Isolation and Partial Characterization of Thermostable Isoperoxidases from Potato (*Solanum tuberosum* L.) Tuber Sprouts

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Peroxidases (POD; EC 1.11.1.7) can cross-link cell wall polymers and may have an impact on the final textural quality of potato tubers. Because heat treatments are important during processing, the thermal properties of isoPODs from soluble and ionically and covalently bound fractions were studied from both potato tubers and sprouts. For both tissues, the ionically bound fraction was the most thermostable; ~20% of POD activity remained after a heat treatment of 10 min at 90 °C (for sprouts). The temperature profile of the ionically bound sprout fraction appeared to be nonlinear and suggested the presence of a very thermostable POD, which still showed activity after a heat treatment at 100 °C. Visualization by using isoelectric focusing confirmed the occurrence of a thermostable isoPOD with an IEP of 9.5, which displayed regeneration of activity after heat inactivation. This cationic POD was further purified by chromatography techniques, and by SDS−PAGE its molecular mass was estimated at 38 kDa.

Keywords: *Potato sprouts; peroxidases; thermostability; isoforms; Solanum tuberosum*

INTRODUCTION

For harvested fruits and vegetables, peroxidases (POD; EC 1.11.1.7) are related to food quality and can contribute to deterioration of stored fruits and vegetables by triggering changes in flavor, texture, and color unless they are inactivated (Haard, 1977; McLellan and Robinson, 1987a). Blanching is the most widely used treatment to inactivate enzymes, thereby preserving food quality during storage and further processing. However, it has been established that PODs are among the most thermostable enzymes and that some are able to recover activity after heat treatment (Naveh et al., 1982; McLellan and Robinson, 1984; Lopez and Burgos, 1995; McEldoon and Dordick, 1996). According to Hammer (1993), blanching of potatoes at 75 °C was not sufficient for total inactivation of POD. This thermostability necessitates a severe blanching treatment, causing a loss of organoleptic quality of food. Moreover, the texture of processed fruits and vegetables is an important quality aspect and is directly related to the mechanical properties of the cell wall. At tissue level, both cell wall strength and cell-to-cell adhesion play a role in determining plant texture (Waldron et al., 1997). For potato, a large extent of cell separation correlates with the so-called "mealiness" of cooked potato tissue (van Marle, 1997). Activation of some cell wall enzymes such as pectin methylesterase (PME; Laats et al., 1997) and POD improves the firmness of the cell wall. Both enzymes can increase the strength of the cell tissue by ionic or covalent binding of cell wall components. Therefore, one strategy to modify texture would be an increase of PME activity leading to a reduction of pectin breakdown. Another strategy would be the augmentation of cross-linking of ferulic acid by increasing the POD activity in plants and, consequently, a reduction of mealiness after cooking.

PODs use H_2O_2 as an electron acceptor and can oxidize a multitude of donor compounds (Hammer, 1993). Many isoforms of plant POD (isoPODs) have been identified (Shannon, 1968). Ranked according to their isoelectric points, isoPODs are roughly classified into an acidic (anionic) group with an IEP range from 3 to 6, a neutral group, and a basic (cationic) group with an IEP range of 8 to 10 (Robinson, 1991). IsoPODs have been purified from different plant materials, and most molecular masses of proteins varied between 40 and 50 kDa (Gaspar et al., 1982). For potato, however, Decedue et al. (1984) isolated four main isoPODs with apparent molecular masses of 48.5−105 kDa. Khan et al. (1981) showed that potato PODs still displayed enzyme activities after 10 min at 70 °C, but they did not investigate the behavior of PODs at higher temperatures, nor did they study the reactivation of PODs. In relation to the role of PODs in determining the processing quality, we report on the nature, putative localization, and thermostability of the respective POD isoforms.

MATERIALS AND METHODS

Plant Material. Tubers and sprouts from potatoes (*Solanum tuberosum* L.) cv. Agria and Bintje were used as a source to prepare POD extracts. After harvesting, tubers were stored at 4 °C in the dark for up to 6 months. Two-month-old sprouts and the tubers from which they originated were collected, washed, freeze-dried, and stored at −20 °C.

Preparation of POD Extracts. Three POD fractions were subsequently extracted according to the method of Gkinis and Fennema (1978) with minor modifications as mentioned below.

