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Quantitative Fate of Chlorogenic Acid during Enzymatic Browning of Potato Juice

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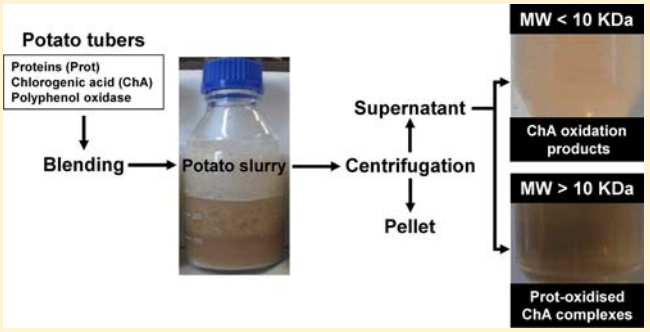
Supporting Information

ABSTRACT: The quantitative fate of chlorogenic acid (ChA) during enzymatic browning of potato juice was investigated. Potato juice was prepared in water without the use of any antibrowning agent (*OX* treatment). As a control, a potato juice was prepared in the presence of $NaHSO_3$ (*S* control). To study the composition of phenolic compounds in potato in their native states, also a potato extract was made with 50% (v/v) methanol containing 0.5% (v/v) acetic acid (*MeOH* control). Water-soluble low molecular weight fractions (LMWFs) and high molecular weight fractions (HMWFs) from *S* and *OX* extracts were obtained by ultrafiltration and dialysis, respectively. Pellets obtained after the *OX* treatment and the *S* and *MeOH* controls were also analyzed for ChA content. Whereas in the *S*-LMWF all ChA was converted to sulfonic acid adducts, no free ChA was found in the *OX*-LMWF, indicating its high reactivity upon enzymatic browning. Analysis of protein in the HMWFs showed a higher content of "reacted" ChA in *OX* (49.8 ± 7.1 mg ChA/100 g potato DW) than in *S* (14.4 ± 1.5 mg ChA/100 g potato DW), as evidenced by quinic acid release upon alkaline hydrolysis. The presence of quinic acid in *S*-HMWF was unexpected, but a mass balance incorporating the ChA content of LMWF, HMWF, and pellet for the three extractions suggested that ChA might have been attached to polymeric material, soluble in the aqueous environment of *S* but not in that of *MeOH*. Size exclusion chromatography, combined with proteolysis, revealed that ChA reacted with patatin and protease inhibitors to produce brown soluble complexes.



KEYWORDS: *UHPLC−MS, Solanum tuberosum, sulfite, enzymatic browning, phenolic acids, patatin, oxidation products*

INTRODUCTION

Enzymatic browning is associated with the oxidation of phenolics, such as hydroxycinnamic acids (HCAs) and their conjugates (HCAcs), to yield quinones, by the action of polyphenoloxidase (PPO) or peroxidase/H_2O_2.[1−7] Chlorogenic acid (ChA) and its isomers, *neo*-ChA and *crypto*-ChA, are the most abundant HCAcs in potato, their amounts commonly ranging from 23 to 350 mg/100 g dry weight, depending on the cultivar.[8−10] ChA consists of a caffeic acid esterified to the C-5 OH group of a quinic acid moiety. Dimers and, to a lesser extent, trimers of HCAs/HCAcs as well as conjugates comprising up to two HCAs/HCAcs and up to two amino acid moieties have been reported as low molecular weight oxidation products. Some of these had absorption in the visible light spectrum.[1,3−7] High molecular weight conjugates of HCAs/HCAcs and proteins have been reported as well, such as between ChA and proteins, sometimes leading to cross-linking of proteins.[5,6] When soy proteins were modified by oxidized caffeic acid or oxidized ChA, an increase in the absorption from 250 to 500 nm was observed compared to unmodified proteins, with the absorption at 400−500 nm being indicative of brown color.[7] Attempts have been made toward the identification of oxidation products upon enzymatic browning of fruits or vegetables, e.g., the study of low molecular weight oxidation products in (cider) apple juice.[4,11,12] In the work cited, several isomers of dimers of ChA, as well as ChA-catechin and ChA-procyanidin B2 conjugates (dimers and oligomers), were found as oxidation products. PPO-catalyzed oxidation of ChA to produce ChA quinone seems to be a key step in the generation of such (homo/hetero) oxidation products.[4,11−13]

So far, studies on enzymatic browning have not focused on the fate of major phenolics in complex mixtures of proteins and phenolics during fruit or vegetable processing. Neither information on possible changes in the chemical identity of phenolics nor information on their quantities, including their presence in insoluble fractions, is available. Hence, it is not known to what extent the oxidized phenolics react with proteins, with free amino acids, with other phenolics, or with themselves. The aim of the present research was to follow the quantitative fate of ChA when potato tubers were homogenized

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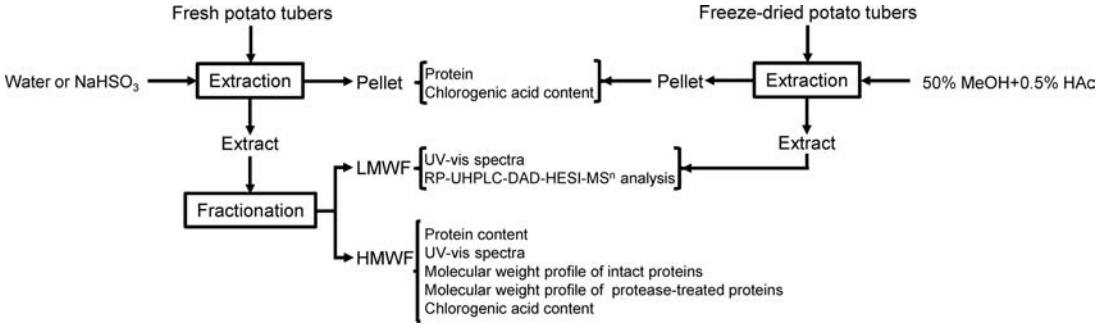

