MiniProtean II (Bio-Rad) electrophoresis kit.

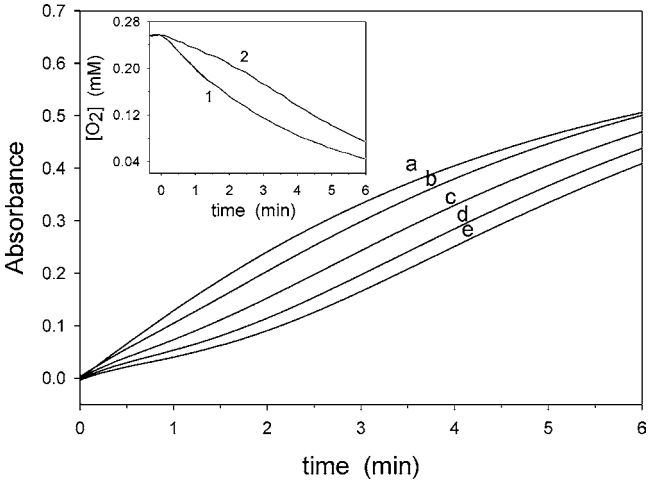


**Figure 1.** Progress curves for the inhibition of mushroom tyrosinase by cucumber extract. The reaction medium contained 6 mM 4tBC in 50 mM phosphate buffer (pH 6.5). Cucumber extract amounts were (**a**) 0, (**b**) 0.1, (**c**) 0.2, (**d**) 0.3, and (**e**) 0.4 mL. The reaction was started by the addition of the enzyme (1 $\mu$g/mL). (Inset) Traces for $O_2$ consumption under conditions of curve **a**, trace **1**, and curve **e**, trace **2**.

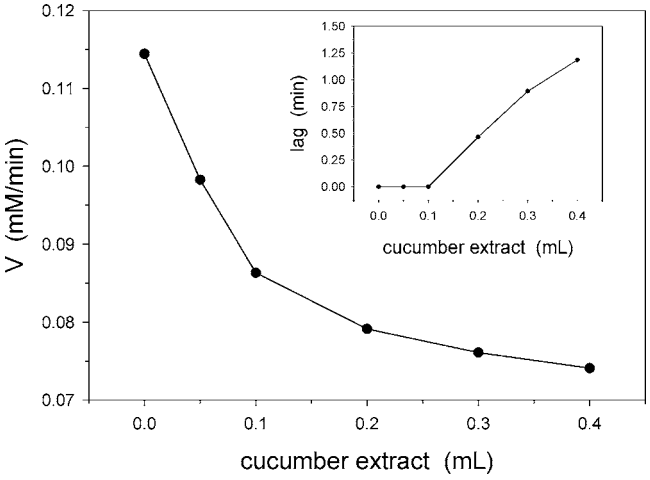


**Figure 2.** Inhibitory effect on the rate and on the lag period (inset) of 4tBC oxidation by mushroom tyrosinase using cucumber extract. The reaction conditions are the same as for the progress curves of **Figure 1**.

**Gel Staining.** After electrophoresis and IEF, the gels were equilibrated into the buffer used to detect the enzymatic activity or prepared to be processed in the Western blot. PPO and PO activities were detected as described by Rodriguez-Lopez et al. (*32*) and Ferrer et al. (*29*), respectively. The staining for PPO activity was carried out with 50 mM sodium phosphate buffer (pH 6.5), 5 mM dopamine, and 2 mM MBTH. The addition of 5 mM tropolone was used for PPO activity inhibition. PO activity was detected with 50 mM sodium acetate buffer (pH 5.0), 1 mM 4MN, and 0.45 mM $H_2O_2$.

**Western Blotting of SDS-PAGE.** Proteins were transferred to PVDF membranes (Bio-Rad) using a Mini Trans-Blot apparatus (Bio-Rad). The transfer was carried out at 4 °C, under constant stirring in 25 mM Tris, 192 mM glycine, and 15% MeOH at pH 8.3 (as transfer solution) at 100 V for 1 h. Once the transfer was finished, the immunodetection was carried out as described by Escribano et al. (*33*) using polyclonal antibodies against PPO from broad bean leaves (a gift from Dr. William H. Flurkey).

## RESULTS AND DISCUSSION

The inhibitory effect of the cucumber extract on PPO activity was assayed by both polarographic and spectrophotometric methods, using 4-*tert*-butyl-catechol as substrate. For these inhibition studies the commercial mushroom tyrosinase was used as an enzyme source because it is readily available.