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So-called soluble PODs were isolated by extracting plant material with a 0.05 M sodium phosphate buffer, pH 6.0. After centrifugation (rotor SS34, 14000 rpm for 60 min), the supernatant was collected. The residue was washed twice with demineralized water and homogenized for 4 min (Ultra-Turax, 9500 rpm) in 1 M NaCl. After centrifugation (rotor SS34, 14000 rpm for 20 min), the supernatant was collected and referred to as the ionically bound POD fraction. The remaining residue was washed twice and similarly homogenized in 0.2 M sodium acetate buffer, pH 5.5, supplemented with 2% pectinase (Fluka) and 1% cellulase (Fluka). The homogenate was incubated for 18 h at 35 °C and centrifuged for 20 min at 14000 rpm, rotor SS34. The supernatant was collected and referred to as the covalently bound POD fraction. The enzyme extracts were stored at −50 °C.

The three POD fractions were assayed for POD activity (see below), and the total protein concentration was determined as described in the microassay procedure of Pierce, which is based on Bradford's method (1976).

Determination of POD Activity. POD activity was determined according to the method of Anao et al. (1990) using 3 mM 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a hydrogen donor and 0.045% H_2O_2 as a hydrogen acceptor in 0.1 M sodium citrate buffer at appropriate pH values. Enzyme activity was determined by monitoring the absorbance at 414 nm and expressed in microkatals per milligram of protein. One katal is defined as 1 mol of substrate transformed by the enzyme in 1 s. The pH dependency of the activity was established between pH values of 2.0 and 6.0 for sprouts and 4.0 and 5.0 for tubers.

Isoform Composition of the POD Fractions. The presence of POD isoforms was visualized by electrophoresis. After ultracentrifugation (Centricon 10, 1950*g*, Amicon) for 2 h at 4 °C, isoforms of the POD fractions were separated by isoelectric focusing using the Phastsystem (Amersham Pharmacia Biotech). The pH gradient of carrier ampholyte was from 3 to 10. Activity staining was performed following the modified Sijmons' method (1986; guaiacol and H_2O_2 as substrates). Gels were incubated for 5 min in 0.5% guaiacol and 0.25% H_2O_2 in 0.1 M sodium citrate buffer, pH 4. Subsequently, gels were incubated for 5 min in a stop solution of 5% acetic acid.

The isoelectric points (IEP) were determined more precisely by isoelectric focusing electrophoresis using large precasted gels (Multiphor system, Precots, pH 3−10, Amersham Pharmacia Biotech) followed by activity staining as described above. In addition, the Bio-Rad Rotofor system was used to measure the IEP of isoPODs of the ionically bound POD fraction. Prior to Rotofor analysis, the ionically bound POD fraction was ultrafiltered using a membrane with a cutoff of 10 kDa to desalt the sample buffer (pressurized stirred cell, Amicon). A 2% ampholyte solution (pH 8−10.5) was used to create a pH gradient. Prefocusing of 1 h was performed before application of the samples. The running conditions were as follows: 2000 V, 12 W for 3 h. Protein concentration after ampholyte removal (precipitation with 1 M NaCl) and POD activity were determined for all collected fractions.

Determination of the POD Thermostability. Thermal inactivation and reactivation of PODs were measured as follows: a 300 μL aliquot, in a 1.5 mL Eppendorf tube, of each fraction (in extraction buffer) was heated for 10 min in a water bath at 70, 80, 90, or 100 °C and immediately cooled on ice. Activity was assayed after 5 min. Samples were subsequently incubated for 2 h at 23 °C, and the POD activity was measured again to test recovery of the activity. The effect of the incubation temperature on the regeneration of activity of the ionically bound POD fraction was measured at three different temperatures (4, 23, and 40 °C). After ultracentrifugation (Centricon 10, 1950*g*, Amicon) for 2 h at 4 °C, isoforms of heat-treated (10 min at 90 °C) POD fractions were separated by isoelectric focusing and stained for POD activity using the Phastsystem (IEF gel pH 3−9, Amersham Pharmacia Biotech).

Purification of the Thermostable isoPOD. Purification of POD was carried out by using several chromatographic techniques. Ion exchange chromatography was performed using a 1 mL Resource S column (Amersham Pharmacia Biotech), which is a strong cation exchanger. The ionically bound POD was ultrafiltered using a membrane with a 10 kDa cutoff (pressurized stirred cell, Amicon) and dissolved in 0.1 M Tricine buffer, pH 8.8. One milliliter was loaded onto the Resource S column. The starting buffer was 0.1 M Tricine buffer, pH 8.8, and the sample was eluted with a salt step gradient of 0.1 M Tricine buffer and 1 M NaCl, pH 8.8. Protein concentration and POD activity before and after a heat treatment for 10 min at 80 °C were determined for each collected fraction.