Figure 1. General scheme for the preparation and analysis of the potato extracts.

with water, without the use of any antibrowning agent. In this respect, the occurrence of ChA in a modified form after processing was studied. Low molecular weight oxidation products as well as high molecular weight oxidation products were investigated in the oxidized potato extract. A potato tuber extract prepared in the presence of sodium hydrogen sulfite, known to inhibit browning by formation of sulfo-*o*-diphenolics and by direct inhibition of PPO,[14,15] served as a control. To assess the parental composition of phenolic compounds in non-oxidized potato, without formation of sulfonic acid derivatives, an aqueous methanolic extract was prepared as well.[16]

MATERIALS AND METHODS

Chemicals. Chlorogenic acid (ChA), ferulic acid, sinapic acid, tyrosine, quinic acid, 37% (v/v) HCl, $NaHSO_3$, mushroom tyrosinase, α-amylase (from *Bacillus licheniformis*), and amyloglucosidase (from *Rhizopus* sp.) were purchased from Sigma-Aldrich (Steinheim, Germany). Proteinase K (number 92905, 825 units/mg) was from Fluka (Steinheim, Germany). UHPLC−MS grade acetonitrile (ACN) was purchased from Biosolve BV (Valkenswaard, The Netherlands). Water was obtained using a Milli-Q water purification system (Millipore, Billerica, MA, USA). All other chemicals were from Merck (Darmstadt, Germany).

Plant Material. Three batches of potato tubers (Nicola cultivar) were purchased in January 2012 from local supermarkets in Wageningen, The Netherlands. Before further processing potato tubers were washed under tap water and dried with paper tissue. Part of the potato tubers was freeze-dried.

Preparation of Brown Potato Extract and Fractionation. To study the reaction products, potato extract was prepared in water (*OX* extract). The procedure, schematically, is shown in Figure 1. Fresh potato tubers (200 g) were diced (0.5−1 cm thick) and immediately homogenized in a household blender with 200 mL of water. Subsequently, the mixture was stirred for 10 min at 4 °C. Next, starch and fibers were left to settle for 10 min at 4 °C. After decanting, the solution was centrifuged (37,000*g*; 30 min; 4 °C). The pellet was extracted three times with 100 mL of water. The four supernatants were combined and subsequently filtered through a 0.45 μm filter (Whatman, Schleicher & Schuell, Dassel, Germany). The filtrate (pH 6.0) represented the *OX* extract, which was immediately processed. Part of the *OX* extract (20 mL) was processed to obtain a low molecular weight fraction (LMWF), while the remainder was used to obtain a high molecular weight fraction (HMWF). The remaining pellet after 4 extractions and centrifugations, rich in starch, was freeze-dried, ground with a mortar and pestle, and kept at −20 °C until further analysis.

Preparation of LMWF involved the adjustment of the pH of the 20-mL aliquot to 3.5 by adding glacial acetic acid. The aliquot was left overnight at 4 °C. The resulting material was centrifuged (9,000*g*; 30 min; 4 °C), and the supernatant was filtered through a 0.45 μm filter (Whatman, Schleicher & Schuell). Remaining soluble proteins were retained by ultrafiltration using regenerated cellulose centrifugal filter units (Millipore, Amicon ultracel YM-10, 15 mL, cutoff 10 kDa, Bedford, MA, USA). The filtrate was denoted LMWF. Afterward, 10 mL of the LMWF was applied onto an activated Sep-Pak cartridge (C18, 6 mL/1 g, Waters, Milford, MA, USA). Next, the cartridge was washed with 5 mL of MQ water, and the compounds retained were eluted with 5 mL of methanol. The methanolic fraction was concentrated using a Savant ISS-110 SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature under reduced pressure. The final volume was adjusted to 500 μL with 50% (v/v) aqueous methanol containing 0.5% (v/v) acetic acid. The concentrated LMWF as well as the LMWF before Sep-Pak were flushed with N_2 and kept at −80 °C until further analysis.

Preparation of HMWF involved removal of phenolics and other low molecular weight compounds by dialysis. Since most of the proteins in potato tubers (98% w/w) have molecular masses higher than 20 kDa,[17,18] extract *OX* was extensively dialyzed against water at 4 °C in cellulose ester dialysis tubes with a cutoff of 12−14 kDa (Medicell International, London, U.K.). The dialyzed *OX* extract was freeze-dried, denoted HMWF, and stored at −20 °C until further analysis.

Preparation of Control Potato Extracts and Fractions. As a control, a potato extract was prepared in the presence of $NaHSO_3$ (*S* extract), and the LMWF, HMWF, and remaining pellet were obtained (Figure 1). The extract *S* was obtained as described above for extract *OX*. The first extraction was done with 200 mL of 400 mg/L $NaHSO_3$ solution, and the subsequent extractions with 100 mL of 200 mg/L $NaHSO_3$ solution. Differently as for *OX*-HMWF, to avoid any further reaction between phenolics and proteins, the *S*-HMWF extract was first dialyzed against 200 mg $NaHSO_3$/L until no further change in the conductivity of the retentate was observed. Subsequently, it was extensively dialyzed against water. The pellet after the fourth extraction was processed as described for the *OX* extract.

