Inactivation of Polyphenol Oxidase by High-Pressure Carbon Dioxide†

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Purified Florida spiny lobster, brown shrimp, and potato polyphenol oxidases (PPOs) exhibited a time-related decline in activity following treatment at 43 °C with high-pressure CO_2 at 58 atm. Kinetic studies showed crustacean PPOs were more vulnerable than potato PPO to high-pressure CO_2 treatment. High-pressure-CO_2-treated and nontreated PPOs varied with respect to electrophoretic protein patterns, isoelectric profiles, and secondary structures.

INTRODUCTION

Undesirable enzymatic browning caused by polyphenol oxidase (EC 1.14.18.1; PPO) on the surface of fruits, vegetables, and seafood products has been of great concern to food processors because the formation of melanin reduces consumer acceptability of these products. It thus has become a challenge for food scientists to find solutions to inhibit and prevent undesirable browning. Many chemicals have been extensively studied for their effectiveness in inhibiting PPO activity (Palmer and Roberts, 1967; Walker, 1975; Madero and Finne, 1982; Golan-Goldhirsh and Whitaker, 1984; Sayavedra-Soto and Montgomery, 1986; Ferrer et al., 1989; Chen et al., 1991b). However, problems related to off-flavor, off-odor, toxicity, and economic feasibility affect the application of these compounds (Eskin et al., 1971).

Supercritical (SC) fluids exhibiting physicochemical properties intermediate between those of liquids and gases have been reported to inactivate peroxidase (Christianson et al., 1984), PPO (Zemel, 1989), and pectinesterase (Arreola, 1990) when carbon dioxide (CO_2) is used as the SC fluid. CO_2 is used as the SC fluid because it is nontoxic, nonflammable, inexpensive, and readily available (Hardardottir and Kinsella, 1988). CO_2 also has a relatively low critical temperature and pressure (Rizvi et al., 1986). Taniguchi et al. (1987) studied the retention of activities for α-amylase, glucose oxidase, lipase, and catalase by SC-CO_2. Although SC-CO_2 has been shown to inactivate PPO, information regarding the inhibitory effect and inactivation kinetics of SC-CO_2 on purified PPO has not been elucidated. This study was thus undertaken to investigate the effect of SC-CO_2 on the inactivation of purified Florida spiny lobster, brown shrimp, and potato PPOs.

MATERIALS AND METHODS

Fresh Florida spiny lobster (*Panulirus argus*) tails were obtained from the Whitney Marine Laboratory (Marineland, FL) and transported in ice to the Food Science and Human Nutrition Department, University of Florida, Gainesville, and stored at −20 °C. Russet potato tubers were purchased from a local supermarket. Nonsulfited fresh brown shrimp (*Penaeus aztecus*) were obtained from a local seafood store. Lobster cuticle, shrimp cephalothorax (head), and potato peel were frozen in liquid nitrogen and ground into a fine powder using a Waring blender. The ground powder was stored at −20 °C until needed.

Extraction and Purification of PPO. PPO was extracted and purified following the procedures of Chen et al. (1991a). Ground lobster, shrimp, or potato powder was added to 0.05 M sodium phosphate buffer (pH 7.2) (1:3 w/v) containing 1 M NaCl and 0.2% Brij 35 (Fisher Scientific Co., Orlando, FL), stirred for 0.5 h at 4 °C, and then centrifuged at 8000*g* (4 °C) for 30 min. The supernatant was dialyzed at 4 °C overnight against three changes of 4 L of distilled water.

