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Effect of detergents, trypsin and unsaturated fatty acids on latent loquat fruit polyphenol oxidase: Basis for the enzyme's activity regulation

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Abstract

The effects of detergents, trypsin and fatty acids on structural and functional properties of a pure loquat fruit latent polyphenol oxidase have been studied in relation to its regulation. Anionic detergents activated PPO at pH 6.0 below critical micelle concentration (cmc), but inhibited at pH 4.5 well above cmc. This behavior is due to a detergent-induced pH profile alkaline shift, accompanied by changes of intrinsic fluorescence of the protein. Gel filtration experiments demonstrate the formation of PPO–SDS mixed micelles. Partial PPO proteolysis suggest that latent PPO losses an SDS micelle-interacting region but conserves an SDS monomer-interacting site. Unsaturated fatty acids inhibit PPO at pH 4.5, the strongest being linolenic acid while the weakest was γ-linolenic acid for both, the native and the trypsin-treated PPO. Down-regulation of PPO activity by anionic amphiphiles is discussed based on both, the pH profile shift induced upon anionic amphiphile binding and the PPO interaction with negatively charged membranes.
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Polyphenol oxidases (PPOs)[2] (EC 1.14.18.1 or EC 1.10.3.2) are ubiquitous plant enzymes that catalyze the OB_{2B}-dependent oxidation of mono- and di-phenols to *o*-quinones. The oxidation of phenolic substrates by PPO is thought to be the major cause of the brown discoloration of many fruits and vegetables during ripening as well as handling, storage and processing [1–4]. This problem, known as enzymatic browning, is of considerable importance to the food industry as it affects nutritional quality and appearance, reduces consumer acceptability and therefore causes significant economic impact, both to primary food producers and to the food processing industry [1,2]. Thus the detailed knowledge of the regulation of this ubiquitous enzyme has practical implications in the food and agriculture industry. Although sub-cellular compartimentation is a generally accepted mechanism of enzymatic browning control [3], substantial experimental evidence point to additional mechanisms of PPO activity regulation.

Many plant PPO preparations from different tissues including leaf [5,6], fruit [7–9] and root [10,11] are said to be latent since an increase in enzyme activity at pH 6.0 or higher occurs when treated with activating agents. Activation of PPO can be achieved either by long exposure to acidic pH [12,13], to sub-cmc concentrations of SDS

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[2] *Abbreviations used:* CA, chlorogenic acid; cmc, critical micellar concentration; CTAB, cetyltrimethylammonium bromide; DPHT, 1,6-diphenyl-1,3,5-hexatriene; Li, linoleic acid; α-Ln, α-linolenic acid; γ-Ln, γ-linolenic acid; Ol, oleic acid; PPO, polyphenol oxidase; SDS, sodium dodecyl sulfate; SDSA, sodium 1-dodecanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SOS, sodium octyl sulfate; SOSA, sodium 1-octanesulfonic acid; TBC, tert-butylcatechol; THF, tetrahydrofurane; TX-100, Triton X-100.

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[6,12], unsaturated fatty acids [8] or incubation with proteases [14,15], among others. Although many studies report on PPO activation by the above mentioned treatments, only a few [16,17] have been conducted using homogeneous preparations of PPO.

PPO genes are generally organized into multigene families [18–21]. Several studies have demonstrated the simultaneous expression of two or more PPO genes in the same tissue at the same developmental stage [22,23]. Moreover, in apricot fruit PPO protein and its catalytic activity are present whatever the fruit age, although its coding gene PA-PPO was transcriptionally active only in the early immature-green fruits [24]. Therefore the occurrence of different PPO gene products in the same plant tissue seems highly probable. Regarding enzyme activity level, non-latent PPO activity has been found both in soluble and particulate fractions of tomato fruits, exhibiting differential kinetic properties [25]. Likewise, in a previous work we demonstrated the co-existence of two PPO activities in loquat fruit, one active and SDS-insensitive found in the soluble fraction, accounting for a 20% of the total PPO activity, and another latent, activable by SDS, present in the particulate fraction and accounting for an 80% of the total PPO activity [26]. The facts described above highlight the importance of using homogeneous preparations of latent PPO to characterize the phenomenon of latency and its relation with the enzyme's activity regulation linked to a specific PPO gene product. Plant PPOs having tetrameric structure composed of 30 kDa subunits have been purified [13,27], described to be latent and activated by acid pH exposure and SDS [17]. However, no reports on the genes encoding these oligomeric PPOs have been published to date.