To assess the composition of phenolics without producing sulfo-*o*-diphenolics,[14] an extract from 2 g of freeze-dried material, of the same potato tuber batch, was obtained in 50% (v/v) aqueous methanol containing 0.5% (v/v) acetic acid (extract *MeOH*) as described elsewhere[16] (Figure 1). This extraction at acidic pH was aimed to precipitate proteins,[19] inhibit PPO activity, and therefore inhibit enzymatic oxidation of phenolics. This extraction is reported to yield full recovery of ChA from potato[20] and is considered to be indicative for the total amount of native phenolics present in LMWF. No HMWF was prepared, because during this acidic extraction proteins were precipitated. The pellet remaining after extraction was processed as described for the *OX* extract.

Extractions *OX*, *S*, and *MeOH* were performed on three independent batches of potato tubers, and their data are reported as averages with standard deviation.

Analysis of LMWF. *UV−vis Spectra.* LMWF was diluted 5 times with water, and the UV−vis spectra were recorded.

RP-UHPLC-DAD-HESI-MS^n Analysis. Tyrosine, (sulfo) HCAs, (sulfo) HCAcs, and HCAs/HCAcs oxidation products were analyzed by reversed phase-ultra high performance liquid chromatography (RP-UHPLC) with diode array detection (DAD) and an in-line heated electrospray ionization (HESI) mass spectrometer (MS^n). The same system was used to quantify unbound ChA in the *OX*-HMWF and *S*-HMWF. Conditions for the UHPLC were according to previous work,[16] except that the flow rate was 300 μL/min and the injected

sample volumes were 2 μL. The eluate of the column was directed to a HESI probe, which in turn was coupled to an LTQ-Velos (Thermo Scientific, San Jose, CA, USA). HESI-MS^n was performed in negative mode in a full-scan mass spectrum mode over a m/z range of 150−1,500 with tune files for tyrosine during the first 3 min and for ChA for the rest of the run. Other settings were Source voltage, 3.5 kV; ion transfer tube temperature, 250 °C; heater source, 230 °C. MS^2 spectra were collected with a collision energy of 35%, with the use of wideband activation. The control of the instrument and data processing were done using Xcalibur 2.1 (Thermo Scientific). Annotation of (sulfo-)HCAs/(sulfo-)HCAcs was done according to previous work.[14,16]

Quantification of (sulfo-)ChA isomers was based on calibration curves for ChA (0.1 to 5 μg/mL, R^2 = 1.000, five data points). Concentrations of sulfo-ChA isomers were expressed as ChA equivalents after correction of the difference between the molar extinction coefficients of ChA and sulfo-ChA[14] by the formula $X = [(Y_{325\ nm} - a)/b](\varepsilon_{ChA}/\varepsilon_{sulfo\text{-}ChA})$; where X = μg ChA/mL; $Y_{325\ nm}$ = absorbance at 325 nm of the sulfo-ChA isomers; a = intercept of the calibration curve of ChA; b = slope of the calibration curve of ChA; ε_{ChA} = extinction coefficient of ChA (18,494 ± 196 M^{-1} cm^{-1}); $\varepsilon_{sulfo\text{-}ChA}$ = extinction coefficient of sulfo-ChA (9,357 ± 395 M^{-1} cm^{-1}). Quantification of 2′-*S*-glutathionyl-ChA was based on calibration curves with ChA, and its concentration was expressed as ChA equivalent. Other caffeic acid conjugates were quantified by means of caffeic acid calibration curves after correction of the difference in molecular weight by the formula $X = [(Y_{325\ nm} - a)/b](MW_{caffeic\ acid\ conjugate}/MW_{caffeic\ acid})$; where X = μg caffeic acid conjugate/mL; $Y_{325\ nm}$ = absorbance at 325 nm of the caffeic acid conjugate; a = intercept of the calibration curve of caffeic acid; b = slope of the calibration curve of caffeic acid; $MW_{caffeic\ acid\ conjugate}$ = molecular weight of the caffeic acid conjugate; $MW_{caffeic\ acid}$ = molecular weight of caffeic acid. Ferulic acid and synapic acid were adopted as standards for quantification of ferulic acid and sinapic acid conjugates, respectively. The latter concentrations were calculated likewise as for caffeic acid conjugates. Calibration lines for caffeic, ferulic, and sinapic acids consisted of five data points at concentrations ranging from 0.05 to 2 μg/mL (R^2 = 0.999, in all cases). Limits of quantification were determined as 10 times the standard deviation of the noise.

Quantification of tyrosine was based on selective reaction monitoring (SRM) during HESI-MS^n. UHPLC conditions were as described above in this section, but HESI-MS^n settings were different. HESI-MS^n analysis during the first 3 min of the run was performed using SRM mode, for which the parent ion of tyrosine (m/z 180) was fragmented to produce the ion m/z 163 as base peak. The ion m/z 163 was used for quantification of tyrosine. Calibration curves were performed with tyrosine (1−8 μg/mL, R^2 = 0.994, five data points). Repeatability was tested by analyzing a solution of 5 μg/mL tyrosine evenly distributed within the sequence of sample analysis (five data points, coefficient of variation = 0.9%).

Analysis of HMWF and Pellet. *Protein Content.* Protein contents of HMWFs and pellets were measured by the Dumas method (conversion factor 6.25[17]) using a Flash EA 111 NC analyzer (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

UV−vis Spectra. HMWFs were prepared in 50 mM sodium phosphate buffer, containing 40 mM NaCl, at pH 7.0 in a ratio of 3 mg of powder to 1 mL of buffer. After centrifugation (18,000*g*; 10 min; 20 °C), samples were diluted with buffer to obtain 0.05 mg protein/mL buffer. Subsequently, UV−vis spectra were recorded.

