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Changes in Green Coffee Protein Profiles during Roasting

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To reveal its flavor, coffee has to be roasted. In fact, the green coffee bean contains all ingredients necessary for the later development of coffee flavor. It is now widely accepted that free amino acids and peptides are required for the generation of coffee aroma. However, the mechanisms leading to defined mixtures of free amino acids and peptides remain unknown. Information pertaining to the identification of precursor proteins is also lacking. To answer some of these questions, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was used to follow the fate of green coffee proteins. Two conditions were considered: roasting and incubation of green coffee suspensions at 37 °C. Coffee beans were observed to acquire the potential to spontaneously release H_2O_2 upon polymerization of their proteins during roasting. Fragmentation of proteins was also observed. Conversely, H_2O_2 was found to control polymerization and fragmentation of green coffee proteins in solution at 37 °C. Polymerization and fragmentation patterns under the two conditions were comparable. These observations suggest that the two conditions under study triggered, at least to some extent, similar biochemical mechanisms involving autoxidation. Throughout this study, a unique fragmentation cascade involving the 11S coffee storage protein was identified. Generated fragments shared an atypical staining behavior linked to their sensitivity to redox conditions.

KEYWORDS: Green coffee; roasting; storage protein; autoxidation; hydrogen peroxide; two-dimensional polyacrylamide gel electrophoresis

INTRODUCTION

Coffee aroma development and coffee browning are essentially considered to be consequences of Maillard reactions (*1*, *2*). These reactions are initiated by the interaction of the carbonyl group of a reducing sugar and the free amino group of an amino acid, a peptide, or even a protein (*3*). After condensation reactions, brown nitrogenous polymers called melanoidins are produced (*4*−*7*).

In the green coffee bean, potential nitrogenous flavor precursors are represented by free amino acids, peptides, and proteins. The protein content is relatively high, representing 8.7−12.2% of the coffee bean dry weight, based on crude nitrogen and corrected for nonprotein nitrogenous components (*8*). Green coffee proteins, the 11S storage protein in particular (*9*), represent an important reservoir for free amino acids and peptides. In comparison, the free amino acid content appears to be rather low, in the range of 0.3−0.6% on a dry weight basis (*10*). There is no information available to suggest that these free amino acids are either required or sufficient for the generation of the coffee aroma. Peptides were isolated from the green bean by Ludwig and colleagues (*11*) in amounts ranging from 0.4 to 0.6% on a dry weight basis. They also identified several endopeptidases. The contribution of these peptides to coffee flavor was suggested by model roasting trials. However, alternative mechanisms and sources of free amino acids and peptides may be considered in relation to flavor generation.

Like other phenols, the chlorogenic acids (CGAs) are prone to oxidation. Coffee beans are one of the richest sources of CGAs [7−10 and 5−7.5% on a dry weight basis for Robustas and Arabicas, respectively (*12*)]. These CGAs are a family of esters formed between defined transcinnamic acids (caffeic and ferulic) and quinic acid. Their tendency to oxidize during maturation of the cherry, postharvest processing, and storage ultimately defines the redox status of the green coffee beans. The actual redox status of the green bean seems to be important for the development of quality in the brew (*13*). During that study, we identified the involvement of oxidative processes in the degradation of the 11S coffee storage protein. We are now interested in the fate of this very same protein during roasting, an essential step for the development of coffee aroma.

Here we provide evidence that the 11S storage protein follows a specific degradation pathway during roasting. Degradation proceeded in a sustained fashion over more than half of the required roasting time. Concurrently, transient redox-active protein fragments were generated. To obtain a better insight into the mechanisms involved, we tried to mimic the observed fragmentation and polymerization processes in solution at 37 °C. Increasing hydrogen peroxide (H_2O_2) concentrations were used to trigger the oxidation of CGAs and other phenols. Similar polymerization and fragmentation patterns of 11S storage protein were obtained under these conditions. The possible contribution

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Table 1. Comparison of the Three Postharvest Treatments Applied to Santos Arabica[a]

method	dry	semidry	wet
type of coffee	natural	semidry	washed
processing			
separation of floaters	yes	yes	yes
separation of greens	**no**	**yes**	**yes**
removal of pulp	**no**	**yes**	**yes**
removal of mucilage	**no**	**no**	**yes**
drying	**yes, with pulp and mucilage**	**yes, with mucilage**	**yes, without pulp and mucilage**
cleaning and destoning	yes	yes	yes
hulling	yes	yes	yes
polishing (removal of silver skin)	no	no	yes
size grading	yes	yes	yes
density separation	yes	yes	yes

[a] Steps that strongly differed between the different processes and were likely to affect two-dimensional protein profiles of the beans are in boldface type.

of autoxidation reactions to the formation of melanoidins will be discussed.

MATERIALS AND METHODS

Origin of Green Coffee Samples. Postharvest Study. The first roasting experiment was performed with a well-specified Arabica (*Coffea arabica*) originating from Brazil (Santos). Samples were processed according to our specifications and provided by Pinhalense S.A., Maquinas Agricolas, Brazil. The three samples kept for analysis differed only by the type of postharvest processing (dry, semidry, or wet; see **Table 1**). Moisture contents for wet-, semidry-, and dry-processed samples were, respectively, 12.1, 10.5, and 11.3% (weight percent).

Roasting and H_2O_2 Incubation Studies. A commercially available green coffee was preferred for subsequent studies. We chose a wet-processed Colombian Arabica.

Protein Profiles of Damaged Beans. Three types of damaged beans were analyzed for comparison with a healthy Arabica from Thailand. These beans belonged to a collection of damaged beans.

Coated beans suffered from drought; they are characterized by a strongly adherent silver skin (origin: Mexico).

"Cardinello beans" are fungus-infested beans, covered with yellowish or reddish spores (origin: Colombia).

Black beans are beans with coffee berry disease (anthracnosis) caused by the fungus *Colletotrichum coffeeanum*, renamed *C. kahawae* (origin: Colombia).

