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UNFOLDING AND REFOLDING OF ACTIVE APPLE POLYPHENOL OXIDASE

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Abstract—For the first time, unfolding (6 M guanidine) and refolding of partially proteolysed purified polyphenol oxidase (PPOr) was achieved, with 88% of activity recovered. Optimal refolding conditions consisted in stepwise dialysis of guanidine treated extracts, the dialysis buffers containing 1 M $(NH_4)_2SO_4$ and 100 μM $CuSO_4$. However, $CuSO_4$ had limited effect on the recovering of PPOr activity, whereas $(NH_4)_2SO_4$ was essential. Concerning the PPO tertiary structure, denaturing conditions (combinations of boiling and reducing agent) used on SDS-PAGE have shown (i) a compact tertiary structure and (ii) the presence of disulfide bonds in PPOr, accounting for the shift between 27 and 41 kDa, and 41 and 42 kDa, respectively. Resistance to proteolytic cleavage was used to study the conformational changes induced by the denaturing treatments. Folded PPOr was resistant to further proteolysis whereas unfolded PPO was totally digested, indicating the role of tertiary structure of PPOr in the resistance to proteases.

INTRODUCTION

Plant polyphenol oxidases (PPO) are copper-containing enzymes catalysing hydroxylation of mono phenols to *o*-diphenols (mono oxygenase, EC 1.14.18.1) and/or oxidation of *o*-diphenols to *o*-diquinones (catecholase, EC 1.10.3.1) [1–3]. The *o*-diquinones generated are highly reactive and may auto oxidize or cross-link to nucleophilic targets such as amino acids and nucleic acids, leading to browning, a widespread and often undesirable phenomenon. Although these reactions have been well studied in technological processes of fruit and vegetable storage [4], the physiological role of PPO is not yet totally elucidated. Despite a possible role in photosynthesis due to the plastidial location of the enzyme [5, 6], recent data suggested the participation of PPO in defense reactions against pathogens and insect pests [7–9].

PPO is a nuclear encoded protein, located in the thylakoïd lumen [10, 11]. Studies on the routing of PPO to chloroplasts have demonstrated a typical thylakoïd lumen targeting [11]. However, inhibition of PPO import into plastids by low concentrations of Cu^{2+} tended to suggest that the intermediate forms have to be unfolded for protein import. Cu^{2+} was suspected to bind to the precursor, preventing complete unfolding [11]. Indeed, many plastidal proteins are maintained partially unfolded *in vivo* by chaperonins during the targeting into the thylakoïds [12, 13].

The most intriguing feature of PPO, particularly apple PPO, is the resistance of PPO activity to SDS and proteinase K incubations, previously studied in our laboratory [14, 15]. The resistance of PPO to SDS allowed detection of the activity on SDS-PAGE, providing the samples are not boiled and reduced. This helpful feature allowed us to show the relationship between the two active forms of apple extracts: the smaller form (27 kDa non-denatured, 42 kDa when denatured) resulted from limited proteolysis of the main form (42 kDa non-denatured, 64 kDa when denatured) [14] and this relationship was further demonstrated with PPOs from different *Prunus* fruits [16].

In this work, the first step was to study the implication of the tertiary structure on the biological activity of apple fruit proteolysed PPO, purified by proteolysis of apple extracts, as previously described [14]. The protein obtained, smaller than the native PPO, was still active and resistant to further proteolysis and it will be further called PPOr. For the first time unfolding and refolding of active PPOr was

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achieved. Clear evidence for the occurrence of a compact tertiary structure and disulfide bonds, in the apple PPOr were provided. Resistance to proteinase was also studied in relation to the different conformational changes of the protein.