Molecular Weight Profile of Unhydrolyzed Proteins. Size exclusion chromatography (SEC) of the HMWFs of *S* and *OX* was performed on an ÄKTAmicro system (GE Healthcare, Uppsala, Sweden). A Superose 12 HR 10/30 column (GE Healthcare) was equilibrated and run using 50 mM Tris/HCl buffer (pH 8.0), containing 150 mM NaCl at a flow rate of 500 μL/min at room temperature. A 100-μL sample (5 mg/mL unhydrolyzed proteins at pH 7.5) was injected onto the column. The eluate was monitored at 280, 320, and 400 nm. Void volume of the column was assessed with blue dextran (2,000 kDa, void volume). Apparent distribution of molecular masses was estimated with ferritin (440 kDa), aldolase (158 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa). The included volume was estimated with glutathione.

Enzymatic Digestion of Proteins and Effect on Molecular Weight Profile. Hydrolysis of the proteins present in the HMWFs of *S* and *OX* was performed by Proteinase K using a pH-stat setup. For this, 8 mL of HMWF solution (5 mg/mL) was preheated to 40 °C in the temperature-controlled pH-stat. Subsequently, the pH was adjusted to pH 7.5 with 0.5 M NaOH. Next, 500 μL of a 10 mg/mL proteinase K solution was added. The pH was kept constant by the pH-stat (pH 7.5) during 24 h of incubation with Proteinase K. The obtained hydrolysate was analyzed with SEC, without any further dilution, as described above.

Estimation of "Reacted" Chlorogenic Acid. Determination was based on the release of quinic acid by alkaline hydrolysis.[21] HMWFs from *OX*, *S*, and *MeOH* (25 mg) were suspended in water and clarified by adjusting the pH to 8 with 0.5 M NaOH. Afterward, the volume was adjusted to 1 mL using water. Next, 1 mL of 1 M NaOH was added. Mixtures were vortexed and then hydrolyzed by incubation at 100 °C during 1 h.[21] After cooling to room temperature, 37% (v/v) HCl was added to the hydrolysates to reach a pH of 3−4. Acidified hydrolysates were centrifuged (9,000*g*; 5 min; 4 °C). The supernatant was kept, and the pellet was washed twice with 0.5 mL 0.1% (v/v) HCl. Supernatants were combined, and final volume was adjusted to 5 mL with water. Aliquots (500 μL) were ultrafiltrated using regenerated cellulose centrifugal filter units (Amicon ultra 0.5 mL, cutoff 10 kDa, Millipore). Blanks, without alkaline treatment, were run to estimate the amount of free quinic acid and free (sulfo-) ChA present in HMWF extract. Hydrolysates and blanks were stored at −20 °C until further analysis.

The pellets from *OX*, *S*, and *MeOH* (100 mg) were each suspended in 20 mM sodium acetate buffer pH 5.2 (1 mL), followed by the addition of 50 μL of 1 mg/mL α-amylase. The suspension was incubated at 80 °C for 1 h and cooled to room temperature. Next, 50 μL of 1 mg/mL α-amylase and 50 μL of 1 mg/mL amyloglucosidase were added to the suspension, which was incubated at 30 °C during 12 h. Two cycles of enzymatic treatment were performed. The destarched suspension was freeze-dried and analyzed for ChA content. Enzyme-treated pellets were analyzed before and after alkaline hydrolysis for their content of free quinic acid and free (sulfo-) ChA.

An Accela UHPLC system (Thermo Scientific) equipped with a pump, an autosampler cooled at 7 °C, and a Hypercarb column (100 mm × 2.1 mm i.d.; particle size 3 μm; Thermo Scientific) at 30 °C was used to determine the quinic acid released. The eluents used were 100% water (eluent A), 100% ACN (eluent B), and 15% (v/v) aqueous formic acid (eluent C). The elution program in terms of A/B/C composition was as follows: 0−5 min, 100/0/0%; 5−6 min, 100/0/0% → 0/25/75%; 6−8 min, 0/25/75%; 8−9 min, 0/25/75 → 50/50/0%; 9−11 min, 50/50/0%; 11−12 min, 50/50/0% → 100/0/0%, and 12−16 min, 100/0/0%. The flow rate was 400 μL/min. Sample volumes of 5 μL were injected. The eluate was directed to a splitter (1:9), with the lowest flow going into the HESI, which was coupled to an LTQ-Velos Pro (Thermo Scientific). Settings for the HESI-MS^n were as described above, except that the system was autotuned with quinic acid. Quantification of quinic acid (retention time 3.55 min) was performed by SRM analysis by monitoring the fragment m/z 85 produced from the fragmentation of the parent ion of quinic acid (m/z 191). The concentrations of ChA in hydrolyzed HMWF and pellet were calculated by means of calibration curves that were performed by hydrolysis of authentic ChA (0.04−20 μg ChA/mL, R^2 = 0.999, 10 data points). The recovery of quinic acid upon alkaline treatment of ChA was 83.2 ± 2.3% (w/w). Free quinic acid content in the blanks was calculated by means of calibration curves with authentic quinic acid (0.02−10 μg quinic acid/mL, R^2 = 0.999, 10 data points). Free (sulfo-) ChA was analyzed in the blanks as described above. Repeatability was tested with a solution of 5 μg/mL alkali-treated ChA, which was analyzed evenly distributed within the sequence of sample analysis (6 data points, coefficient of variation = 2.0%).