Coffee Sample Preparation. *Roasted Samples.* Sixty grams (Santos Arabica) and 250 g (Colombian Arabica) of green beans were roasted at ~240 °C in a laboratory-scale commercial drum roaster (Probat, BRZ 2). The temperature was adjusted by regulating the heating current and the flow of air through the roasting drum.

Samples were drawn at different roasting times and ground (Retsch type ZM1 mill) under liquid nitrogen before analysis. To estimate the roasting level, the Neuhaus Colortest was applied by using a Neuhaus electronic reflectometer. A sample of roasted and ground coffee (ground on an Olympia mocca express mill set at 5.5), evenly distributed on a circular surface (diameter = 7 cm), is illuminated by a monochromatic infrared source. The reflected light is expressed in Colortest numbers (CTn), based on a linear scale ranging from 0 (black) to 200 (white). Two minutes of roasting time elicited a weak browning, which was outside the calibration curve and could not be determined.

Moisture/dry matter losses and CTn were recorded at definite roasting times for the Santos Arabica samples (see **Table 2**).

Green Coffee Suspensions. Colombian green coffee beans were milled under liquid nitrogen in a Retsch type ZM1 mill. Aliquots (1 g) were suspended and incubated (24 h at 37 °C with agitation in the presence of air) in either 10 mL of Milli-Q grade water (Millipore) or H_2O_2 aqueous solutions (H_2O_2 concentrations: 97.9 μM, 979 μM, 9.79 mM, 97.9 mM, and 0.97 M). After incubation, the suspensions were centrifuged (10 min at 1000 rpm) and the supernatants freeze-dried. The remaining pellets were washed with 10 mL of Milli-Q grade water and freeze-dried as well. Proteins of the freeze-dried powders were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

Table 2. Moisture and Dry Matter Losses (Boldface Numbers) and Color Reflectance Values (in Parentheses) of Santos Arabica Samples (Dry-, Semidry-, and Wet-Processed) during Roasting[a]

	roasting time			
processing	2 min	4 min	6 min	8 min
dry	**3** (nd)	**7** (185)	**13** (139)	**20** (47)
semidry	**2** (nd)	**7** (187)	**12** (132)	**20** (55)
wet	**2** (nd)	**7** (192)	**12** (136)	**19** (51)

[a] Moisture and dry matter losses are indicated in percent (boldface numbers). Color test numbers (CTn, in parentheses) were determined as described under Materials and Methods. When browning was too weak, CTn could not be determined (nd).

Fluorometric Hydrogen Peroxide Assay. After roasting, 7.5 g of milled coffee was added to 150 mL of boiling Milli-Q grade water. After a short stirring, brews were left at room temperature in the dark. After 30 min of incubation, 1 mL aliquots were removed, acidified (35 μM HCl), and left on ice for 1 h to promote precipitation. After centrifugation (minifuge, 10 min at 14000 rpm), H_2O_2 was determined in the supernatant by the homovanillic acid/horseradish peroxidase (HVA/HRPase) method as reported by Stadler et al. (*14*, *15*). Fluorescence was recorded at λ_{ex} = 314 nm and λ_{em} = 425 nm with a Hewlett-Packard spectrofluorometer. For the standard curves a 3% stock solution of H_2O_2 (Aldrich, Buchs, Switzerland) was used and diluted accordingly with Milli-Q grade H_2O.

Two-Dimensional Polyacrylamide Gel Electrophoresis. *Apparatus.* Isoelectric focusing (IEF) separation was performed with an ISOBOX (Hoefer Scientific Instruments, San Francisco, CA) connected to a MultiDrive XL power supply from Amersham Pharmacia Biotech (Europe GmbH). For the high-voltage run, a Consort power supply E 752 was used. The ISOBOX was modified so that the tray could accommodate Immobiline DryStrips (Amersham Pharmacia Biotech, Europe GmbH). SDS-PAGE was performed on vertical slab gels (160 × 200 × 1.5 mm) using a Protean II xi 2-D multicell (Bio-Rad). The gels were cast in a Protean II multigel casting chamber (Bio-Rad) using a model 395 gradient former (Bio-Rad). The gels were run using Bio-Rad's 3000xi power supply. Gels were scanned with the Personal Laser Densitometer (Molecular Dynamics, Sunnyvale, CA) and analyzed on a SUN Sparc Station 10 and Melanie II software from Bio-Rad.

Reagents. Reagents were from the following sources: Resolyte 3.5−10 from BDH (Poole, U.K.); acrylamide 2X from Serva (Heidelberg, Germany); piperazine diacrylamide (PDA), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), and ammonium persulfate from Bio-Rad (Glattbrugg, Switzerland); glycine and sodium dodecyl sulfate (SDS) from Fluka; tris(hydroxymethyl)aminomethane, 1,4-dithioerythritol (DTE), and urea from Merck; 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) from Sigma; and naphthalene-

disulfonic acid disodium salt (NDS) from Kodak. All other chemicals were of analytical grade.

Sampling. To follow the evolution of protein profiles during roasting, 30 coffee beans corresponding to each selected roasting time were milled under liquid nitrogen and extracted [200 mg in 3 mL of solubilization/lysis buffer (64 mM DTE, 65 mM CHAPS, 9 M urea, 35 mM Tris)].

For the analysis of water-soluble compounds generated during the roasting process (water-soluble extracts), 1 g of milled coffee was mixed with 0.1 g of polyvinylpolypyrrolidone (PVPP) and suspended in 1.5 mL of Milli-Q grade H_2O. After incubation (2−3 h at room temperature), the water extract was centrifuged and the supernatant (300 μL) mixed with the solubilization/lysis buffer (2.7 mL).

Freeze-dried suspensions were used as such for extraction (200 mg in 3 mL of solubilization/lysis buffer).

Healthy and diseased green coffee samples were milled under nitrogen and extracted (200 mg in 3 mL of solubilization/lysis buffer).