RESULTS AND DISCUSSION

PPOr denaturation and renaturation

In this work, we describe for the first time the conditions of renaturation of apple PPOr after denaturation with 6 M guanidine hydrochloride (GdmCl). In the presence of 6 M GdmCl, PPOr was totally inactivated. Dialysis in the recovery buffers without addition of any salt, did not result in recovery of PPOr activity. The addition of $CuSO_4$ alone in the recovery buffers was not efficient in restoring PPO activity, whereas a recovery of 88% was obtained when $(NH_4)_2SO_4$ and $CuSO_4$ were used together in the recovery buffers. Interestingly, the absence of $CuSO_4$ had a limited and not significant effect on the PPO recovery when $(NH_4)_2SO_4$ was added (75% versus 88%).

Copper salts have already been used in purification protocols of PPO to lower the loss of activity during dialysis, which is a key step during PPO purification [8]. It may be assumed that during unfolding of apple PPO, the copper atoms of PPO are not extensively lost. The use of ammonium sulfate is often recommended during renaturation of proteins, because it limits inter-molecular aggregations [17]. Other work reported ammonium sulfate as a key element in renaturation conditions [18, 19]. From a dynamic point of view, it has been shown that proline isomerization from *trans* to *cis*, during refolding, is accelerated with ammonium sulfate during the formation of folding intermediates [20].

Refolding can also be analysed on SDS-PAGE, since unfolded and refolded species have different electrophoretic mobilities. Control and renatured PPOr were analyzed on immunoblots (Figure 1), under partially denaturing conditions (without boiling and reducing). The PPOr was 27 kDa and a faint band of 41 kDa was stained (Figure 1 lane 1). This inactive 41 kDa form was previously described as corresponding to the same protein unfolded [15]. Such an artefactual denaturation could occur during the concentration steps of purification. The renatured PPOr was 27 kDa, as expected, and no denatured form was visible, demonstrating that refolding was highly efficient and that the 41 kDa form in the standard extract did correspond to the denatured PPOr (Figure 1 lane 2). The lesser intensity of the band in lane 2 could be due to some loss of protein during ultrafiltration, prior to electrophoresis. In our experiments, the removing of GdmCl, even followed by acid or basic shocks, always resulted in spontaneous refolding of the protein (data not shown). Thus, for further

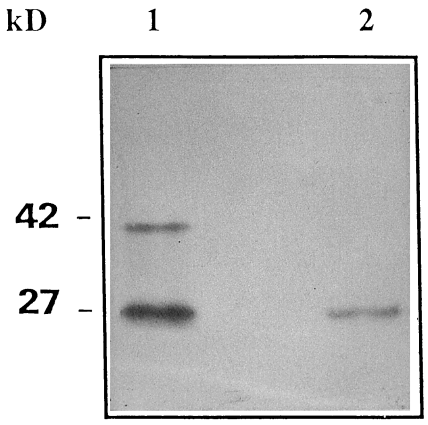

Fig. 1. SDS-PAGE immunoblot of purified proteolysed PPO, before and after dentaturation-renaturation cycle. Lane 1, purified proteolysed PPO, lane 2, purified proteolysed PPO after a dentaturation-renaturation cycle. About 5 μg of PPO were used.

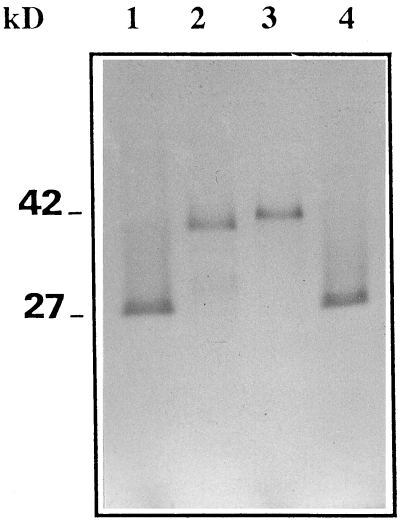

Fig. 2. SDS-PAGE immunoblot of purified proteolysed PPO under different denaturing conditions. Lane 1, PPO neither heated or reduced, lane 2 PPO heated 3 min at 100°, lane 3, PPO heated 3 min at 100° and reduced with 0.03% (v:v) β-mercaptoethanol, lane 4, PPO incubated 20 min at 25° with 0.3% (v:v) β-mercaptoethanol. About 10 μg of PPO were used for each condition.

experiments, unfolding was performed by heating samples 3 min at 100°C as suggested elsewhere [17].