Statistical Analysis. Data were reported as the means with their standard deviation. Quantities of phenolics in *MeOH* extract, *S*-LMWF, and *OX*-LMWF were analyzed using a one-way ANOVA as a completely randomized design with three replications. Means of phenolics in *MeOH* extract, *S*-LMWF, and *OX*-LMWF were compared by Tukey's test ($P < 0.05$). Data from *S*-HMWF and *OX*-HMWF were compared by Student's *t* test ($P < 0.05$). Means of ChA in the mass balance were compared by Tukey's test ($P < 0.05$).

RESULTS AND DISCUSSION

Color of the Potato Extracts. When potato extract was prepared in water (*OX* extract), a reddish coloration was observed in the low molecular weight fraction (LMWF), while a brown color was observed in the high molecular weight fraction (HMWF) (inserts in Figure 2). In contrast, colorless

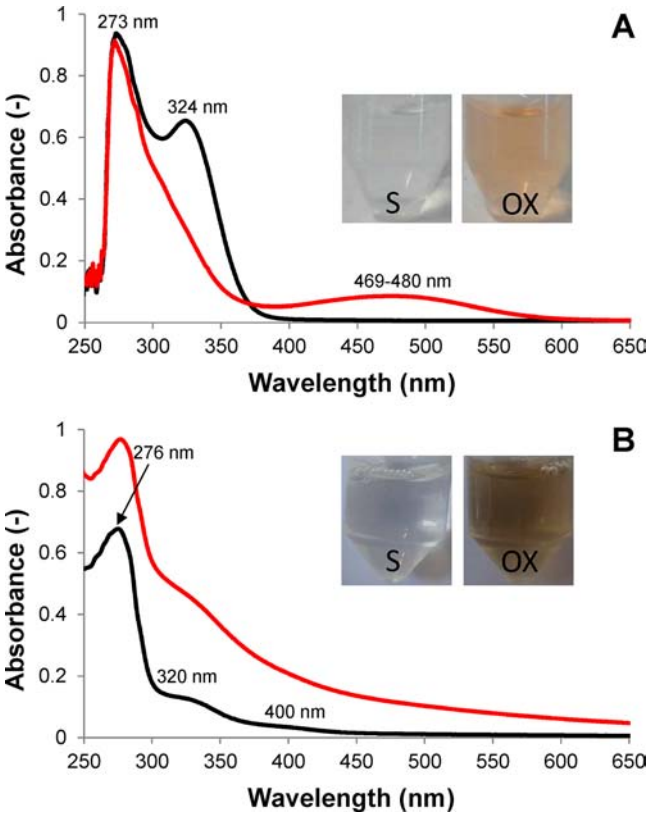

Figure 2. (A) Low molecular weight fraction (LMWF) of potato extracts prepared in the presence of $NaHSO_3$ (black line, *S*) and in water (red line, *OX*), both 5× diluted. Inserts show pictures of undiluted *S*-LMWF and *OX*-LMWF. (B) High molecular weight fraction (HMWF) of potato extracts prepared in the presence of $NaHSO_3$ (black line, *S*) and in water (red line, *OX*), both 0.5 mg protein/mL. Inserts show pictures of *S*-HMWF and *OX*-HMWF, both 2.0 mg protein/mL.

LMWF and HMWF were obtained when the extraction was performed in the presence of $NaHSO_3$ (*S* extract) (Inserts in Figure 2). The UV−vis spectra of *S*-LMWF showed absorption maxima at 273 and 324 nm (Figure 2A). The first maximum corresponded to the free aromatic amino acids tyrosine and tryptophan, reported to be present in potato.[20] The second maximum corresponded to sulfo-chlorogenic acid (sulfo-ChA) isomers that are expected to be present in *S*.[14] In the *OX*-LMWF there was an increase in the absorption in the visible region with a maximum at 469−480 nm (Figure 2A) when compared to *S*-LMWF. The absence of a maximum of absorption at 324 nm (as expected for ChA and its isomers) in *OX*-LMWF indicated a depletion of the ChA population.

The UV−vis spectrum of the *S*-HMWF (Figure 2B) showed that it had a maximum of absorption at 276 nm, indicative of the presence of tyrosine and tryptophan, present in proteins in significant quantities.[22] Shoulders observed in *S*-HMWF at 320 nm and at 400 nm indicated the presence of oxidized phenolics.[7] *OX*-HMWF showed similar maxima/shoulders of absorption as those observed in *S*-HMWF, but with higher values. Furthermore, *OX*-HMWF showed absorption at 450−650 nm, indicative of browning, while absorption for *S*-HMWF in that region of the spectrum was negligible (Figure 2B). These results, therefore, suggested that both low and high molecular weight oxidation products were generated during enzymatic browning of potato extracts.