Crude PPO preparation was further purified using a nondenaturing preparative polyacrylamide gel electrophoresis (PAGE) system. Equipment utilized included a gel tube chamber (Model 175, Bio-Rad Laboratories, Richmond, CA) and a power supply (Model EPS 500/400, Pharmacia LKB Biotechnology Inc., Piscataway, NJ). A 1-mL aliquot of crude enzyme extract (lobster, shrimp, or potato) was applied to each of the eight gel tubes (1.4-cm i.d. × 12-cm length) containing 5% acrylamide/0.13% bis(acrylamide) gel and subjected to a constant current of 10 mA/tube in an electrode buffer (pH 8.3) containing 5 mM Tris-HCl and 38 mM glycine (Sigma Chemical, 1984). PPO was visualized using a specific enzyme–substrate staining method (Chen et al., 1991a); 10 mM DL-β-3,4-dihydroxyphenylalanine (DL-Dopa) in 0.05 M sodium phosphate buffer (pH 6.5) was used as substrate. After the migration of the enzyme relative to the dye front (R_f) was determined using one of the eight gels, the remaining gels were sectioned at the determined R_f. The enzyme was eluted from the gel by homogenization in distilled water utilizing a Dounce tissue grinder (Wheaton, Millville, NJ). The homogenates were filtered through a Whatman No. 4 filter paper, pooled, and concentrated using an Amicon stirred cell (Model 8050, Amicon Co., Danvers, MA) fitted with a YM 10 filter. Concentrated PPO was dialyzed overnight (4 °C) against two changes of 4 L of distilled water.

Protein Quantitation and Enzyme Purity Determination. The protein content of PPO preparations was quantitated using the Bio-Rad protein assay kit with bovine serum albumin (Sigma) as standard. Enzyme purity was examined using a mini sodium dodecyl sulfate (SDS) polyacrylamide gel [7.5% acrylamide/0.2% bis(acrylamide)] system (Mini-Protean II dual slab cell; Bio-Rad Laboratories, 1985a). PPO (20 μg of protein/well) was loaded into the sample well of a polyacrylamide gel of 7 × 8 cm (1-mm thickness), and electrophoresis was carried out at 200 V in an electrode buffer (pH 8.3) containing 17 mM SDS, 25 mM Tris-HCl, and 0.19 M glycine for 35 min. The purity of enzyme preparations was examined by comparing gels stained with 10 mM DL-Dopa in 0.05 M sodium phosphate buffer (pH 6.5) and then with a Commassie Blue R-250 (Eastman Kodak Co., Rochester, NY) solution.

Enzyme Activity Assay. PPO activities were measured by adding 60 μL of enzyme preparations to 840 μL of 10 mM DL-Dopa in 0.05 M sodium phosphate buffer (pH 6.5) and monitored at 25 °C for 5 min. Initial velocity was determined as ΔA_{475nm}/min, and one unit of PPO activity was defined as an increase in absorbance of 0.001/min at 25 °C. Unless otherwise specified, experiments were replicated three times.

Effect of High-Pressure CO_2 on PPO Activity. The apparatus used for the study of PPO inactivation by high-pressure CO_2 is shown in Figure 1. Coleman grade CO_2 (99.99% pure, Liquid Air Co., Walnut, CA) was connected to a high-pressure

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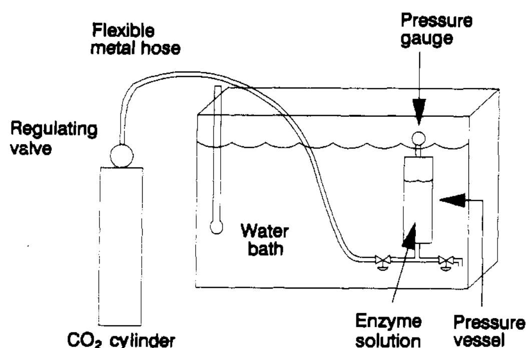

Figure 1. Apparatus for the study of polyphenol oxidase inactivation by high-pressure CO_2.

resistant stainless steel vessel (volume = 100 mL) equipped with valves through a metal hose. After the vessel was immersed into a water bath maintained at 43 °C, a constant pressure of 58 atm (850 psi) inside the vessel chamber was achieved by adjusting the pressure-regulating valve. For each study, 80 mL of lobster (0.15 mg of protein/mL), brown shrimp (0.27 mg of protein/mL), or potato (0.09 mg of protein/mL) PPO preparation was placed in the vessel. After all treatment parameters were at equilibrium, the vessel was removed from the water bath and an 8-mL aliquot was removed to determine PPO activity. Each time after sampling, the vessel was immediately replaced into the bath. Sampling was done every minute during the first 5 min and 5 min thereafter.