Samples collected after cation exchange chromatography exhibiting a thermostable POD activity were concentrated by ultrafiltration (Centricon 10, 1950*g*, Amicon), and 1 mL was applied to a Superdex 75 preparative grade (pg) column (Amersham Pharmacia Biotech). Sodium citrate buffer of 0.1 M, pH 4, was used to elute the sample using an isocratic gradient. Protein concentration and POD activity were determined for every collected fraction. The presence of polypeptides in FPLC fractions was sought by SDS−PAGE electrophoresis (Multiphor system, Excel gel SDS, gradient 8−18, Amersham Pharmacia Biotech). Gels were stained with silver nitrate according to the manufacturer's instructions (Amersham Pharmacia Biotech).

RESULTS

Cellular Distribution of Potato PODs. Intracellular and cell wall-bound PODs from stored potato tubers and tuber sprouts were separated by differential extraction procedures. For potato tubers, the pH activity profile of each POD fraction showed a maximum at pH 4.5 (Figure 1B), whereas the maximum pH was 4.0 for all POD fractions of potato sprouts (Figure 1A). At the optimal pH, the ionically bound POD fraction of tubers showed the highest specific activity of ∼91 μkat/mg of protein (Figure 1B). Similarly, the ionically bound POD fraction of potato sprouts contained the highest specific activity of ∼1570 μkat/mg of protein (Figure 1A). Potato sprouts contained ∼18−35-fold higher levels of POD activity as compared to tubers. Due to low concentration of POD in tubers, potato sprouts were used as a source for further studies of PODs.

Isoform Composition of the POD Fractions. The occurrence of POD isoforms was visualized by using isoelectric focusing analysis followed by activity staining (Figure 2). The soluble POD fraction showed an anionic isoform with an IEP of ∼3 and a cationic isoform with an IEP of ∼10. The covalently bound POD fraction showed anionic isoforms with IEP values between 3 and 6 and a cationic isoform with an IEP of ∼10. The ionically bound POD fraction displayed a neutral isoform and a cationic isoform with an IEP of ∼10.

Rotofor fractionation confirmed that the ionically bound POD fraction contained two main isoforms. Isoform 1 had an IEP of ∼7 and isoform 2, which is more basic, had an IEP of ∼10 (Figure 3). However, the Rotofor system has the disadvantage that, after electrofocusing, the samples are contaminated with ampholytes, which interfere with protein concentration measurements by use of Coomassie Brilliant Blue. The IEP of the cationic isoPOD of the ionically bound fraction was determined more precisely by using the Multiphor system, which presents a higher resolution of separation (Amersham Pharmacia Biotech). Isoelectric focusing revealed a band with POD activity at pH 9.5.

Heat Stability of PODs. Two cultivars, Agria and Bintje, were investigated to determine the thermostability of PODs. Several heat treatments were applied to the fractions isolated from potato sprouts. It appeared

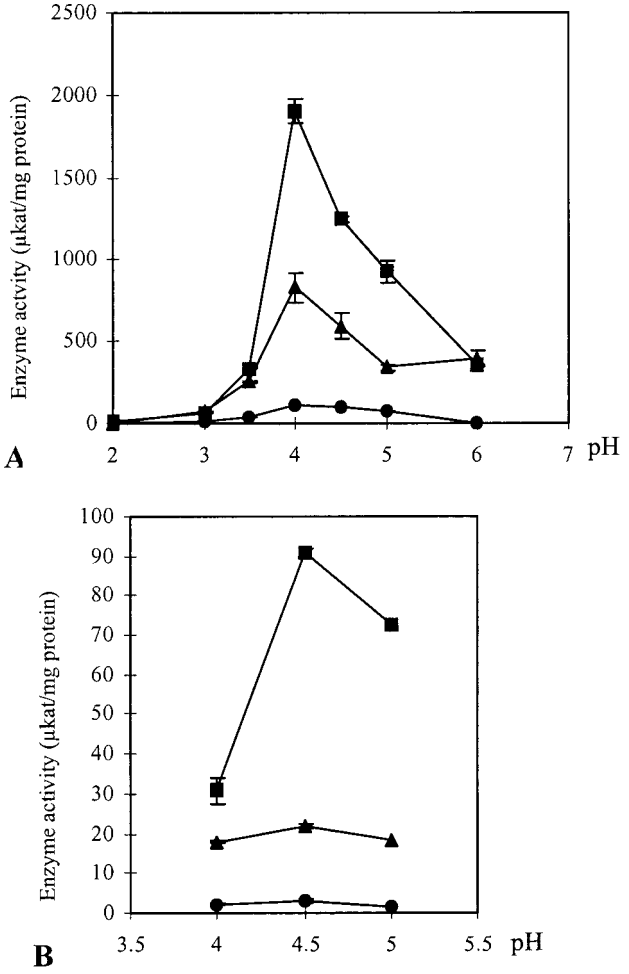

Figure 1. Specific enzyme activities at different pH values of POD fractions extracted from potato sprouts (A) and from potato tubers (B): soluble fraction (●), ionically bound fraction (■), and covalently bound fraction (▲). Assays were performed using 3 mM ABTS and 0.045% H_2O_2 in 0.1 M sodium citrate buffer.