Phenolics in Low Molecular Weight Fractions. *Composition of Phenolics in MeOH and Low Molecular Weight Fraction of S.* The *MeOH* extract contained ChA isomers, caffeic acid, and caffeoyl putrescine (Table 1, Figure 3). In contrast, in *S*-LMWF, ChA isomers and caffeoyl putrescine were found as their sulfonic acid derivatives (Table 1, Figure 3). Furthermore, a glutathionyl adduct was observed as well (**21**). Parent ion $[M - H]^-$ = 658 and the base peak *m/z* 385 after fragmentation were consistent with this compound being a glutathionyl-caffeoyl quinic acid adduct.[15] The 2′-C position in ChA quinone (produced upon PPO catalyzed oxidation of ChA) is the most activated and, therefore, more prone to nucleophilic attack.[14] Accordingly, **21** was tentatively annotated as 2′-*S*-glutathionyl-ChA. Tyrosine, together with ferulic and sinapic acid containing compounds, were constituents of both extracts (Table 1, Figure 3). Tyrosine (**1**), ChA (**4**), and caffeic acid (**6**) were identified by the combination of their retention times, UV spectra, and MS^n data. These data coincided with those of the authentic standards. MS^n and UV data of other compounds, including the sulfonic acid derivatives, matched those found in previous work.[14,16]

Quantification of HCAs/HCAcs in the *MeOH* extract (Table 2) revealed that caffeic acid-containing compounds represented 94% (w/w) of the total content of HCAs/HCAcs, while ferulic acid- and sinapic acid-containing compounds accounted for 6% (w/w). In the *MeOH* extract a total amount of ChA isomers of 80.6 ± 10.6 mg/100 g DW potato was found, which is comparable to literature data.[9,20] When the amounts of sulfo-ChA isomers and 2′-*S*-glutathionyl-ChA were expressed as ChA, a total value of 78.4 ± 2.6 mg/100 g potato DW (Table 2) was found in *S*-LMWF. The amount of tyrosine in the *S*-LMWF (55.2 ± 7.9 mg/100 g potato DW) was markedly lower than that in the *MeOH*-LMWF (110.2 ± 13.8 mg/100 g potato DW), which might be caused by the formation of sulfonic acid derivatives of the *o*-diphenol 3,4-dihydroxyphenylalanine (DOPA), formed upon hydroxylation of tyrosine by PPO and the subsequent reaction of sulphite with DOPA quinone. Such a derivative would elute close to the void volume and would escape detection with the conditions applied.

Composition of Phenolics in Low Molecular Weight Fraction of OX. ChA isomers, caffeic acid, and caffeoyl putrescine were not detected in *OX*-LMWF. At 325 nm the RP-UHPLC chromatogram of *OX*-LMWF (Figure 3) was dominated by compound **22**, co-eluting with tyrosine. When recording the chromatogram at 478 nm (chromatogram not shown) still a peak was observed, which could not be attributed to tyrosine. Compound **22**, having a maximum of absorption at

Table 1. Retention Times, MS, and UV−vis Data of HCAs/HCAcs, Sulfonic Acid Conjugates, and Oxidation Products in the Low Molecular Weight Fraction of Potato Extracts

no.	t_R (min)	MS (m/z)	MS^2 (m/z)[a]	UV−vis λ_{max}	tentative identification
1	2.43	180	163, 119, 136, 93	226, 275	tyrosine
2	10.36	353	191, 179[b], 135[b], 173, 161[b]	225, 240sh, 300sh, 324	3-*O*-caffeoyl quinic acid
3	10.60	249	249, 135[b], 207, 179[b], 161[b]	225, 294sh, 317	caffeoyl putrescine
4	11.86	353	191, 179[b], 161[b], 135[b], 173	226, 240sh, 305sh, 326	5-*O*-caffeoyl quinic acid
5	12.08	353	173, 179[b], 191, 135[b], 161[b]	226, 240sh, 305sh, 326	4-*O*-caffeoyl quinic acid
6	12.69	179	135[b], 179[b], 161[b]	225, 305sh, 323	caffeic acid
7	13.42	367	191, 173, 193, 178, 134	225, 300sh, 325	5-*O*-feruloyl quinic acid
8	14.06	309	193, 178, 149, 133, 115, 134	225, 305sh, 326	feruloyl malate
9	14.91	328	310, 295, 135, 149, 175	226, 294sh, 320	feruloyl octopamine
10	15.77	429	249, 205, 385, 179, 223	225, 311	sinapic acid conjugate
11	3.61	433	191, 353[b], *241*, 161[b], *259*, 179[b], 135[b]	225, 246, 295, 327sh	sulfo-caffeoyl quinic acid isomer
12	5.76	433	*259*, *215*, 161[b], *241*, 415, 387, 433, 179[b], 301, 191, 135[b], 353[b]	225, 240, 305sh, 327	sulfo-caffeoyl quinic acid isomer
13	6.64	433	*241*, *259*, 387, 415, 433, 301, 191, 161[b], 179[b], 353[b]	225, 250, 295, 323	sulfo-caffeoyl quinic acid isomer
14	7.35	329	329, 249, 161[b], 135[b], *215*	224, 294sh, 320	sulfo-caffeoyl putrescine isomer
15	9.01	433	301, 387, 433, *241*, *259*, 415, 191, 161[b], *215*, 179[b], 353[b]	225, 245sh, 295sh, 324	sulfo-caffeoyl quinic acid isomer
16	9.44	433	301, *259*, *241*, 387, 415, 161[b], 135[b], 191, 433	225, 289sh, 310	sulfo-caffeoyl quinic acid isomer
17	9.80	329	329, 249, *241*, 161[b], *215*, 135[b]	225, 295sh, 322	sulfo-caffeoyl putrescine isomer
18	9.93	433	*259*, 161[b], *241*, 387, *215*, 415, 191, 301, 353[b], 179[b], 135[b], 433	225, 280sh, 315	sulfo-caffeoyl quinic acid isomer
19	10.63	433	301, 387[b], *241*, 161, 387	225, 240sh, 305sh, 329	2′-sulfo-4-*O*-caffeoyl quinic acid
20	10.95	433	*259*, 161[b], *241*, 387, *215*, 415, 135[b], 179[b], 191 433, 301, 353[b]	225, 240sh, 305sh, 329	2′-sulfo-5-*O*-caffeoyl quinic acid
21	11.31	658	385, 466 (NL 192)[d], 529, 272, 193, 191	230, 250sh, 327	2′-*S*-glutathionyl-5-*O*-caffeoyl quinic acid
22	2.41[c]	192	148, 111, 120	475	dopachrome
23	10.48	461	167, 353[b], 191, 152	230, 250sh, 286, 460sh	caffeoyl quinic acid oxidation product
24	10.89	192	148	225, 316	oxidation product
25	10.94	396	178, 222 (NL 174), 173, 191	230, 294, 513	caffeoyl quinic acid oxidation product
26	12.68	489	429, 191, 179[b]		caffeoyl quinic acid oxidation product
27	13.92	561	501, 479, 369 (NL 192), 516, 191, 179[b]		caffeoyl quinic acid oxidation product
28	14.40	523	313, 331 (NL 192), 287, 269, 191, 173	230, 240sh, 309, 420sh	caffeoyl quinic acid oxidation product
29	15.06	523	331 (NL 192), 287, 313, 269, 191, 173	231, 280, 320sh, 420sh	caffeoyl quinic acid oxidation product