Following equilibration to ambient temperature, the PPO activity was determined as previously described. The activity of a non-gas-treated control heated at 43 °C was also determined as previously described. Percentage of relative activity was determined as $(E_t/E_0) \times 100$, where E_t was PPO activity at time t and E_0 the activity of PPO without heat and CO_2 treatment. The pH change resulting from CO_2 treatment during the course of study was monitored using a pH meter.

pH Control Study. Enzyme mixture containing 1 mL of lobster PPO preparation and 3 mL of 0.05 M $NaHCO_3$ buffer (pH 5.3) was heated in a water bath maintained at 43 °C. After 30 min of incubation, the mixture was instantly removed from the water bath and put into a 0 °C ice chest. Following equilibration to room temperature, a 60-μL aliquot of PPO solution was added to the microcuvette containing 840 μL of 10 mM DL-Dopa in 0.05 M sodium phosphate buffer (pH 6.5). The reaction was monitored at 475 nm (25 °C) for 5 min. For control sample, $NaHCO_3$ buffer was replaced by an equivalent volume of 0.05 M sodium phosphate buffer (pH 6.5).

Kinetics of PPO Inactivation. The reaction rate constant (k) for PPO inactivation in the presence or absence of CO_2 treatment was determined by measuring the maximal initial rate and plotting the logarithmic value of (V_t/V_0) vs time (t). V_t represents the activity of high-pressure-CO_2-treated PPO at time t and V_0 the original activity of nontreated PPO (Segel, 1976). The negative slope of the equation equals the reaction constant for PPO inactivation.

Mass Balance of CO_2-Treated and Nontreated PPO. CO_2-treated PPO solution (1.5 mL) was centrifuged in an Eppendorf 5415 microcentrifuge (Brinkmann Instruments Inc., Hamburg, Germany) at 13 000 rpm for 30 min. After the supernatant was removed, the pellet was redissolved in 0.5 mL of 0.05 M sodium phosphate buffer (pH 6.5), and the protein contents of both portions were quantitated. The combined protein contents from both portions were then correlated to that of an equal volume (1.5 mL) of nontreated PPO.

The protein patterns of CO_2-treated and nontreated PPO were also examined using the mini SDS polyacrylamide gel [7.5% acrylamide/0.2% bis(acrylamide)]. Twenty-five-microliter supernatant (5 μg of protein) and pellet (15 μg of protein) portions of CO_2-treated and nontreated (15 μg of protein) PPO were separately loaded into the sample well, and electrophoresis was carried out as previously described (Bio-Rad Laboratories, 1985a). Following electrophoresis, the gel was stained with the Bio-Rad silver stain kit (Bio-Rad Laboratories, 1987). The SDS-6H kit (Sigma) containing carbonic anhydrase (29 000), egg albumin (45 000), bovine albumin (66 000), phosphorylase B (97 400), β-galactosidase (116 000), and myosin (205 000) was used for protein molecular weight standards.

Polyacrylamide Gel Isoelectric Focusing. A gel mixture containing 5% acrylamide/0.13% bis(acrylamide), 5% glycerol, and 6.2% ampholyte (Pharmalyte 3-10, Pharmacia LKB Biotechnology) was degassed for 5 min. After the addition of 5% (v/v) fresh ammonium persulfate and 0.1% (v/v) *N,N,N′,N′*-tetramethylethylenediamine (TEMED), the gel mixture was poured into 16- × 20-cm slab gel plates assembled with a 0.75-mm comb and allowed to polymerize for 1.5 h according to the *Protein II Slab Cell Instruction Manual* (Bio-Rad Laboratories, 1985b). Following the removal of the comb, buffer containing 0.2% (v/v) Pharmalyte 3-10 and 5% (v/v) glycerol was overlayed onto the polymerized gels and allowed to sit for 1 h. Prefocusings at constant voltages of 200 (15 min), 300 (30 min), and 400 V (30 min) were alternately carried out after the overlaying buffer was changed. PPO preparation (50 μg of protein) was then loaded onto the sample well, and electrofocusing was performed at a constant voltage of 400 V for 17 h. The gel was fixed with the fixative solution [sulfosalicylic acid/trichloroacetic acid/methanol 4:12.5:30 (v/v)] and then stained with Coomassie Blue R-250. The isoelectric point (p*I*) of PPO was determined by comparing the R_f value of the sample with those of protein standards (broad p*I* kit, pH 3-10, Pharmacia) containing amyloglucosidase, p*I* 3.50; soybean trypsin inhibitor, p*I* 4.55; β-lactoglobulin, p*I* 5.20; bovine carbonic anhydrase, p*I* 5.85; human carbonic anhydrase B, p*I* 6.55; horse myoglobin acidic band, p*I* 6.85, basic band, p*I* 7.35; lentil lectin acidic band, p*I* 8.15, middle band, p*I* 8.45, basic band, p*I* 8.65; and trypsinogen, p*I* 9.30.