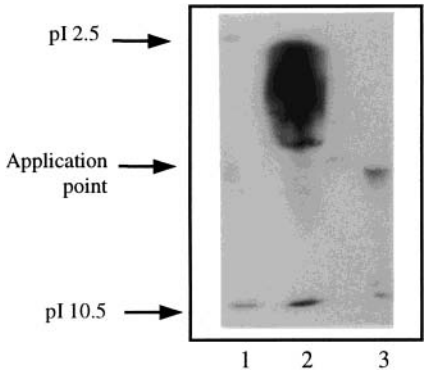

Figure 2. Isoelectric focusing analysis with staining for POD activity of fractions from sprouts using 0.5% guaiacol and 0.25% H_2O_2 as substrates: (lane 1) soluble POD fraction; (lane 2) covalently bound POD fraction; (lane 3) ionically bound POD fraction.

that ~80% of POD activity of the ionically bound POD from cv. Agria remained after a heat treatment at 70 °C for 10 min, whereas 60% of POD activity was remaining for the same fraction from cv. Bintje (Figure 4). Even after a drastic heat treatment such as 10 min at 90 °C, the ionically bound POD from cv. Agria still showed POD activity (~20%, Figure 4). Cultivar Agria, which was shown to contain the most thermostable

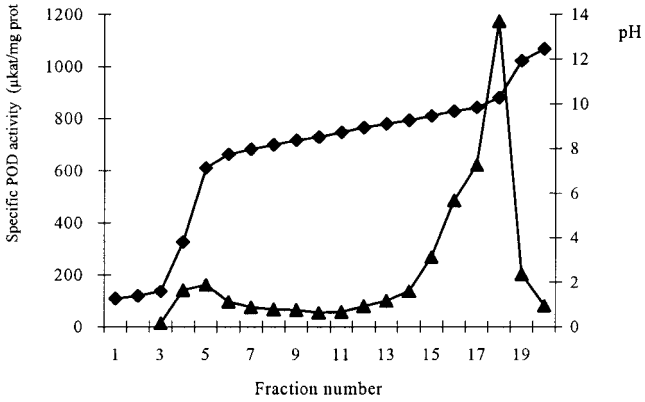

Figure 3. Preparative isoelectricfocusing (Rotofor system, Bio-Rad) of the ionically bound POD fraction in pH 8−10.5 gradient: (▲) POD activity; (◆) pH gradient.

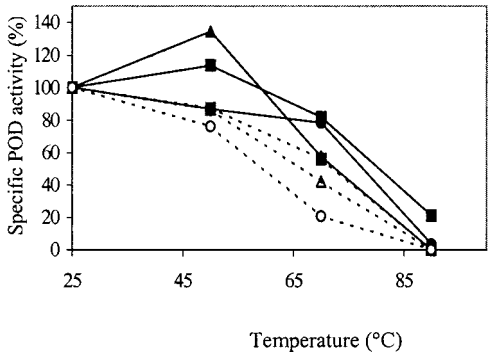

Figure 4. Comparison of the residual POD activities of potato sprouts after heat treatments of 10 min at 50, 70, and 90 °C for the cultivars Agria (solid symbols) and Bintje (open symbols): (△/▲) soluble fraction; (□/■) ionically bound fraction; (○/●) covalently bound fraction.

POD, was further analyzed by comparing thermostable POD properties between sprouts and tubers. Incubation of 10 min at 80 °C inactivated all enzyme activity of POD fractions from potato tubers (Figure 5B,D,F). The ionically bound POD fraction of tubers, which was heat treated at 80 °C for 10 min, showed a regeneration of its enzyme activity up to 5% after 2 h of incubation at 20 °C (Figure 5D). For potato sprouts, however, a heat treatment of up to 100 °C did not totally inactivate PODs of the ionically bound fraction (Figure 5C). After different heat treatments, reactivation of the ionically bound fraction was assayed by measuring enzyme activity after incubation for 2 h at 20 °C. Between 10 and 60% of the POD activity was regained.