[a]Ions are written in order of intensity, the first one is the base peak in MS^2. Those ions diagnostic of SO_3 attached to the aromatic ring of caffeic acid are in italic. [b]Fragments related to the presence of chlorogenic acid or caffeic acid. [c]Coelution with tyrosine. [d]NL: neutral loss. Underlined values are related to a neutral loss (NL) of quinic acid or to ions expected for quinic acid.

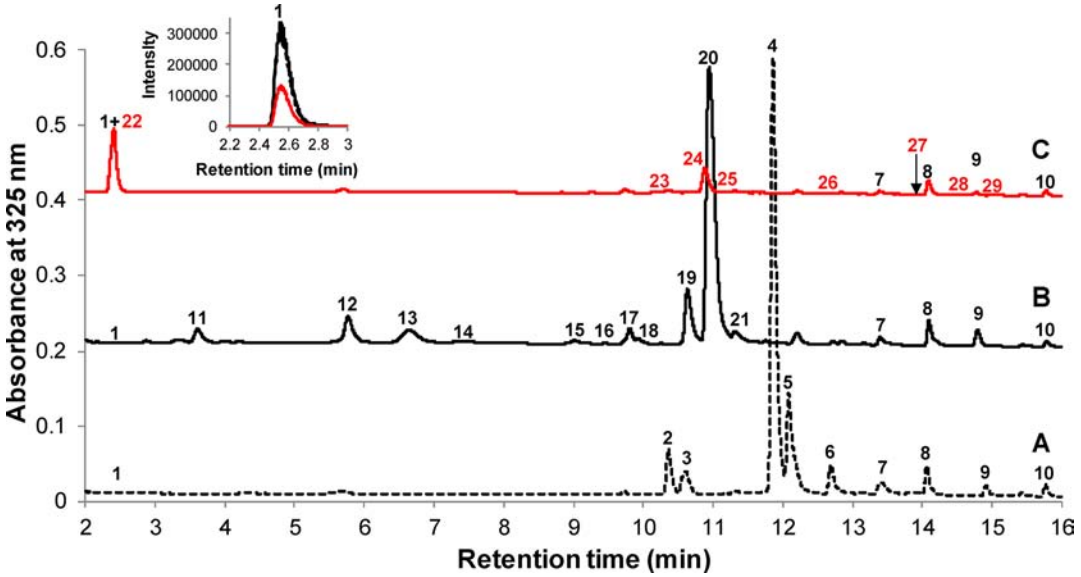

Figure 3. UHPLC profile recorded at 325 nm of the low molecular weight fraction of undiluted extracts prepared in 50% (v/v) methanol plus 0.5% (v/v) acetic acid (black dotted line, **A**), $NaHSO_3$ (black solid line, **B**), and water (red solid line, **C**). Compounds **1**−**29** correspond to those in Table 1. Insert shows the UHPLC-HESI-MS-SRM chromatograms from 2.2 to 3 min of the ion m/z 163.

478 nm, was then considered to be responsible for the reddish color observed in *OX*-LMWF (insert in Figure 2A). MS analysis revealed that compound **22** had a molecular parent ion of m/z 192, which in MS^2 gave the fragments m/z 148 ([M − CO_2 −

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Table 2. Quantification of Compounds Found in the Low Molecular Weight Fraction of Potato Extracted with 50% (v/v) methanol + 0.5% (v/v) Acetic Acid (*MeOH*), with Aqueous $NaHSO_3$ (*S*), and with Water (*OX*)