Disulfide bonds of PPOr

Samples were loaded on a 12% SDS-PAGE and detected by immunoblotting (Figure 2). Heating at 100°C resulted in a single band of 41 kDa (Figure 2 lane 2). When PPO was boiled and reduced by addition of β-mercaptoethanol, a small shift was visible, from 41 to 42 kDa (Figure 2 lanes 2 and 3). This shift in the electrophoretic mobility indicates the

occurrence of disulfide bonds in the PPOr, as it was described for native PPO [15, 21]. This result is consistent with the analysis of the amino acid sequences of PPO already described, particularly apple PPO [22]. The deduced polypeptidic sequence obtained from a PPO cDNA expressed in wounded apple tissues revealed the occurrence of six cysteine residues located mainly in the N-terminal half of the protein, indicating the possibility of three disulfide bonds [22]. The putative disulfide bonds should strengthen a region of at least 350 amino acids, comprising the two copper-binding regions of the protein and leaving the C-terminal third of the polypeptide sensitive to proteolytic cleavage, as demonstrated for broad bean PPO by Robinson and Dry [23]. During the proteolysis of native apple PPO, a peptide representing one third of the apparent *Mr* is removed. This peptide, is also supposed to be a regulatory domain for the pH response, as suggested by Marquès *et al*. [15]. Concerning this residual protein, PPOr, we now show that a change in the tertiary structure can lead to a shift from 27 to 41 kDa, and reduction of internal disulfide bonds modify the electrophoretic mobility from 41 to 42 kDa.

Incubation with β-mercaptoethanol at 25° did not affect the electrophoretic mobility and no band corresponding to the release of a smaller peptide was detected (Figure 2 lane 4). In the non-denatured sample (Figure 1, lane 1), the upper faint band had the same *Mr* as the heat treated PPO, 41 kDa (Figure 2, lane 2). This form obtained by spontaneous denaturation (when handling the samples) still contains disulfide bonds and could therefore be refolded, as shown above (Figure 1, lanes 1 and 2).

Conformational changes and resistance of PPOr to proteolysis

Incubations with proteinase K of PPOr previously treated with β-mercaptoethanol at 25° did not produce any noticeable change in the electrophoretic mobility and in the intensity of the band, compared with the standard PPOr (Figure 3 A, B and C, lanes 1 and 4). These results indicate that a possible reduction of internal disulfide bonds does not induce an important modification in the tri dimensional structure of the protein, provided no chaotropic agent was used. By contrast, the two denaturing treatments used (heating and heating/reducing) led to extensive digestion of PPOr by proteinase K since immuno staining of the corresponding bands is very faint (Figure 3 A, B and C, lanes 2 and 3). The dose effect was clear for both treatments; PPOr was totally proteolysed and the corresponding band completely disappeared with a 100-fold dilution of proteinase K (Figure 3 C, lanes 2 and 3) whereas faint bands and lower intermediary bands were still visible with the 1000-fold dilution (Figure 3 A, lanes 2 and 3). At the high dilutions of proteinase K, the band corresponding to the heat treated PPOr was more visible than the heated and reduced PPOr (Figure 3 A, lanes 2 and 3), indicating that the disulfide bonds in the denatured protein still have little effect on proteinase susceptibility. Heating the PPOr was not sufficient to totally denature the protein, and thus the conformation only due to the presence of disulfide bonds could confer a light resistance to proteinase K (Figure 3 A and B, lanes 2 and 3).

Our experiments have provided evidence concerning the key role of the tertiary structure in the resistance of PPOr to proteases. Kinetics of proteolysis of native PPO have shown that apple PPO is totally proteolysed into a smaller, still active protein within 1 hr, with very low levels of proteinase [14]. This behaviour towards proteolysis could be ubiquitous, since it was shown for broad bean leaf PPO [23], various *Prunus* fruit PPOs [16] and even for tyrosinase, a very similar enzyme [24, 25].