Consistent with the results of enzyme activity measurements after heat treatment, the thermostable isoform(s) of the ionically bound POD fraction was (were) visualized after isoelectric focusing by staining for enzyme activity (Figure 6). The cationic POD isoform with an IEP of 9.5 appeared to be the only reactive polypeptide in the gel after heat treatment of each POD fraction. Figure 7 shows this ionically bound POD gradually reactivated in the course of time. It appeared that the incubation temperature had an effect on the amount of enzyme reactivation. The reactivation phenomenon could be enhanced up to 60% when the incubation temperature after the heat treatment was increased from 4 to 23 °C. From these results, we concluded that the cationic POD isoform from the ionically bound fraction is primarily responsible for thermostability of PODs in potato sprouts.

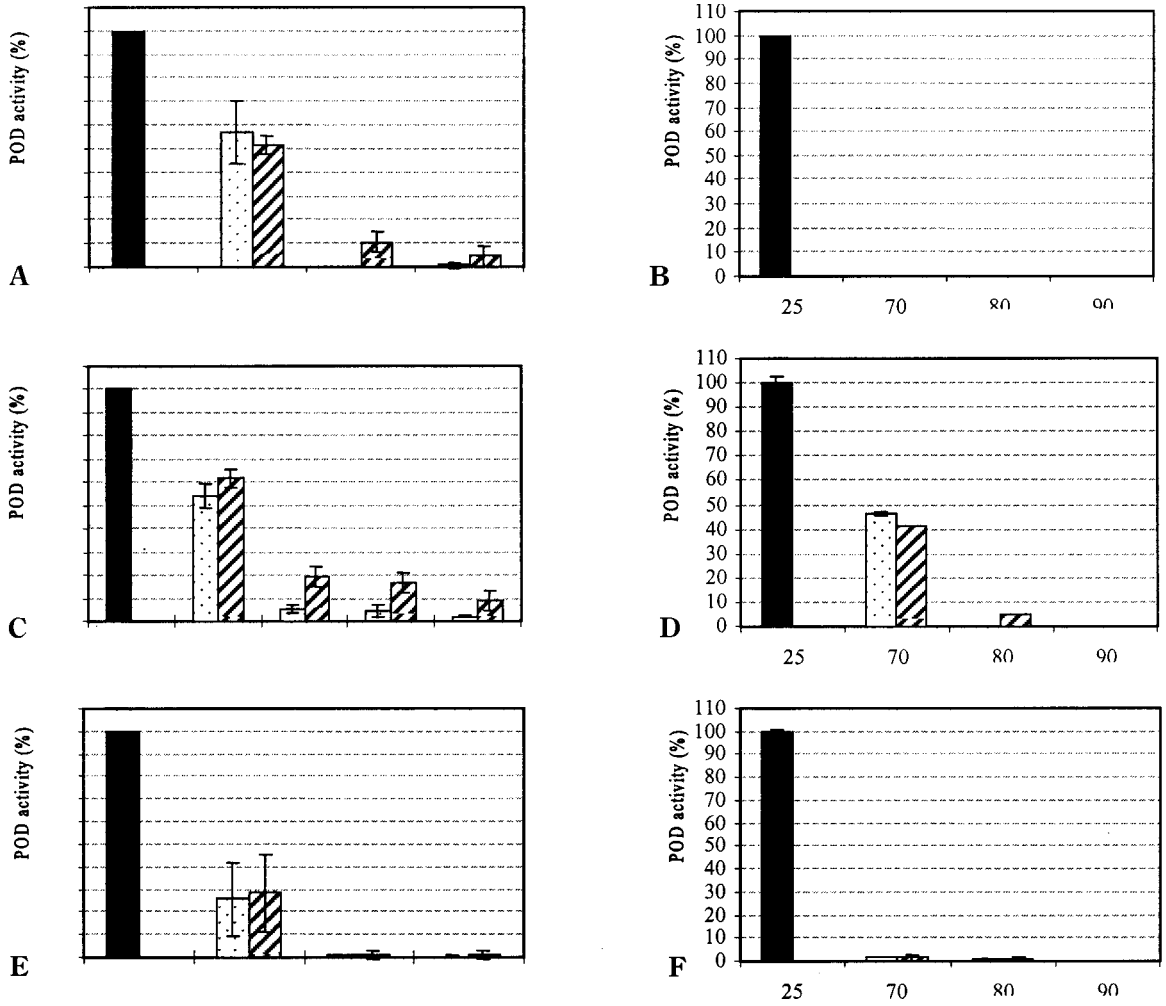

Figure 5. Residual POD activity and reactivation after different heat treatments (for 10 min) of the POD fractions extracted from potato sprouts (A, C, E) and from potato tubers (B, D, F): (A, B) soluble fraction; (C, D) ionically bound fraction; (E, F) covalently bound fraction; measurement without heat treatment (■), after heat treatment (⊡), and after incubation for 2 h at 20 °C (⧄).