Spectropolarimetric Analysis of PPO. Circular dichroic (CD) spectra of CO_2-treated and nontreated PPO were scanned at the far-UV range (250–200 nm) using a Jasco J-20 automatic recording spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) and a Suprasil (Helma Cells) cuvette with 1.0-cm light path. Four milliliters of PPO (15 μg/mL) in 0.05 mM sodium phosphate buffer (pH 6.5) was used as sample, and the measurement of CD spectra was carried out at ambient temperature. Secondary structure calculations were performed by computer analysis of the CD spectra using the SSE program (Japan Spectroscopic, 1985) with myoglobin, cytochrome *c*, ribonuclease A, lysozyme, and papain as CD references.

Study of Restoration of PPO Activity. To examine the reactivation ability of PPO following CO_2 treatment, a portion of CO_2-treated sample was stored at −20 °C in a microcentrifuge tube for 6 weeks. After thawing and equilibrating to ambient temperature (~25 °C) for 15 min, the pH of the PPO solution was measured and its enzyme activity determined as previously described. Following the assay, the PPO solution was then again frozen at −20 °C until needed. For this study, the assays were performed weekly and percentage of relative activity was determined as $(ER_t/ER_0) \times 100$, where ER_t represents the activities of CO_2-treated PPO stored at time t and ER_0 the original activity of nontreated PPO. PPO control samples without CO_2 treatment were similarly studied.

RESULTS AND DISCUSSION

In this study, the purified lobster, brown shrimp, and potato PPOs with enzyme activities of 3750, 740, and 5400 units/mg of protein, respectively, were verified by the substrate (DL-Dopa) and Coomassie Blue staining methods.

Effect of High-Pressure CO_2 on Lobster, Brown Shrimp, and Potato PPO Activity. Heating of lobster and brown shrimp PPO at 43 °C for 30 min caused some loss of activity (Figure 2a,b). Such treatment, however, caused only 5% loss of potato PPO activity (Figure 2c). No protein precipitation occurred in these treated samples.

The treatment of these PPOs with high-pressure (58 atm) CO_2 at 43 °C, however, caused a dramatic loss of enzyme activity (Figure 2). Lobster, brown shrimp, and potato PPOs after treatment for 1 min retained only 2 (ΔA_{475nm}/min = 0.001 vs 0.083), 22 (0.010 vs 0.046), and 45% (0.240 vs 0.540) of the original activity, respectively.

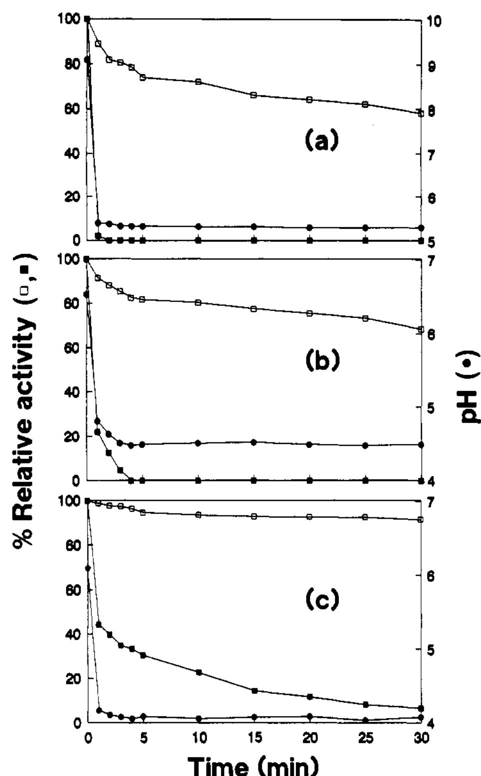

Figure 2. Effect of high-pressure (58 atm) CO_2 treatment at 43 °C on changes in pH (●) and enzyme activity (■) of (a) Florida spiny lobster, (b) brown shrimp, and (c) potato PPOs. The treatment at 43 °C on PPO activity in the absence of CO_2 (□) was also included.