When the diphenolase activity of tyrosinase was assayed by using 4tBC as substrate, the reaction immediately reached a steady-state rate (**Figure 1**, curve **a**). The addition of different amounts of the cucumber extract to the reaction media resulted in the appearance of a lag period in the formation of a colored product, followed by a low rate of this formation (**Figure 1**, curve **e**).

PPO activity was also determined by measuring the oxygen consumption of the reaction to ascertain the real inhibition of the enzyme activity in which the rate of the quinone formation is slower and, therefore, the rate of oxygen consumption as well. That way it is possible to discard the reaction of the formed quinones with the cucumber extract. The polarographic assays demonstrate that the addition of cucumber extract to the assay medium (**Figure 1** inset, curve **2**) resulted in an appearance of a lag period and in a decrease in the rate of oxygen uptake of the same percentage (35%) as when the reaction was followed spectrophotometrically.

**Figure 2** shows that the increase of the amount of cucumber extract in the reaction media provoked a decrease of the steady-state rate together with the appearance of lag periods in its presence (**Figure 2** inset), both parameters being evaluated as indicated under Materials and Methods.

In addition, preincubation of the enzyme with the cucumber extract in the absence of the substrate did not decrease the enzyme activity significantly, suggesting that the compounds are inhibitors rather than inactivators of the enzyme (*20*).

The attempt to establish the possible components of the cucumber extract responsible for the tyrosinase activity inhibition led us to investigate the chemical structure of the cucumber components described in the bibliography. Several papers have been published about the presence of volatile aldehydes (*34*−*36*) in cucumber, the major component being *trans,cis*-2,6-nonadienal, followed by *trans*-2-nonenal (*37*). In addition, a series of $\alpha,\beta$-unsaturated aldehydes of olive oil were found to inhibit tyrosinase activity; one of them was *trans*-2-nonenal (*25*). For all of these reasons, the commercial aldehyde, *trans,cis*-2,6-nonadienal, was tested to determine its tyrosinase inhibitory activity.

Although the assays were limited by the low solubility in a water solution of these aldehydes, *trans,cis*-2,6-nonadienal showed a dose-dependent inhibitory effect on the oxidation of 4tBC by tyrosinase. The aldehyde group is generally known to react with biologically important nucleophilic groups such as sulfhydryl, amino, or hydroxyl groups. The formation of a Schiff base with a primary amino group in the enzyme has been proposed as being responsible for the inhibitory effect of these aldehydes (*20*, *24*). The inhibition kinetic of this compound was analyzed by a Lineweaver−Burk plot as shown in **Figure 3**. The three lines, obtained from the uninhibited enzyme and from the two different concentrations of *trans,cis*-2,6-nonadienal, intersected on the horizontal axis. This result indicates that *trans,cis*-2,6-nonadienal exhibited a noncompetitive inhibition for 4tBC oxidation by mushroom tyrosinase. The $K_I$ obtained in the these conditions was 3.4 mM. Kubo et al. (*25*) characterized a series of $\alpha,\beta$-unsaturated aldehydes, (2*E*)-alkenals, from olive oil as tyrosinase inhibitors, and their results indicated that (2*E*)-alkenals are also noncompetitive inhibitors. Spectrophotometric and polarographic assays were carried out to discard the chemical reaction of *trans,cis*-2,6-nonadienal with 4tBC or the *o*-quinone formed (results not shown).

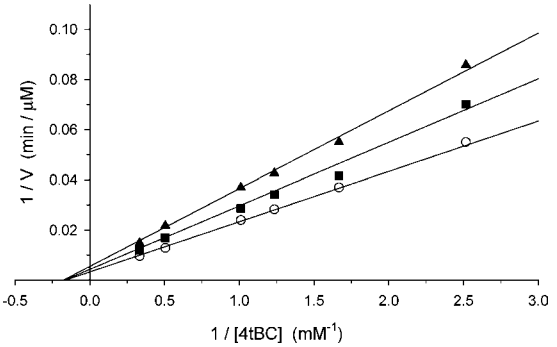


**Figure 3.** Lineweaver–Burk plots for the inhibition of mushroom tyrosinase reaction with 4tBC by *trans*,*cis*-2,6-nonadienal. The reaction medium contained (1.0 mL) 4tBC in 50 mM phosphate buffer (pH 6.5) and mushroom tyrosinase (1.5 $\mu$g/mL). *trans*,*cis*-2,6-Nonadienal concentrations were (○) 0.0, (■) 1.5, and (▲) 2.3 mM.

The next aim was to detect PPO activity in the cucumber skin, following the method of enzyme extraction described under Materials and Methods. Miller et al. (*26*) described that PPO activity was present only in the cucumber skin, whereas PO activity was highest in the skin but was also found in carpel and pericarp tissues.