PPO has recently been more and more implicated in defense reactions, using defense reaction signals such as methyl jasmonate or systemin as PPO activators [9], wounding [26]. In a plant-pathogen interaction, decompartmentation of infected cells is a classical event. In this context, it may be assumed that, if PPO is implicated in defense reactions, rapid proteolysis could take place. The resulting PPOr would be resistant to the action of proteases and would have a wide optimum pH spectra [15]. This form could thus be considered as the protein implicated *in vivo* in the defense mechanisms against pathogens. In this hypothesis, it appears that PPOr could be an interesting model in the study of the physiology of the defense reactions, as regards the structure-related characteristics of the protein.

EXPERIMENTAL

Plant material

Apples (*Pyrus malus* L. cv Granny Smith) picked in June were kindly provided by the Centre Expérimental Horticole de Marsillargues (CEHM, Hérault, France) and stored at −80°.

Enzyme extraction

All steps were carried out as described in ref. [14], at 4°. Briefly, 300 g of apples were disrupted in a Waring Blender with 400 ml of a grinding buffer (Na-Pi 0.1 M pH 7.3, 300 mM sorbitol, 40 mM Na ascorbate). The homogenate was filtrated through 2 layers of cheesecloth and centrifuged (6000 *g*, 10 min). The pellets enriched in thylakoïd membranes [23, 27] were resuspended in the same buffer and pelleted (6000 *g*, 10 min). The resulting washed thylakoïd membrane pellets were stirred 30 min with 0.1 M Na Pi buffer pH 7.3, 1.5% (v/v) Triton X-114, 40 mM Na ascorbate and centrifuged (40 000 *g*, 20 min). The supernatant was made 8% Triton X-114 and submitted to temp-induced phase partitioning [28]. After centrifugation (3000 *g*, 10 min) the colorless supernatant phase

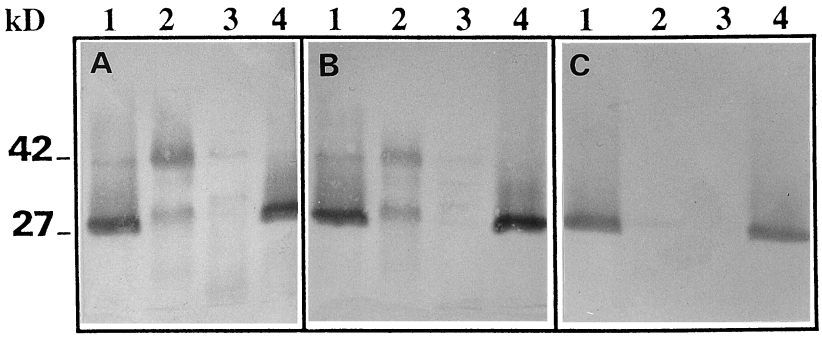

Fig. 3. Proteinase K digestion of PPO after different denaturing conditions. Proteolysis was performed on the stacking gel during 3 min and then proteins were seperated, blotted and immunostained. A, 1:1000 dilution of proteinase K (3 mU.ml^{-1}), B, 1:500 dilution of preoteinase K (6 mU.ml^{-1}), C 1:100 dilution of proteinase K (0.03 U.ml^{-1}), Lane 1, PPO neither heated nor reduced, lane 2, PPO heated 3 min at 100°, lane 3, PPO heated 3 min at 100° and reduced with 3% (v:v) β-mercaptoethanol, lane 4, PPO incubated 20 min at 25° with 0.3% (v:v) β-mercaptoethanol. About 15 μg of PPO were used for each condition.

enriched in PPO activity was recovered and the pellet containing mostly pigments was discarded.