Extended treatment of lobster and brown shrimp PPOs for more than 1 min caused a complete loss in activity. For these two PPOs the treatment for 10 and 15 min, respectively, caused protein precipitation. CO_2 treatment thus caused PPO denaturation and the loss of lobster and brown shrimp activity. The results also showed that brown shrimp PPO was slightly more resistant than lobster PPO to high-pressure CO_2 treatment at 43 °C, and potato PPO was the most resistant among all PPOs. Potato PPO eventually lost 91% of its original activity after treatment for 30 min (Figure 2c). Suzuki and Taniguchi (1972) studied the effect of pressure on heat denaturation of various proteins and enzymes. These authors concluded that protein denaturation resulting from hydrostatic effect only occurred when pressures went above 1000–3000 atm. Thus, pressure effect alone was not the determining factor that caused protein denaturation of PPO molecules in this study.

Lobster PPO was more susceptible to high-pressure CO_2 than atmospheric CO_2 when heated at 43 °C; treatment of this PPO for 20 min with atmospheric CO_2 (1 atm) at 43 °C did not cause a complete loss in activity (Chen et al., 1991c). In comparison with the studies of Christianson et al. (1984) and Taniguchi et al. (1987), it also appears that lobster PPO was more vulnerable to high-pressure CO_2 than corn germ peroxidase, α-amylase, glucoamylase, β-galactosidase, glucose oxidase, glucose isomerase, lipase, thermolysin, alcohol dehydrogenase, and catalase.

High-pressure CO_2 treatment at 43 °C for 1 min caused drops in pH from 9.1 to 5.4 for lobster PPO, from 6.5 to 4.8 for brown shrimp PPO, and from 6.1 to 4.2 for potato PPO (Figure 2). The treated lobster, brown shrimp, and potato PPOs were then constant at pH 5.3, 4.5, and 4.1, respectively, throughout the experiment. This study shows that the high-pressure-CO_2-treated PPO has a similar time-related pH change profile as that of atmospheric CO_2-treated PPO (Chen et al., 1991c). Using the pH control study, we did not observe any occurrence of protein precipitation. We found that lobster PPO under an environmental pH of 5.3 still retained 35% of its original activity after being heated at 43 °C for 30 min. Weder and co-workers have shown that supercritical CO_2 treatment could decrease L-arginine content of solutions (Weder, 1984, 1990; Weder et al., 1992). These workers proposed that, under a lower pH environment, protein-bound arginine can easily interact with CO_2, forming a bicarbonate complex (Weder et al., 1992). Therefore, in addition to its pH-lowering effect, CO_2 may directly bind to the enzyme and cause loss in activity.

Kinetics of PPO Inactivation. The reaction constants (k) for lobster, brown shrimp, and potato PPO inactivation at 43 °C were determined to be 1.6×10^{-2}, 9.4×10^{-3}, and 2.5×10^{-3} min^{-1}, respectively. Under the same heating conditions in the presence of CO_2 (58 atm), the k values for brown shrimp and potato PPO were changed to 0.98 and 0.69 min^{-1}, respectively. The k value for lobster PPO was expected to be greater than those of brown shrimp and potato PPO since no enzyme activity was detected after lobster PPO was exposed to CO_2 at 43 °C for 1 min. Potato PPO was thus more resistant than lobster and brown shrimp PPOs to high-pressure CO_2 treatment at 43 °C.

The results also showed that the combined treatment of heat and high-pressure CO_2 enhanced the inactivation of brown shrimp and potato PPOs by 104-fold (0.98 min^{-1}/9.4×10^{-3} min^{-1}) and 276-fold (0.69 min^{-1}/2.5×10^{-3} min^{-1}), respectively, than by heat alone.