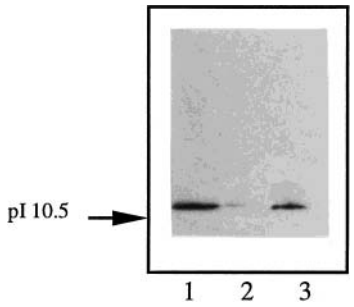

Figure 6. Isoelectric focusing analysis with staining for activity of POD fractions from sprouts using 0.5% guaiacol and 0.25% H_2O_2 as substrates; ionically bound POD fraction of potato sprouts: (lane 1) without heat treatment; (lane 2) after incubation for 10 min at 90 °C; (lane 3) after incubation for 2 h at 20 °C after heat treatment.

Purification of the Thermostable isoPOD. To further study the thermostable properties of the POD, a purification was initiated using potato sprouts from cv. Agria as a source. Analysis by cation exchange chromatography revealed two major peaks containing POD activity (Figure 8A). The first peak corresponds to acidic isoPODs being negatively charged at pH 8.8, which did not bind to the column. The second peak corresponds to the basic isoPODs, which bound to the column, and was eluted with a salt gradient (12.5%). The latter peak displayed a specific POD activity 20-fold as high as compared to the first peak. Apparently, this purification step by cation exchange chromatography, with a purification factor of 3.6, is very efficient, as demonstrated by the rise of specific activity of the basic isoPODs. This indicates that many cell wall proteins without POD activity, and which are ionically bound to cell walls, were removed (Figure 8A). After a heat treatment for 10 min at 80 °C, this cationic fraction retained ~30% of its POD activity. A recovery of POD activity up to 40% was observed after an incubation of 2 h at room temperature (Figure 8B). Polypeptide composition of this fraction was analyzed by SDS−PAGE followed by silver staining. Figure 9A shows that this basic fraction still contained two major polypeptides with molecular masses of ~43 and ~38 kDa. To further purify this fraction, the samples were analyzed by gel permeation chromatography. The chromatographic profile displayed one major peak that contained POD activity. After this last purification step, no thermostability could be determined due to the low level of POD activity in the collected samples. However, the fraction collected after purification by gel filtration was assayed by SDS−PAGE electrophoresis and displayed one band

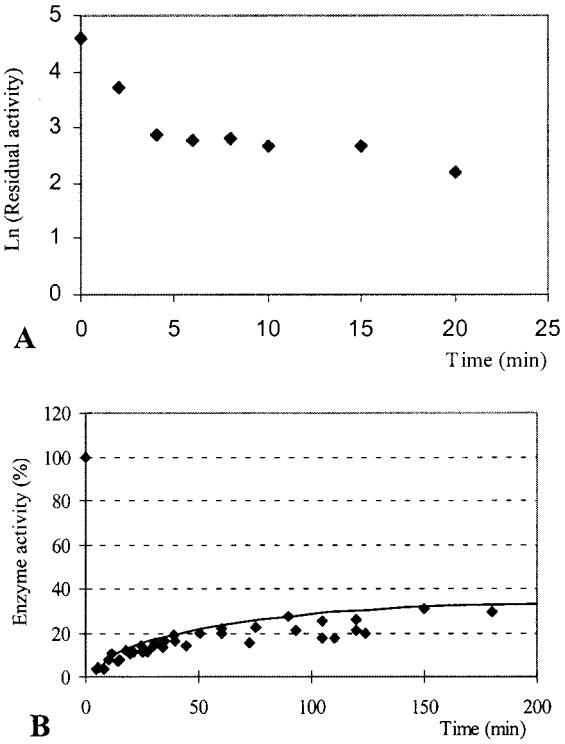

Figure 7. (A) Residual POD activity for potato sprouts (cv. Agria) after different periods of heat treatment at 80 °C. (B) Recovery of enzyme activity for potato sprouts (cv. Agria). Ionically bound POD fraction was incubated for 3 h at 23 °C after heat treatment for 10 min at 90 °C.