The enzymes were extracted from cucumber skin. The extraction was carried out by subcellular fractionation, obtaining both the soluble and the membrane fractions. The soluble fraction was partially purified with ammonium sulfate (35−85%). Due to the high chlorophyll content, the membrane fraction was resuspended with Triton X-114 and partially purified by temperature phase partitioning to remove these pigments and phenolic compounds.

To measure PPO activity in both membrane and soluble fractions, several PPO substrates such as dopamine, catechol, 4tBC, 4-methylcatechol, and dopa were used, and the PPO activity was not detected. No PPO activity was found even when SDS was added to the reaction medium to disprove that the enzyme was in its latent state, as has been described for PPO of other sources (*7*−*10*). When a very high concentration of substrate (0.1 M catechol) was used, the addition of the enzyme extract showed a slight increase of the substrate oxidation. To check that PPO was the enzyme involved in this enzymatic reaction, a PPO inhibitor, tropolone (*17*), was added to the reaction medium, and the enzymatic activity registered spectrophotometrically. Tropolone is structurally analogous to ortho-diphenolic substrates of PPO, as well as an effective copper chelator. Furthermore, tropolone is a substrate of peroxidase in the presence of $H_2O_2$ (*38*), and it is therefore an agent that allows differentiation between PPO and PO (*39*). The enzymatic activity turned out to be the same as that without tropolone; therefore, PPO was not the enzyme responsible of the catechol oxidation. However, the presence of 0.33 mM $H_2O_2$ in the reaction medium resulted in an increase of 50 times in the reaction rate. Therefore, the PO enzyme was responsible for catechol oxidation. This control was not performed by Miller et al. (*26*), and therefore this would account for the low level of apparent PPO activity that they observed because they did not differentiate PPO from PO. Furthermore, PO activity was not inhibited by adding different amounts of cucumber extract to the reaction medium.

A partially denaturing SDS-PAGE was used to detect PPO and PO in the enzyme extract from the cucumber skin (**Figure 4**). As can be observed, the soluble fraction showed two active bands with apparent $M_r$ values of 62 and 43 kDa, the latter being the most active when the gel was stained with dopamine and

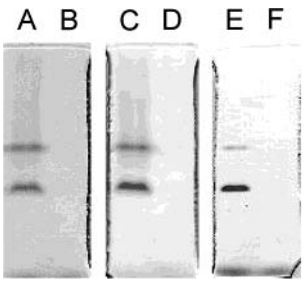


**Figure 4.** Partially denaturing SDS-PAGE of the soluble fraction (lanes **A**, **C**, and **E**) and the membrane fraction (lanes **B**, **D**, and **F**). Lanes **A** and **B** were stained with dopamine and MBTH. Lanes **C** and **D** were stained with dopamine, MBTH, and tropolone. Lanes **E** and **F** were stained with 4MN and $H_2O_2$. Seven micrograms of protein was applied in each well. The specific conditions are described under Materials and Methods.

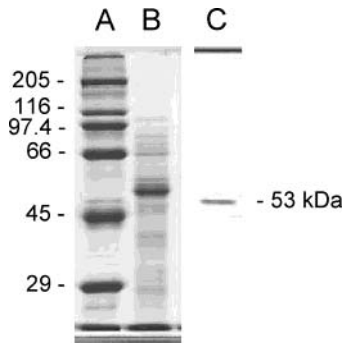


**Figure 5.** Totally denaturing SDS-PAGE (**B**) and immunodetection (**C**) of PPO from soluble fraction. Lane **A** shows molecular markers. Seven micrograms of protein was applied in each well. The gel staining was carried out with Coomassie Blue.

MBTH (**A**). To clarify, if these bands corresponded to either PPO or PO activity, the staining was also carried out in the presence of tropolone (**C**). In this case, the same two bands were also revealed, which indicated that they could correspond to PO activity. These two PO isoforms were also detected when the gel was stained with 4MN and $H_2O_2$ (**E**). However, the membrane fraction did not show any band when the gel was stained with any of the substrates (**B**, **D**, and **F**). Therefore, neither of the enzymatic activities, PPO or PO, was detected in the membrane fraction.

A totally denaturing SDS-PAGE and protein staining with Coomassie Blue (**Figure 5**, lane **B**) revealed a clearly predominant $M_r$ band of 55 kDa in the soluble fraction.

A Western blot from these gels was carried out to detect the presence of PPO using polyclonal antibodies against PPO from broad bean leaves (**Figure 5**, lane **C**). As can be seen on the immunoblotting, only a single band was found with a $M_r$ of 53 kDa, a molecular mass that has also been described for PPO from other sources (*40*, *41*). These results support the assumption that the enzyme PPO is present in the cucumber skin, but some compounds of this fruit inhibit its activity. At this point, it should be taken into account that the cucumbers were cultivated without the addition of any pesticide that could cause the PPO inhibition.