Protein Extraction. Each coffee homogenate, freeze-dried suspension (200 mg), or water extract (300 μL) was mixed with 3 mL (2.7 mL for water extracts) of solubilization/lysis buffer. Extraction was allowed to proceed for at least 2−3 h at room temperature with occasional vortexing. The Santos Arabica samples were, however, further extracted overnight at 4 °C. Extracts were then centrifuged (20000*g*, 30 min, 15 °C) and supernatants either used immediately for electrophoresis or stored at −20 °C until use.

Electrophoresis. (a) Standard Gels. The 2D-PAGE was performed with immobilized pH gradient (IPG) strips (Immobiline DryStrip gels, Amersham Pharmacia Biotech; see ref *16*) as described by Hochstrasser et al. (*17*).

Santos Arabica protein extracts (40−60 μL) were loaded onto rehydrated IPG strips with a nonlinear pH gradient (Immobiline DryStrip, pH 3−10NL, 18 cm). Otherwise, 50 μL of extracts was loaded onto the IPG strips. In the case of roasted coffee water extracts, narrow-range IPG strips were used (Immobiline DryStrip, pH 4−7, 18 cm).

A 300 V potential was applied and increased linearly to 3500 V within 3 h and then overnight at 5000 V (total of ∼100 kVh). The temperature was maintained at 10 °C. The IPG were then equilibrated in 100 mL of 0.05 M Tris-HCl buffer, pH 6.8, containing 6 M urea, 2% (w/v) SDS, 30% (w/v) glycerol, and 129 mM DTE. After 12 min, the procedure was repeated with another 100 mL of equilibration buffer containing 135 mM iodoacetamide and 250 μL of bromophenol blue solution (0.5% w/v) instead of DTE (*18*). After equilibration, the IPG strip gels were transferred to 9−16% gradient polyacrylamide gels (*18*, *19*). SDS-PAGE was performed at 40 mA per gel at a constant temperature of 8 °C. Then the gels were washed, fixed, and silver-stained as described elsewhere (*19*).

(b) Gelatin Gels. To identify proteins that do not stain with silver (negative staining), a modified 2D-PAGE procedure was applied. Gelatin was added to the polyacrylamide matrix to generate a dark background against which negatively staining proteins appear as white spots. Gelatin A (15 mg %) was allowed to copolymerize with the PDA−polyacrylamide gel. After the second dimensional run and prior to staining, the gels were washed in 2.5% Triton X-100 (30 min, room temperature) and then in Tris-HCl buffer (50 mM, pH 7.4; 5 mM $CaCl_2$) at 40 °C during 120 min. The gels were then silver-stained as usual.

Phenol Staining. To reveal polyphenols on two-dimensional gels, Folin−Ciocalteu's reagent (Sigma) was used. After electrophoresis, gels were fixed in 12% trichloroacetic acid, washed in H_2O, and incubated with Folin−Ciocalteu's solution according to Jennings' method (*20*) until a blue coloration appeared.

RESULTS AND DISCUSSION

Identification of 11S Storage Protein in Two-Dimensional Gels. The coffee storage protein (11S) was characterized by Rogers et al. (*9*). Its physiological role is to provide a source of amino acids and nitrogen for seed germination. This protein accounts for ∼45% of total proteins in the endosperm tissue, representing 5−7% of coffee bean dry weight (*9*). Under reducing conditions, the mature precursor form generates one high molecular weight subunit (the α-component, ∼32 kDa) and one low molecular weight subunit (the β-component, ∼22 kDa). Isoforms of each subunit constitute the α- and β-arm of each subunit (**Figures 2** and **3**). On the basis of Rogers's publication, storage protein subunits can easily be recognized on two-dimensional protein profiles of coffee.

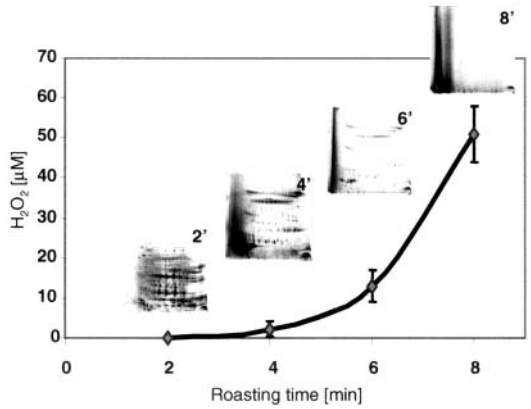

Figure 1. Changes in green coffee protein profiles during roasting followed by 2D-PAGE (Immobiline DryStrip, pH 3–10NL). Santos Arabica green coffee was roasted for 2, 4, 6, and 8 min before analysis. H_2O_2 was measured in the respective brews as described under Materials and Methods ($n = 3$, combining values obtained with three different postharvest treatments).