The activation by SDS has been correlated with conformational changes of the protein in accordance with concomitant changes in the intrinsic protein fluorescence [6,17] and ellipticity [17]. The effect of SDS has been described as a change of the enzyme behavior with respect to pH rather than as an activation, consisting of an enzyme's pH profile shift of ca. 1.5–2 U towards alkaline pH [28], thus it was proposed the use of the term 'activation by SDS' be restricted to a range of pH values [29].

Partial proteolysis was early recognized as one of the typical activating treatments [12]. Known plant PPO genes are nuclear and encode polypeptides sized between 65 and 70 kDa [30]. Cleavage of the N-terminal transit peptide responsible for chloroplast and thylakoid targeting yields a mature protein of approximately 60 kDa [15,18,19,21,31–33]. Loquat fruit latent PPO purified to homogeneity by us corresponded to a 59 kDa monomer [26]. Nucleotide sequence analysis indicates that the mature protein comprises a large domain of ca. 40 kDa that contains two highly conserved copper-binding regions, required for PPO activity [19,32], and a C-terminal domain which extends for ca. 16 kDa has not a clearly assigned function, although it might have a role in activation. It has been shown that the 60 kDa purified broad bean leaf PPO proteolyzed by trypsin treatment, was converted into a catalytically active 40 kDa form that conserved just a residue of sensitivity to SDS treatment [15]. A C-terminal extension domain, found in the cDNA sequence of sweet potato catechol oxidase but not in the purified, mature form, has been modeled on the basis of three dimensional structural data of mature catechol oxidase and of hemocyanin from giant octopus [34]. These authors propose that the C-terminal extension likely acts as a shield precluding the access of substrate to the active site and either limited proteolysis, that removes this domain or detergents that may induce changes in the orientation of such region, both would lead to activation. In spite of the well documented effect of trypsin on latent PPO, no one endogenous proteolytic activity acting in a similar fashion has been reported so far.

Among all the latent PPO activating agents described until now, only free fatty acids might have a possible biological significance in plant tissues. These were first described as activators of a crude chloroplast preparation of broad bean leaf PPO [35]. More recently, the long exposure of a partly pure preparation of pear fruit PPO to fatty acids resulted in a slight increase in activity [8].

In order to gain insight in mechanisms of PPO activity regulation, apart from sub-cellular compartimentation, in this paper we have studied the effect of activating agents—detergents, trypsin and fatty acids—on both structural and functional properties of a homogeneous latent PPO purified from loquat fruits [26]. Conformational changes in latent PPO induced by the presence of SDS were assessed by intrinsic fluorescence changes of the protein. It has been showed that long chain alkyl sulfates and alkyl sulfonates, being activators of PPO at pH 6.0, or higher, inhibit PPO at pH 4.5, to an extent mainly dependent on chain length. From gel filtration experiments in the presence of detergents it has been demonstrated the formation of mixed micelles of latent PPO with SDS with a defined stoichiometry. Eventually, unsaturated fatty acids are shown to inhibit PPO at pH 4.5. Taking into consideration the loquat fruit juice pH and the pH stability of the natural substrate chlorogenic acid, results are interpreted in terms of down-regulation of PPO activity by anionic amphiphiles through induction a pH profile-shift and by physical uptake of PPO by negatively charged surfaces.

Materials and methods

Biological materials

Loquat fruits (*Eriobotrya japonica*, cv. Algerie) were picked ripe from trees cropped in the experimental orchards of Cooperativa Agrícola de Callosa d'En Sarria, located in a semi-arid zone in Alicante (Spain). The fruits were kept at −20 °C until use.

Reagents

Sodium dodecyl sulfate (SDS), sodium octyl sulfate (SOS), sodium 1-dodecanesulfonic acid (SDSA), sodium 1-octanesulfonic acid (SOSA), Triton X-100 (TX-100), cetyltrimethylammonium bromide (CTAB), oleic

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