Enzyme purification

The phase partitioning supernatant was made 0.7% SDS (w/v), and the pH increased to 8. The proteolysis started with addition of 0.1 U.ml^{-1} of proteinase K at 37° during 90 min. The proteolysed extract was then loaded on a DEAE-cellulose column equilibrated with 0.1 M Na-Pi buffer pH 7.3, 3.5% (v/v) glycerol, 0.03% (v/v) aprotinin, and PPO was eluted with the same buffer supplemented with 0.2 M NaCl. The most active fractions were pooled, dialysed and constituted the highly purified PPOr.

Protein determination

Protein content was determined by the method of ref. [29], using bovine serum albumin as standard.

Enzyme assay

PPO activity (EC 1.10.3.1) was measured at 30° in 2.5 ml of Na-Pi buffer pH 6, 20 mM 4-methyl catechol. The A_{410} was monitored immediately after mixing. One unit of PPO activity was defined as the amount of enzyme which gives a change of 1 A unit per min under the specified conditions.

Protein unfolding and refolding (denaturation-renaturation)

PPO unfolding was realized by adding guanidine hydrochloride (GdmCl) to purified extracts (20 μg.ml^{-1} protein), to a final conc of 6 M [17] and incubated 30 min at 25°.

Renaturation was performed by stepwise dialysis of denatured solutions: the first step consisted in dialysis at room temp. during 12 hr, against a Na-Pi buffer 0.1 M pH 7.3 containing 2 M GdmCl (recovering buffer 1). In a second step, dialysis was performed at 4° during 24 hr against a Na-Pi buffer 0.1 M pH 7.3 (recovery buffer 2). Dialysis buffer vols of buffer 2 were calculated to give a residual GdmCl concentration not exceeding 60 mM. The effect of $CuSO_4$ and/or $(NH_4)_2SO_4$ on PPOr recovery was tested, by adjusting both recovery buffers 1 and 2 with 100 μM $CuSO_4$ and/or 1 M $(NH_4)_2SO_4$. Control conditions consisted in dialysis of purified extracts (without GdmCl) in the same conditions of duration, salt concns and temp., in a Na-Pi buffer 0.1 M pH 7.3. Recovery of PPOr activity was assayed by spectrophotometric measurement as described above.

Electrophoresis

SDS-PAGE was conducted using the discontinuous gel system [30]. The separating gels (5.8 × 8.2) contained 12% polyacrylamide. The partially denaturing conditions consisted of a 4-fold dilution of the samples with a (4X) Tris HCl buffer 62.5 mM pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS and 1% (v/v) bromophenol blue. Under these conditions, samples were not boiled. Samples could also be (i) boiled 3 min with the same buffer without reducing agent (heat-denatured samples), or (ii) incubated with 0.3% (v/v) 2-mercaptoethanol at 25° during 20 min, or (iii) boiled and reduced with the sample buffer containing 0.3% (v/v) β-mercaptoethanol (totally denatured samples). After these treatments, samples were loaded on 12% SDS gels.

Proteinase K digestion after denaturing treatments

After the several denaturing treatments cited above, proteinase K digestion was performed [31]. 100, 500, 1000-fold dilutions of the proteinase K stock soln (43 U.ml^{-1}) were made, and 1 μl was added to 15 μl of PPOr soln, just before electrophoresis. Running was stopped for 3 min to allow proteolysis in the stacking gel before re-run.

Protein immunoblotting

After separation by SDS-PAGE, proteins were transferred onto Immobilon P (Millipore). The electroblotting device was the Milliblot™—SDE Transfert System (Millipore), run at room temp., at 2.5 mA.cm^{-2} for 35 min with the buffers given in the instructions (anode 1 buffer: 0.3 M Tris, 10% (v/v) MeOH pH 10.4; anode 2 buffer: 25 mM Tris, 10% (v/v) MeOH, pH 10.4; cathode buffer 25 mM Tris, 40 mM 6-aminohexanoïc acid, 20% (v/v) MeOH pH 9.4). For PPO detection, the rabbit anti-PPO serum previously obtained in our laboratory [14] was diluted 1:1000. This polyclonal serum was raised against the PPOr, first described in ref [14]. The secondary antibody, anti-rabbit IgG, was alkaline phosphatase conjugated.

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