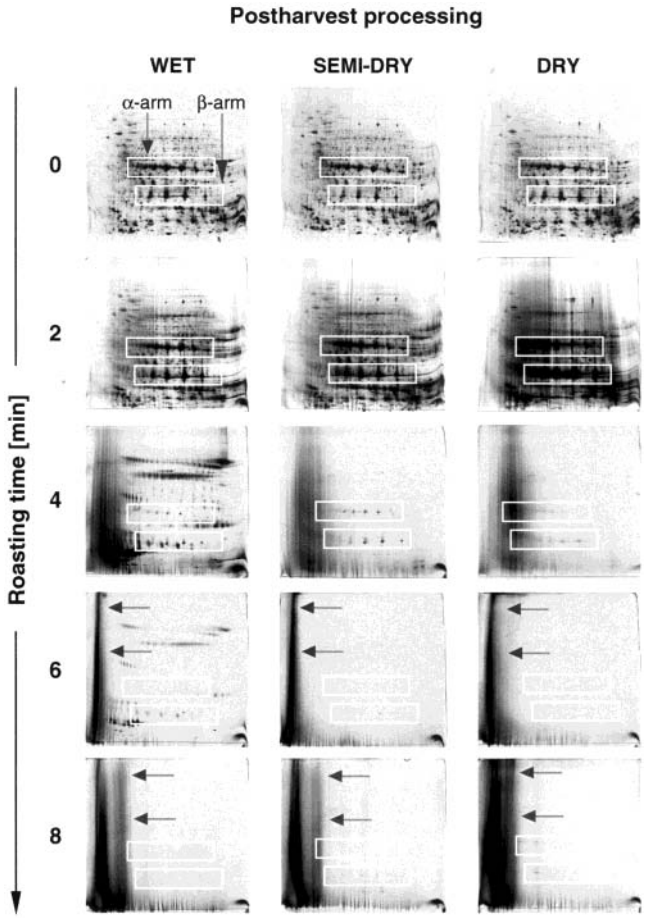

Figure 2. Impact of postharvest processing on coffee protein profiles during roasting. Profiles of Santos Arabica were followed by 2D-PAGE (Immobiline DryStrip, pH 3–10NL). Three processing methods were compared: wet, semidry, and dry. Storage protein subunits of the high (α-arm) and low (β-arm) molecular weight subunits are in rectangles. An acidic curtain of polymers appeared during roasting (arrows).

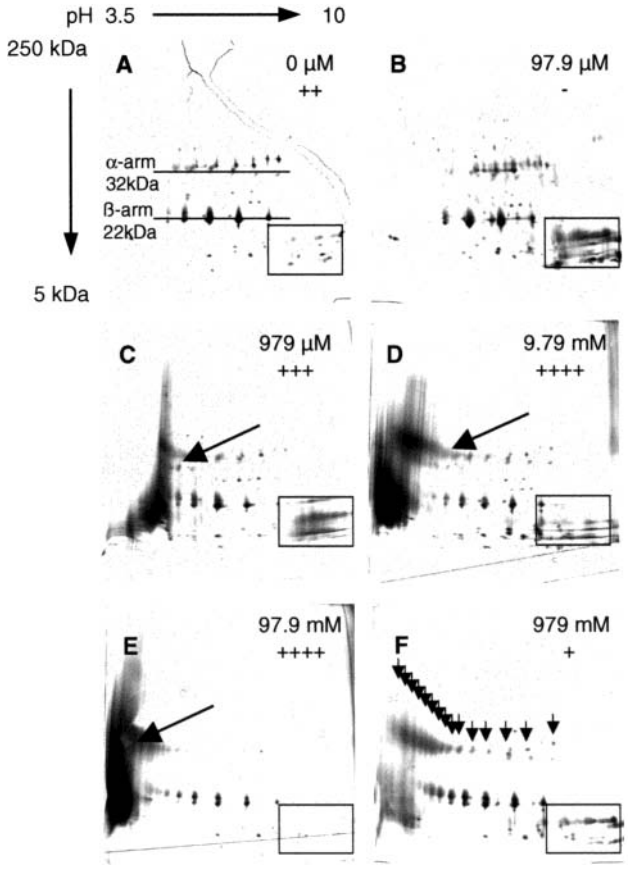

Figure 3. Changes in green coffee protein profiles in green coffee suspensions, water-soluble fraction. Arabica (Colombia) protein profiles were followed by 2D-PAGE (Immobiline DryStrip, pH 3–10NL). One gram of milled green coffee was suspended in 10 mL of H_2O_2 (37 °C, 24 h). Range of H_2O_2 concentrations tested: 0 μM (A) to 979 mM (F). The acidic curtain of polymers is indicated by an arrow. In (F), the progression of storage protein isoforms into the acidic polymeric curtain can be clearly observed (small arrows). Browning of the freeze-dried suspensions was scored on a relative scale from – (no browning) to ++++ (maximal browning).

Hydrogen Peroxide Release and Polymerization of Storage Protein Isoforms. The generation of H_2O_2 in aerated coffee solutions has been described by numerous investigators (*14*, *21*, *22*). Aqueous suspensions of green coffee beans do not generate H_2O_2. The ability to form H_2O_2 is acquired during roasting (*22*; **Figure 1**). This spontaneous reaction is called autoxidation and is primarily attributed to various polyphenols. Temperature, pH, and dissolved oxygen (O_2) are decisive factors that control autoxidation kinetics and H_2O_2 formation. Autoxidation contributes to the sequential reduction of molecular O_2 generating reactive oxygen species (ROS): superoxide radical ($O_2^{\bullet -}$), H_2O_2, and the hydroxyl radical ($OH^{\bullet}$) (*23*−*27*). In the absence of superoxide dismutase, $O_2^{\bullet -}$ is consumed by a pH-dependent dismutation reaction that forms H_2O_2. The rate of dismutation is maximal near pH 4.7 (*28*), a value close to what is usually measured in coffee solutions (pH 5−6).

Under our conditions, a 4-min roast (CTn 185−192, **Table 2**) was required to detect the formation of H_2O_2 (**Figure 1**). Formation of H_2O_2 then steadily increased during roasting, reaching a 50 μM concentration at a roasting time of 8 min. Simultaneously, we observed the formation of polymers in the acidic region of the two-dimensional gels (pH 4−5.3) in the form of a dark silver-stained curtain (**Figures 1** and **2**). The polymers seamlessly occupied the molecular weight range (5−250 kDa) covered by the acrylamide gradient. On the IPG strips (first dimension), a band corresponding to these polymers could be easily located due to its original brown color, clearly indicating their involvement in the browning process. After 8 min of roasting (CTn 132−139), the polymeric curtain was strongest and all protein spots had disappeared. Nonstorage proteins disappeared within the first 4 min of roasting (except for the wet-processed sample that displayed many new transient spots). Storage protein isoforms were slower to disappear.