Mass Balance of CO_2-Treated and Nontreated PPO. For lobster and shrimp PPOs, the combined protein contents in the supernatant and pellet portions of the treated samples were close to those of their respective nontreated controls (data not shown). No protein precipitation was detected in the pellet portion of CO_2-treated potato PPO. The combined protein patterns of the supernatant and the pellet portions of CO_2-treated lobster PPO matched those of the nontreated control (Figure 3). The results thus indirectly suggest that CO_2 treatment could denature enzyme molecules and cause loss of catalytic function.

Polyacrylamide Gel Isoelectric Focusing. Nontreated lobster, brown shrimp, and potato PPOs on IEF gels only showed one protein band with an isoelectric point (pI) of 6.0. The PPOs upon high-pressure CO_2 treatment, however, all showed more than one protein band, including one with a pI of 6.2 (data not shown). This finding was further verified by a study of CO_2-treated lobster PPO and nontreated control subjected to a Sephadex G-100 (Pharmacia) column. Thus, CO_2 treatment at 58 atm could possibly cause the ionization of dissociable groups in PPO proteins as suggested by Suzuki and Taniguchi (1972).

Spectropolarimetric Analysis of PPO. The CD spectra at far-UV range of nontreated and high-pressure-CO_2-treated lobster, brown shrimp, and potato PPOs are given in Figure 4. The negative ellipticity between 207 and 220 nm of the untreated controls was quite different from that of CO_2-treated PPO. Results show CO_2 treatment caused conformational changes in the secondary structures (α-helix, β-sheet, β-turn, and random coil) (Table I). Lobster and brown shrimp PPOs showed the most noticeable alterations in the composition of α-helix and random coil. In contrast, only minor alteration in secondary structure occurred in high-pressure-CO_2-treated potato PPO (Table I). This difference in the responsiveness of the secondary structure to high-pressure CO_2 could

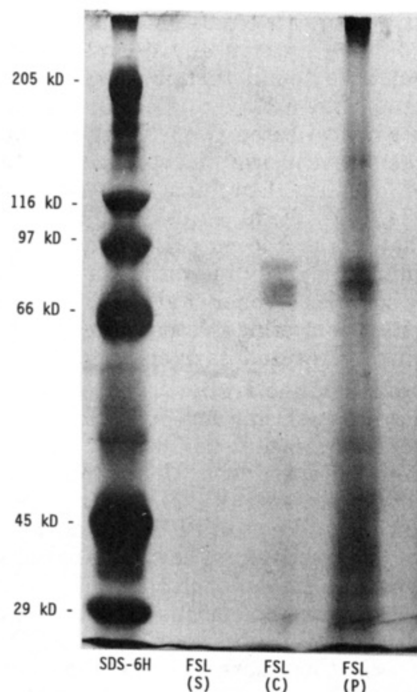

Figure 3. SDS–PAGE profiles of Florida spiny lobster (FSL) PPO. FSL (C) is the nontreated PPO, while FSL (S) and FSL (P), respectively, represent the supernatant and pellet portions of high-pressure (58 atm)-CO_2-treated PPO following centrifugation.

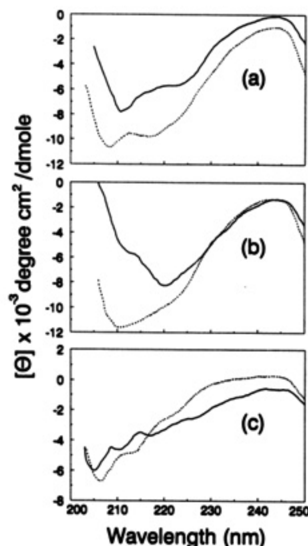

Figure 4. Circular dichroic spectra of (a) Florida spiny lobster, (b) brown shrimp, and (c) potato PPOs before (···) and after (—) high-pressure CO_2 treatment. The circular dichroic spectra of PPO were scanned at the far-UV (250–200 nm) range. Four milliliters of PPO (15 μg/mL) in 0.05 mM sodium phosphate buffer (pH 6.5) was analyzed at ambient temperature.

account for the previous finding that potato PPO was more resistant than lobster and shrimp PPOs to such treatment. Suzuki and Taniguchi (1972) reported that under the high-pressure environment conformational changes in protein and enzyme molecules are likely to occur. This was in agreement with the findings of Miller et al. (1981), who proposed that the pressure-induced effect from SC-CO_2 treatment could cause changes in protein backbone structure and subunit dissociation and thus inactivate the enzyme.