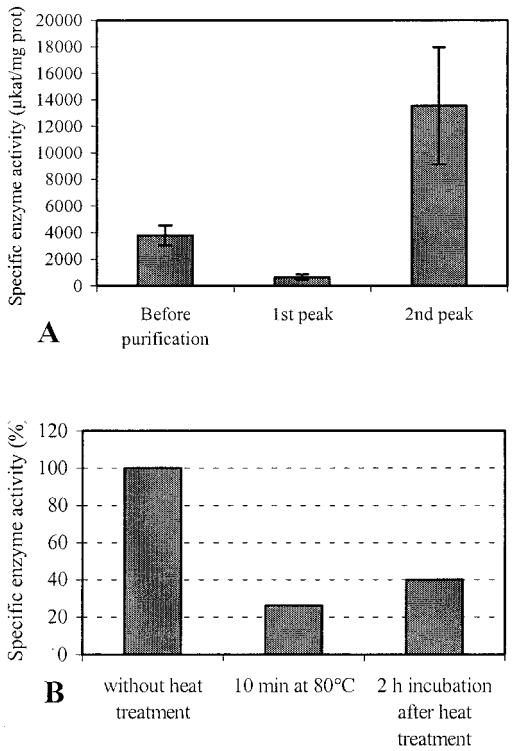

Figure 8. (A) Specific POD activity of the two fractions displaying a peak at 280 nm after cation exchange chromatography. (B) Representative analysis of the residual enzyme activity and reactivation after heat treatment at 80 °C for 10 min of the cationic isoform of the ionically bound POD fraction of potato sprouts obtained by purification with Resource S column.

with a molecular mass of 38 kDa (Figure 9B), representing the thermostable POD.

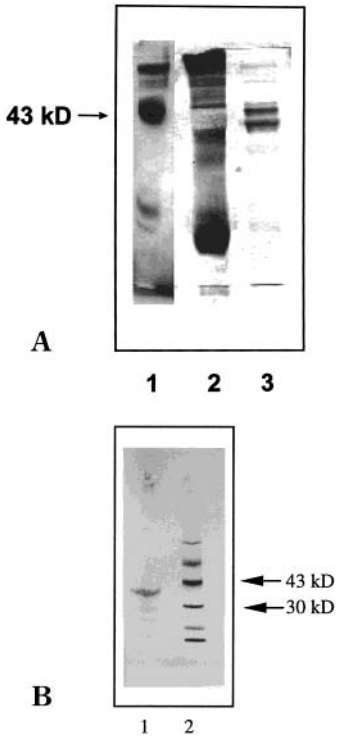

Figure 9. SDS−PAGE followed by silver staining of the cationic fraction of ionically bound PODs of potato sprouts: (A) purification with a Resource S column [(lane 1) low molecular mass markers (14.4−94 kDa); (lane 2) first peak corresponding to acidic and neutral proteins including isoPODs; (lane 3) second peak corresponding to basic proteins including isoPODs]; (B) purification with a Superdex 75 preparative grade column [(lane 1) purified fraction; (lane 2) low molecular mass markers (14.4−94 kDa)].

DISCUSSION

Characterization and Purification of PODs in Potato Tubers and Sprouts. Extraction of three isoPODs fractions (soluble, ionically bound, and covalently bound) allowed us to compare our results to most PODs studies, although this denomination is not rigorous. As Robinson (1991) explained, the soluble POD fraction may still contain suspended intracellular membranes, probably with bound PODs, and location of cationic PODs in the cell wall may be an artifact due to cell breakage. Optimal pH values for potato PODs were established to be 4.0 and 4.5 for sprouts and tubers, respectively. These values were slightly lower as compared to those in studies of Mihalyi and Vamos-Vigyazo (1975) and Khan et al. (1981) on potato tubers, who determined an optimal pH of 5.0. The optimal pH did not change after heat treatment in accordance to the work of the same authors. However, Lopez and Burgos (1995) mentioned that release of hemin from enzyme (which leads to loss of POD activity) was pH dependent and occurred most rapidly below pH 5. Comparison of specific POD activities between sprouts and tubers showed that potato sprouts contained ∼18−35-fold higher enzyme levels as compared to potato tubers (Figure 1). Moreover, it appeared that the ionically bound fraction of potato sprouts contained the highest specific POD activity, as compared to the soluble and the covalently bound POD fraction (Figure 1). A similar result has also been found for potato tubers by Khan et al. (1981). Goldberg et al. (1983) showed a similar distribution of POD activity for poplar. However, Robinson et al. (1989) found that the soluble fraction of grapes displayed the highest POD activity. It has been

often described (Robinson, 1991; Lagrimini et al., 1997) that no structure−function relationship can be established for PODs. However, we assume that cell wall-associated PODs can be one of the determinants of food texture.

The results of our isoelectric focusing study showed anionic isoforms for the covalently bound and soluble POD fractions. Cationic isoforms were observed for each POD fraction. The cationic isoPODs as shown for potato sprouts are compatible with the results of Robinson et al. (1989) for grapes and suggest that the most basic isoPODs are mainly bound to the cell wall by ionic bonds. The ionically bound POD fraction, which was extracted from cv. Agria potato sprout and has a basic IEP (9.5), was demonstrated to be the most thermostable (Figures 4 and 5). The investigated thermostable isoPOD was partially purified by cationic ion exchange chromatography. The thermostability was confirmed by staining techniques, and this fraction was further purified by gel filtration. The only collected fraction that showed POD activity displayed one major band on SDS−PAGE with a molecular mass of ~38 kD, which is in accordance with other studies on potato (Decedue et al., 1984), soybean (Gillikin and Graham, 1991), and tobacco (Gazaryan and Lagrimini, 1996).