On the basis of our data, the inhibitory action of some cucumber compounds against tyrosinase activity may be an indication of the benefits of the use of cucumber in cosmetic products and its possible use in food to prevent browning.

The study was extended to characterize the other enzyme involved in browning, peroxidase. PO activity was measured spectrophotometrically in both the soluble and membrane fractions, but the enzyme was found only in the soluble fraction.

**Table 1.** $K_m$ and $V_m$ Values for Soluble PO Activity

| substrate | $K_m$ (mM) | $V_m$ ($\mu$M/min) |
|---|---|---|
| 4MN | 0.2384 | 15.82 |
| $H_2O_2$ | 0.1518 | 12.19 |

[a] The reaction media contained different 4MN concentrations and 1.5 mM $H_2O_2$ or different $H_2O_2$ concentrations and 1 mM 4MN, in 50 mM sodium phosphate buffer, pH 5.0, at 25 °C and 0.016 mg/mL of protein.

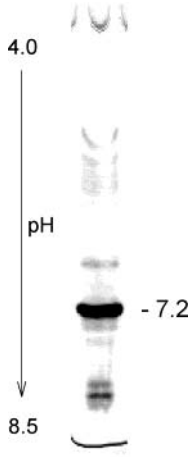


**Figure 6.** IEF of the soluble fraction. The gel staining to detect PO isoforms was carried out as indicated under Materials and Methods.

This fraction was subjected to ammonium sulfate fractionation (35−85%), and a recovery of 86% was obtained, but the degree of purification was negligible. However, this treatment did allow us to obtain a clarified and concentrated enzymatic solution, which was appropriate for further electrophoretic studies.

The kinetic characterization of PO from the partially purified soluble fraction using 4MN and $H_2O_2$ as substrates permitted the determination of the $V_m$ and $K_m$ values for both substrates (**Table 1**). There are no studies concerning the kinetic characterization of soluble PO from cucumber fruit, but if we compare the findings with those obtained in other vegetable sources such as beet root (*34*), the $K_m$ value for $H_2O_2$ was identical, whereas the $K_m$ for 4MN was half of what has been observed in beet root.

IEF is a useful electrophoretic method for studying PO isoenzymes. A lot of researchers have identified multiple PO isoenzymes using this technique: 42 isoenzymes in horseradish (*42*), 18 in pea (*43*), and 2 in spring cabbage (*13*). For this reason, the soluble fraction was analyzed by IEF (pH 3.5−10), and it showed a complex isoenzyme pattern (**Figure 6**). The staining of PO activity on the gels revealed the presence of several acidic isoenzymes with a p*I* in the range between 5 and 6, a basic isoenzyme, and one principal neutral isoenzyme of p$I = 7.2$. Repka and Fischerova (*44*) studied the PO isoenzyme pattern in different cucumber organs, finding a set of acidic PO isoenzymes, which were strongly regulated and related to the defense response. These anionic POs seem to play a central role in the synthesis of secondary metabolites, such as lignins (*45*). On the other hand, the basic PO isoenzymes are located in vacuoles and therefore are colocalized in the plant cell with most of the phenolic substrates involved in enzymatic browning (*46*). In grapes, a basic PO isoenzyme oxidizes the flavonols, quercetin and myricetin, in vacuoles (*47*).

In summary, PPO activity was not detected in cucumber skin extract, but its presence was revealed by Western blot. This observation suggests that PPO is present in cucumber skin but is not able to express its activity. This assumption could be supported by the inhibitory effect of a cucumber extract on mushroom tyrosinase. Furthermore, the commercial aldehyde, *trans,cis*-2,6-nonadienal, described as a major volatile compound of cucumber, was characterized as a noncompetitive inhibitior of mushroom tyrosinase. However, another enzyme responsible for browning, PO, was measured in cucumber skin and did not suffer inhibition by cucumber extracts. According to our research, PO might be the enzyme to monitor this process in cucumbers rather than PPO.

## ABBREVIATIONS USED

PPO, polyphenol oxidase; PO, peroxidase; 4MN, 4-methoxy-α-naphthol; MBTH, 3-methyl-2-benzothiazolinone hydrazone; PMSF, phenylmethanesulfonyl fluoride; 4tBC, 4-*tert*-butylcatechol.

## ACKNOWLEDGMENT

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## NOTE ADDED AFTER ASAP POSTING

In the version of this paper published 11/20/03 on the Web, the DGICYT grant number was incorrect. The correct version was posted 11/20/03.

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