Restoration of Enzyme Activity of CO_2-Treated PPO. Non-CO_2-treated PPO controls gradually lost their activity as time proceeded; nearly 50% of the original

Table I. Secondary Structure Estimates of Non-CO_2-Treated and High-Pressure-CO_2-Treated Florida Spiny Lobster, Brown Shrimp, and Potato Polyphenol Oxidases (PPOs) from Far-UV Circular Dichroic Spectra[a]

PPO		% of secondary structure			
		α-helix	β-sheet	β-turn	random coil
lobster	nontreated	24.4	26.2	21.4	29.9
	CO_2-treated	19.7	25.9	15.2	39.3
brown shrimp	nontreated	20.1	22.3	15.2	42.4
	CO_2-treated	29.6	18.9	18.2	33.3
potato	nontreated	14.8	34.6	28.4	22.2
	CO_2-treated	17.8	35.9	25.9	20.4

[a] The circular dichroic spectra of PPO were scanned at the far-UV (250–200 nm) range. Four milliliters of PPO (15 μg/mL) in 0.05 mM sodium phosphate buffer (pH 6.5) was analyzed at ambient temperature.

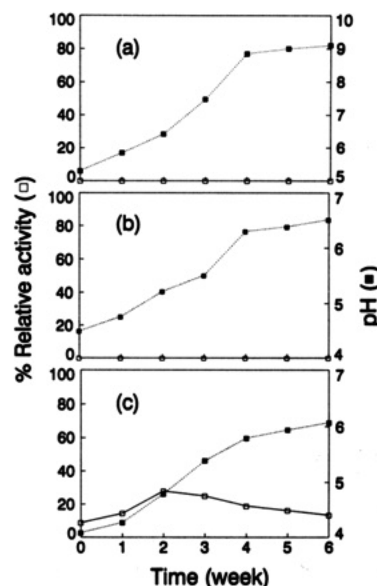

Figure 5. Restorative ability of high-pressure-CO_2-treated (a) Florida spiny lobster, (b) brown shrimp, and (c) potato PPO activity (□) and the pertinent environmental pH changes (■) during frozen storage.

activity was lost after storage over 5 weeks (data not shown). The loss in PPO activity of control samples during storage was apparently due to the freezing effect. For lobster and brown shrimp PPOs treated with 58 atm of CO_2 at 43 °C, no restoration of activity resulted upon the 6 weeks of frozen storage. However, the pHs of these two systems came back from 5.3 to 9.1 and from 4.5 to 6.5, respectively (Figure 5a,b). Similar changes also occurred with potato PPO; the pH was increased from 4.1 to 6.1 over the 6 weeks (Figure 5c).

Unlike the other two PPOs, the CO_2-treated potato PPO regained 28% of the original activity (ΔA_{475nm}/min = 0.151 vs 0.540) during the first 2 weeks of frozen storage (Figure 5c). The activity then gradually decreased as the storage time was increased. This phenomenon was similar to the previous observation that 10% of enzyme activity was restored during the first week of storage after lobster PPO was subjected to atmospheric (1 atm) CO_2 at 33 °C (Chen et al., 1991c), though the enzyme systems used in these two studies were different.

Summary and Conclusion. Purified Florida spiny lobster, brown shrimp, and potato polyphenol oxidases showed a decrease in activity with time when subjected to high-pressure CO_2 (58 atm) treatment at 43 °C. Lobster and shrimp PPOs were more vulnerable than potato PPO to high-pressure CO_2. Studies using gel electrophoresis

showed that there were differences in the isoelectric profiles and protein patterns between the CO_2-treated and non-treated PPO. Spectropolarimetric analysis revealed that CO_2 treatment caused changes in the secondary structures of the PPO.

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