Thermostability of isoPODs. Thermostable POD properties were compared between sprouts and tubers. Our measurements indicated that sprout PODs were more thermostable than either of the tuber fractions (Figure 5). Thermostability was tested for two cultivars, Agria and Bintje. Our study showed that the ionically bound POD fraction from potato sprouts of cv. Agria contained the most thermostable isoPOD and appeared to be more heat stable than has been generally found for PODs (Rasmussen et al., 1997), which highlights the thermostable feature of the studied PODs. The thermostable isoPOD as revealed by isoelectric focusing was cationic and exhibited reactivation of its POD activity (Figures 5C and 6). In accordance with our results, thermostability and reactivation of the ionically bound POD fraction have also been shown for orange by McLellan and Robinson (1984). However, McLellan and Robinson (1987a) showed that there was no regeneration of any POD isoforms of spring cabbage, and Robinson et al. (1989) found that the soluble POD fraction of the Ohane grapes was more heat stable as compared to the ionically bound POD fraction. Moreover, López-Serrano and Ros (1996) showed that a basic isoPOD of strawberry exhibited a thermostable behavior after a canning process, which could be involved in browning reactions. Therefore, thermostability of PODs is a complex phenomenon, which varies according to the plant and the POD localization. Three hypotheses have been presented, postulating how the protein structure can resist thermal denaturation. First, cationic isoPODs have a strong affinity for negatively charged polymers such as pectins, which increases the stability of the bound enzyme and might explain its partial thermostability in vivo. Second, isoPODs can be glycosylated, thereby increasing the overall protein stability (Vieille and Zeikus, 1996; Pomar et al., 1997). Indeed, increased thermostability of PODs by addition of sugars was previously shown by Chang et al. (1988). Third, Robinson et al. (1989) mention that thermostable properties may be due to the formation of aggregates of the thermostable POD conferring an increased thermostability. In addition, Lu and Whitaker (1974) showed that in the presence of salt the thermostability of horseradish POD decreases by dissociation of aggregates.

The thermal deactivation curve appeared to be nonlinear (Figure 7A), suggesting the presence of several groups of POD isoforms which significantly differed in thermostability. The same profile has been found by many authors on various vegetables and fruits (Naveh et al., 1982; McLellan and Robinson, 1987b; Ganthavorn et al., 1991). Moreover, Forsyth et al. (1999) described three phases occurring during a short heat treatment for a nonlinear heat inactivation, which is similar to our results (Figure 7A). Lu and Whitaker (1974) attributed the deviation from first-order kinetics to the presence of several isozymes with different heat stabilities. This hypothesis might be a likely explanation of the behavior of the ionically bound POD fraction before purification of the most thermostable isoPOD. However, McLellan and Robinson (1981) and Forsyth et al. (1999) observed that even after purification of isoPODs, the deactivation curves were still described as nonlinear. Therefore, they concluded that the phenomenon is complex and postulated that POD can present different aggregation states which exhibit distinct thermostabilities. Adams (1997) concluded that regeneration of POD activity was the cause of nonlinear first-order kinetics of the thermal inactivation.

Hypotheses were raised to try to clarify mechanisms of thermostability and enzyme reactivation of POD activity. Gaspar et al. (1982) mentioned that the heme environment influenced the physicochemical properties and catalytic activity of PODs. In that respect, McEldoon and Dordick (1996) showed that in the presence of $CaCl_2$, the heme was more tightly bound for the heat stable soybean peroxidase than for horseradish peroxidase, which is consistent with a thermodynamically more stable enzyme. Earlier, Tamura and Morita (1975) tried to explain the mechanism of POD denaturation by comparing it with that of other hemoproteins such as myoglobin and cytochrome *c*. They established indeed that POD denaturation consisted of three different processes: dissociation of protohemin from the holoprotein, degradation or modification of protohemin, and conformation changes of the apoenzyme. They also showed that POD activity was restored after the addition of hemin. Therefore, the heme complex within the enzyme plays a crucial role in the catalytic properties by allowing a reversible conformation change due to its reintegration.

In conclusion, we demonstrated that POD from potato sprouts of cv. Agria was the best source to extract a thermostable POD (38 kDa, IEP = 9.5). To further study the impact on texture related to processing, a molecular genetic study on this isoform will be initiated.

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