In wine, the oxidation of phenolic compounds is autocatalytic due to regenerative polymerization leading to higher molecular weight polymers with increasing reduction potential (*29*). Improved resonance stabilization of phenolates and semiquinones provided by the highly conjugated structure of the growing polymer likely explains the increasing reduction potential toward O_2. In coffee solutions, a similar mechanism may account for the release of increasing amounts of H_2O_2 in relation to the degree of roasting. Conversely, it suggests that roasting may trigger a forced autoxidation process involving coffee polyphenols. This type of reaction may start long before the onset of pyrolysis. The extent of autoxidation is bound, however, to O_2 availability. There is no information available describing oxygen levels in the green coffee bean. Rolletschek et al. (*30*) showed that the embryo in legume seeds develops in a hypoxic environment. In fact, seeds have developed specific mechanisms to prevent anoxia. One can assume that some O_2, if not much, must be present in green coffee. Nonsymbiotic hemoglobins, which seem to be widely distributed among plants, may also act as O_2 carriers, releasing O_2 at lower partial pressure (*31*). One should also consider the possible regeneration of consumed O_2, a mechanism that would promote sustained oxidative cycling with a minimal amount of O_2. At high temperature, electron acceptors other than O_2 may be activated and radical-driven reactions favored. In fact, negatively staining proteins, identified as markers of oxidative activity in the green bean (*13*), can be traced up to 4−5 min of roasting (**Figure 6**). This observation strongly suggests that some type of oxidative activity affecting polyphenols is maintained during the first half of the roasting process.

Storage protein isoforms stained positively with Folin−Ciocalteu's reagent after 2D-PAGE of green coffee beans (data not shown), suggesting that the 11S storage protein forms adducts with polyphenols already before roasting. Its tendency to stain negatively after incubation in aqueous H_2O_2 solutions (**Figure 4**) supports this suggestion. Hence, a few autoxidative cycles of protein-attached polyphenols would be sufficient to draw 11S storage protein isoforms into a phenolic polymeric structure. Due to the significant moisture level of green beans (~12% in weight percent), molecular components of the green bean have a chance to migrate and interact during roasting. Loss of moisture proceeds slowly, and moisture level never reaches zero during roasting (*32*, *33*), probably due to the concomitant formation of condensation water.

It is clear that the pyrolysis of phenols (caffeic acid in model systems; *15*) and Maillard reaction products (*34*), be it in the presence or absence of O_2, generate compounds and polymers that can later form H_2O_2 by autoxidation. These reactions, however, proceed at high temperature. Thermal decomposition of pure CGA including decarboxylation and concomitant release of CO_2 starts at ~210 °C (*35*). Da Porto et al. (*32*) observed a sharp release of CO_2 at ~200 °C, a temperature corresponding to a 6 min roast under their roasting conditions (drum roasting, 10-min total roasting time). Considering our roasting experiment and the dry-processed Santos Arabica sample in particular

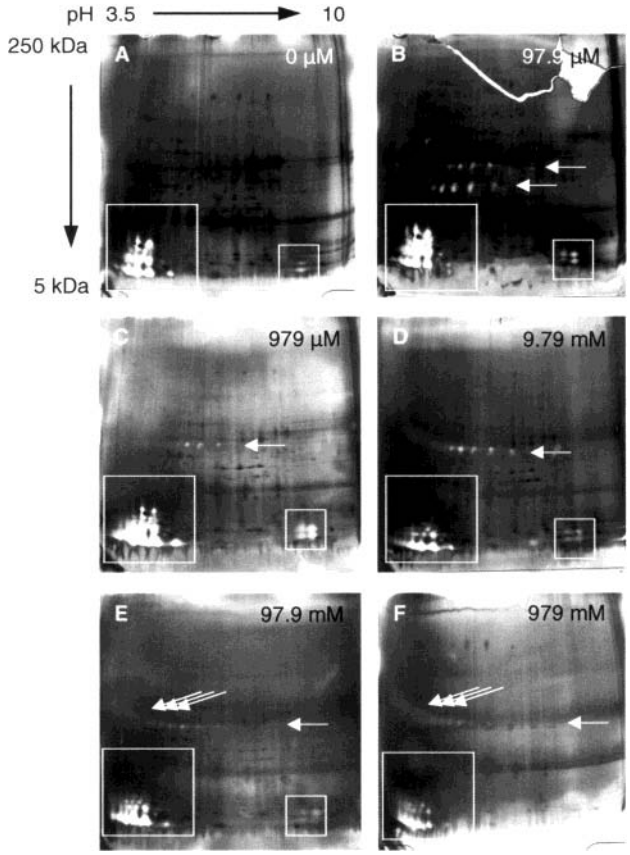

Figure 4. Same as **Figure 3**, but gelatin gels. Negatively staining protein fragments are in boxes. Storage proteins, especially the α-arm isoforms, tended to stain negatively in the presence of H_2O_2 (single arrows). The polymerization pathway of the α-arm isoforms could be followed at very high H_2O_2 levels (E, F; triple arrows).

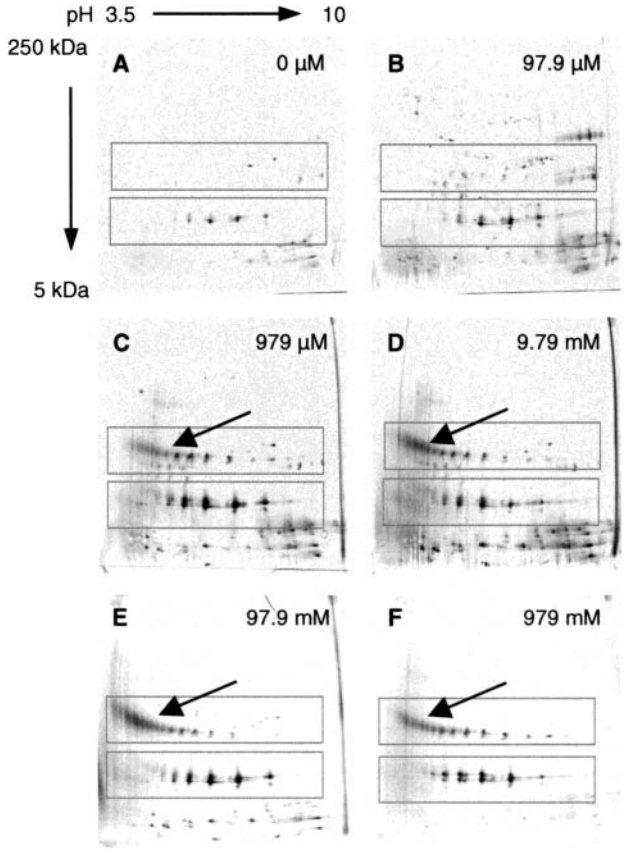

Figure 5. Same as **Figure 3**, but the water-insoluble fraction. No large acidic curtain of polymers could be observed, suggesting that nascent insoluble polymers (single arrow) become soluble upon further polymerization (compare with **Figure 3**). As observed for the water-soluble proteins, the α-arm isoforms appeared to be more reactive than the β-arm isoforms.

(**Figure 2**), we observed clear signs of polymerization after only 2 min of roasting. This time corresponds to an early stage, where pyrolytic decomposition of CGAs is unlikely to occur. Leloup et al. (*36*) showed that as much as 80% of all CGAs were degraded after 4 min of roasting under their conditions (240 °C, 10-min total roasting time). Nucleophilic addition reactions involving quinones (already present in the green bean or generated during roasting), proteins, and carbohydrates may explain, to some extent, the synchronized disappearance of CGAs and proteins. Incorporation of proteins into the polymeric matrix may, however, also proceed via ester, ether, and peroxy links.

Derivatization of proteins with CGAs is accompanied by a change of their isoelectric point toward lower pH values (*37*, *38*), potentially explaining the migration of storage protein isoforms into the acidic polymeric curtain. Storage protein subunits (α-arm and β-arm; **Figure 2**) and the polymeric curtain stained positively with Folin−Ciocalteu's reagent (data not shown), suggesting a significant role for polyphenols in the polymerization process.

Changes in Green Coffee Protein Profiles during Roasting, Impact of Postharvest Processing. Except for the analysis of coffee water-soluble compounds during roasting, we did not use any PVPP to remove phenols from our samples. In a previous paper (*13*) we have already observed that oxidation of coffee samples resulted in fuzzy protein profiles. This could be ascribed to oxidation of various polyphenols, either free or protein bound, which may react with the amide carbonyl of the polyacrylamide matrix or with companion molecules by nucleophilic addition. Consequently, quinoproteins are prevented from migrating properly toward their final position in the two-dimensional gel. After 2 min of roasting (**Figure 2**), we observed similar smearing of protein spots, suggesting that oxidation and polymerization of polyphenols occurred. Strong differences among the three postharvest processes were also obvious.

Beans alone are required for the preparation of coffee. Skin, pulp, mucilage, parchment, silver skin (to some extent), and water must be removed before storage and roasting. That is what postharvest processing is all about. Three main processes commonly used in the coffee world are dry, semidry, and wet (see **Table 1** for details). The dry process is the oldest and simplest: it consists of drying the cherries, most usually in the sun, before hulling. The uneven and slow maturation of coffee cherries (6−8 and 9−11 months for the species of economic value, *C. arabica* and *C. canephora*, respectively) results in the coexistence of green (usually unripe), red (ripe), and dark red (over-ripe) cherries on the same tree. Strip harvesting, which is typical for dry-processed cherries, does not include any form of classification or selection. Dry-processed coffee is consequently composed of beans representing various maturation stages. During the wet and semidry processes, pulping is the essential operation by which the soft pulpy part of the cherry together with the skin is removed. Pulping machines, to be effective, have to be fed with cherries of the right texture corresponding to ripe cherries and must not be fed with immature beans.

Dry processing is known to be much more aggressive than wet and semidry processing. The dry process elicits oxidative

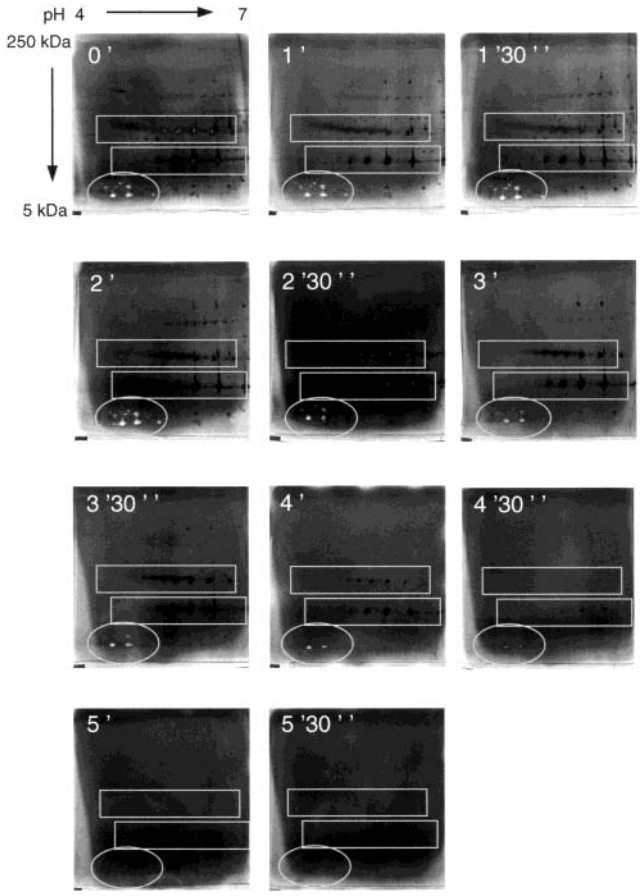

Figure 6. Changes in green coffee protein profiles during roasting followed by 2D-PAGE (Immobiline DryStrip, pH 4–7). Gelatin gels were used to trace negatively staining protein fragments. The water-soluble fraction was analyzed. Total roasting time of Arabica (Colombia) sample was 9 min. Negatively staining spots were observed during the first half of the roasting process (ovals). Negatively staining fragments and storage protein isoforms (gray boxes) vanished in a synchronized fashion.

stress and activates polyphenol oxidases. Dry-processed samples also contain unripe cherries, which appeared to be more prone to oxidation than ripe cherries (*13*). Preliminary pulping excludes the use of unripe cherries in the two other processes. The wet process also ensures optimal protection against oxidation and top quality. Large amounts of clean water are, however, required (40 L/kg of dry parchment coffee). Hence, a mechanical system was developed in Central America to remove the mucilage from the beans. This ecological process requires only ~1 L/kg of dry parchment coffee and largely preserves the original quality of the coffee beans. The semidry process was developed in Brazil with the aim to reduce costs and water pollution. Contrary to the wet method, it does not include a fermentation step and beans that are still surrounded by mucilage are dried. Accordingly, protein spots in the semidry-processed sample were sharp, nearly comparable to those in the wet-processed sample. These data suggest that the redox status of the green bean does influence the course and kinetics of melanoidin formation during roasting.

Changes in Green Coffee Protein Profiles in H_2O_2 Green Coffee Suspensions. H_2O_2 can be considered as a marker for autoxidation. At the same time, it may trigger autoxidation. Accordingly, we wanted to address the following question: can we induce polymerization of storage protein isoforms under mild conditions (37 °C), where pyrolysis and Maillard reaction do not occur? We decided to use H_2O_2 as an oxidizing agent. Green coffee suspensions were incubated during 24 h in aqueous H_2O_2 solutions covering a wide range of concentrations. Water-soluble and water-insoluble fractions of these suspensions were analyzed separately (**Figures 3**−**5**).

*Water-Soluble Fractions (**Figures 3** and **4**).* At high concentrations, H_2O_2 promoted the formation of an acidic curtain of nascent polymers that clearly integrated the isoforms of the α- and β-arms into their structure (**Figure 3C**−**F**). Browning was observed when polymerization occurred. Storage protein subunits migrated toward the acidic end of the gel, suggesting that strong derivatization precedes polymerization. No polymerization was observed at low H_2O_2 concentration or when H_2O_2 was omitted (**Figure 3A**,**B**).

An increase of neutral to basic low molecular weight proteins was observed as a result of protein degradation during incubation (**Figure 3**). When gelatin was added to the separating gel of the second-dimensional run, two populations of low molecular weight (10−20 kDa) negatively staining proteins were identified (**Figure 4**). Under oxidative stress, storage protein subunits seem to be forced into either polymerization (see negative staining of α-arm in **Figure 4E**,**F**, arrows) or fragmentation (see negative staining of α-arm and fragments in **Figure 4B**, single arrows).

In a previous study (*13*), we identified oxidative stress as the cause for negative staining. This behavior was attributed to the conversion of phenol-proteins into quinone-proteins. During silver staining, quinones may collect the electrons supposed to reduce protein-bound silver ions to black molecular silver. In the presence of H_2O_2, even storage protein subunits tended to stain negatively (**Figure 4B**−**F**), suggesting that these molecules share some specific structural and functional properties with negatively staining fragments. The ability of these fragments to withstand polymerization and to remain as discrete spots on the gel may be conferred by partial or total saturation of addition sites on the quinonic rings. At the same time, stabilization of the quinonic state is achieved, because regeneration of phenols by addition reactions cannot proceed.

Water-Insoluble Fractions. Insoluble material separated into sharp spots (**Figure 5**). A strong acidic shift of storage protein subunits in the presence of H_2O_2 was also observed. No acidic polymeric curtain could be observed, suggesting that insoluble storage protein isoforms (denatured?) become soluble upon polymerization. Rawel et al. (*38*) observed that oxidative conjugation of CGAs with bovine serum albumin strongly altered the secondary structure of the protein. Conformational modifications became more significant upon further derivatization. It is clear that these modifications may have a strong influence on the solubility of any protein, leading to either reduced or enhanced solubility according to the type of protein (*38*). The grafting of an insoluble protein on a soluble polymeric structure may also contribute to the masking of exposed hydrophobic sites and solubilize the protein.

Changes in Green Coffee Protein Profiles during Roasting (Gelatin Gels). Any specific and reproducible protein degradation process should be considered as a potential source for coffee-specific nitrogenous flavor precursors. Consequently, we decided to trace negatively staining fragments during roasting by using gelatin gels. Similar fragments were observed in their usual acidic location. Nonstorage proteins disappeared first. After 4−5 min of roasting, storage protein subunits and negatively staining proteins vanished in a synchronized fashion. It can be deduced from this observation that the 11S storage protein is the best candidate precursor of negatively staining fragments. When double staining was performed (first silver

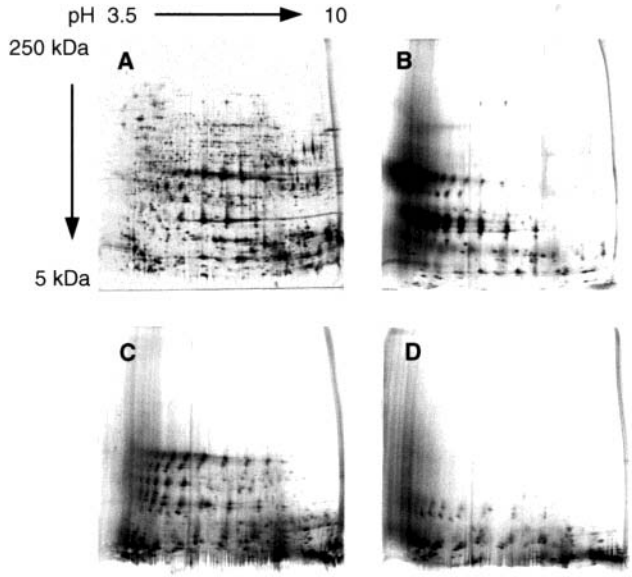

Figure 7. Oxidative burst in coffee: healthy versus damaged beans were analyzed by 2D-PAGE (Immobiline DryStrip, pH 3–10NL): (A) healthy green coffee beans (mature, wet-processed Arabica from Thailand); (B–D) damaged beans [(B) coated beans, beans that suffered from drought, they are characterized by a strongly adherent silver skin (origin: Mexico); (C) Cardinello beans, fungus-infested beans covered with yellowish or reddish spores (origin: Colombia); (D) black beans, beans with coffee berry disease (anthracnosis) caused by the fungus *Colletotrichum coffeeanum*, renamed *C. kahawae* (origin: Colombia)].

and then Coomassie blue), the negatively stained areas turned blue, indicating that there was no artifact (data not shown). In a few cases, however, the blue stain did not appear, suggesting that in situ fragmentation of the corresponding proteins did occur at some point of the staining process.

In green coffee H_2O_2 suspensions, negatively staining proteins accumulated; this was not observed during roasting (**Figures 4** and **6**). Extra energy provided by the roasting process may favor a complete and sustained fragmentation process, avoiding accumulation. **Figure 6** illustrates the fate of water-soluble proteins during roasting. In total extracts (data not shown), negatively staining proteins and storage proteins could be traced up to 5.5 min of roasting. Considering a medium roast (~9 min of roasting under our conditions), one can assume that sustained and complete degradation of storage protein subunits proceeded over more than half of the required roasting time. Similar results were also obtained with Robusta coffee samples (data not shown).

The mechanisms driving the fragmentation process are unclear. Negative staining indicates that oxidation may contribute or even be necessary to achieve fragmentation of 11S storage protein along this pathway. Negative staining may also be a coincidental feature that has nothing to do with the fragmentation mechanism. Pyrolysis and radical-driven fragmentation are well-known processes that certainly degrade proteins during roasting. In our previous study performed at 37 °C, aerobic conditions were required to observe degradation of the 11S storage protein and release of free amino acids (*13*). The similarity of the degradation pathways adopted by the 11S storage protein under extremely divergent physicochemical conditions points to a similar degradation mechanism. Polyphenol adducts perhaps protect the storage protein against pyrolysis and radical-driven degradation and force it into an atypical degradation pathway.

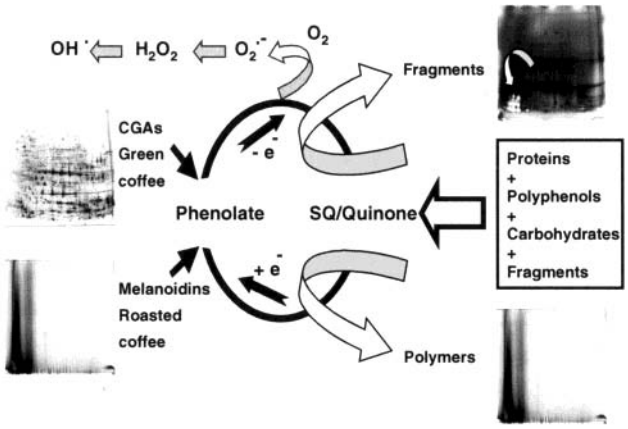

Figure 8. Proposed reaction scheme of autoxidative mechanisms in coffee. For details, refer to the text.

Considerations in Relation with the Physiology of Seeds. Because similar fragmentation and polymerization processes were observed at both high (roasting) and low temperatures (37 °C, in solution), the question about their possible physiological relevance is open. It is well-known that plants respond to pathogen infection by an oxidative burst (*39*). Release of ROS is also associated with germination, the developmental stage most sensitive to pathogen infection (*40*). The major ROS building the oxidative response in plants is H_2O_2 (*41*, *42*), which also appears to be a regulator of the reactions studied here. Two-dimensional protein profiles of beans that had suffered from water stress or infection by fungi clearly displayed similar fragmentation and polymerization profiles of the 11S storage protein (**Figure 7**). Accordingly, the oxidative burst that produces high levels of ROS may also be associated with protein polymerization and fragmentation reactions in seeds. This leads us to suggest that the coffee storage protein, which is devoted to the nutrition of the young seedling, may undergo oxidative fragmentation rather than exclusive enzymatic hydrolysis in the course of germination. This last point however deserves further investigation.

In this study, we showed that the 11S storage protein subunits are integrated into the polymeric structure of melanoidins formed during roasting. In parallel, negatively staining protein fragments are released. According to dynamic aspects of our data, they most likely derive from the 11S storage protein. Our data suggest that polyphenols bound to subunits of the storage protein contribute to their characteristic behavior and the formation of melanoidins. Similar features, polymerization, fragmentation profiles, and atypical staining, were also observed in green coffee suspensions incubated at 37 °C. This suggests that mechanisms other than pyrolysis and the Maillard reaction may play a role during roasting, in particular during the first half of the process. We propose that autoxidation of polyphenols contributes to the formation of melanoidins. Our observations coincide with data obtained by microbiological and metal-chelating affinity characterization of melanoidins, indicating that coffee melanoidins are not just of the Maillard type but also of the phenol type (*43*−*45*). The availability of O_2 appears to be the limiting factor for autoxidation to proceed in the intact bean. Even though it is almost certain that some O_2 is present in the bean, one should be aware that quinones that were generated in the green bean can still undergo one cycle of nucleophilic addition during roasting and in the absence O_2.

Polymerization reactions likely to occur during roasting, be they by autoxidation, pyrolysis, or the Maillard reaction, share the potential to generate compounds with the capacity to

generate H_2O_2 in solution. We observed that some coffee extracts can generate H_2O_2 concentrations well above 1−2 mM after a couple of hours. The involvement of autoxidation cannot be questioned here.

In **Figure 8**, we propose a reaction scheme of autoxidation in coffee. Two entries are proposed: the melanoidins, which are known to undergo spontaneous autoxidative processes in solution, and the polyphenols/CGAs of green coffee, which might enter the cycle by a heat/roasting- or H_2O_2-driven mechanism. Redox cycling promotes nucleophilic addition reactions and ROS generation. Fragmentation may also be involved in this type of redox cycling, but this remains to be confirmed.

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