		mg/100 g DW[a]		
no.	compound	*MeOH*	*S*	*OX*
1	tyrosine	110.2 ± 13.8a	55.2 ± 7.9b	20.8 ± 5.4c
	Quinic Acid Containing Compounds			
2	3-*O*-caffeoyl quinic acid	6.6 ± 1.2	ND	ND
4	5-*O*-caffeoyl quinic acid	57.2 ± 6.9	ND	ND
5	4-*O*-caffeoyl quinic acid	16.8 ± 2.8	ND	ND
total caffeoyl quinic acid isomers		80.6 ± 10.6	ND	ND
7	5-*O*-feruloyl quinic acid	0.6 ± 0.0a	0.2 ± 0.0b	0.1 ± 0.0c
	Other Hydroxycinnamic Acids			
3	caffeoyl putrescine	3.4 ± 0.5	ND	ND
6	caffeic acid	2.2 ± 0.3	ND	ND
8	feruloyl malate	2.5 ± 0.3a	1.4 ± 0.2b	0.8 ± 0.1c
9	feruloyl octopamine	1.0 ± 0.2	<0.03[b]	<0.03[b]
10	sinapic acid conjugate	1.4 ± 0.3a	0.3 ± 0.0b	0.2 ± 0.0c
	Sulfocaffeoyl Quinic Acid Isomers Expressed As Chlorogenic Acid			
11	sulfocaffeoyl quinic acid isomer		2.8 ± 0.2	
12	sulfocaffeoyl quinic acid isomer		5.5 ± 0.4	
13	sulfocaffeoyl quinic acid isomer		5.4 ± 0.3	
15	sulfocaffeoyl quinic acid isomer		0.7 ± 0.0	
16	sulfocaffeoyl quinic acid isomer		0.1 ± 0.1	
18	sulfocaffeoyl quinic acid isomer		1.1 ± 0.1	
19	2′-sulfo-4-*O*-caffeoyl quinic acid		10.3 ± 0.6	
20	2′-sulfo-5-*O*-caffeoyl quinic acid		51.7 ± 3.0	
total sulfocaffeoyl quinic acid isomers expressed as chlorogenic acid			77.6 ± 2.3	
21	2′-*S*-glutathionyl-5-*O*-caffeoyl quinic acid expressed as chlorogenic acid		0.8 ± 0.3	
14, 17	sulfo-caffeoyl putrescine isomers		NQ	

[a]Values within the same row with different letters show significant differences (Tukey's test, $P < 0.05$). Data are expressed as the mean with standard deviation. ND: not detected by MS. NQ: not quantified due to lack of standards. [b]Below limit of quantification: 0.03 mg feruloyl octopamine/100 g potato DW.

$H]^-$), m/z 120, and m/z 111 (see Figure S1 of Supporting Information). The ion m/z 120 might be due to the neutral loss of CO from the quinoidal ring of the ion m/z 148 (Table 1). On the basis of the maximum absorption at 478 nm and MS^n data, compound **22** was tentatively annotated as dopachrome. This annotation was further confirmed by preparing dopachrome via incubation of tyrosine and tyrosinase.[23] Retention time, UV−vis maxima, parent ion, and daughter ions of the product formed matched those of **21**. To our knowledge, no mass fragmentation data have been reported for dopachrome. Compound **24**, with parent ion m/z 192 and UV maximum at 316 nm, was present in neither the *MeOH* extract nor in *S*-LMWF. Furthermore, **24** showed neither absorption in the visible spectrum nor fragments characteristic to caffeic acid or quinic acid. Compound **24** was considered to be an oxidation product, but its exact identity remains unclear.

To screen for trace oxidation products of ChA, *OX*-LMWF was concentrated by Sep-Pak. Compounds **23**, **25**, **28**, and **29** showed absorption between 420 and 513 nm. This indicates that they were co-responsible, although to a lesser extent than dopachrome, for the red color of *OX*-LWMF. It has been shown[1,4] that during oxidation of ChA and caffeic acid the aromatic ring can sometimes be modified leading to a fragmentation pattern different from that characteristic of HCAcs. With this in mind, we screened not only for negative ions (m/z 179, 161, 135) and neutral losses (180, 162 amu) of caffeic acid-containing compounds,[16] but also for negative ions (m/z 191, 173) and neutral losses (192, 174 amu) characteristic to quinic acid-containing compounds. When applying this strategy, compounds **23** and **25**−**29** were found (Table 2, Figure 3) and therefore are considered to be chlorogenic acid oxidation products.

Potato tubers contain amino acids, phenolics different from ChA, and glutathione [24] that can react with ChA quinone to produce heteroadducts as has been shown in *in vitro* systems.[4,11,15] Nevertheless, parent ions (m/z ranging from 396 to 523) of compounds **23** and **25**−**29** did not match those of such heterodimers. When the *OX*-LMWF was screened for glutathyionyl adducts ($[M - H]^- = 658$) in the selected ion monitoring mode (SIM), only a trace amount of 2′-*S*-glutathionyl ChA (**21**) was found, a compound that was detected neither in the UV chromatogram nor in the full MS scan of *OX*-LMWF. It could be that unstable low molecular weight homo- or heteroadducts of ChA were formed, which have reacted further. Compounds **22**−**29** were found neither in *MeOH* nor in *S*-LMWF, indicating the antibrowning effectiveness of these extractants. Neither ChA dimers (parent ion in negative mode m/z 705) nor hydrated-ChA dimers (m/z 723) were found in the brown potato extract, and also not when those parent ions were screened in the SIM mode. ChA dimers have been found during *in vitro* experiments in which ChA was oxidized by fungal tyrosinase.[4] ChA dimers (m/z 705) and hydrated-ChA dimers (m/z 723) have been found in oxidized cider apple juice.[4]

ChA isomers, caffeic acid, caffeoyl putrescine, and tyrosine reacted upon enzymatic browning of potato extract (Figure 3). A stronger decrease in the amounts of ChA and caffeic acid compared to tyrosine (Table 2) was found in the oxidized potato extract. This indicated a higher reactivity of PPO toward ChA and caffeic acid than toward tyrosine, consistent with the literature.[25] Dopaquinone might react with proteins in a nonenzymatic way, leading to brown polymers known as eumelanin.[26] Furthermore, proteins containing surface-exposed tyrosine might also be oxidized by PPO and contribute to browning of proteins as suggested by others.[5] The high molecular weight oxidation products of tyrosine were considered to be outside the scope of the current research and were not investigated further.

Chlorogenic Acid in High Molecular Weight Fraction. *"Reacted" ChA Content of High Molecular Weight Fractions.* Because no ChA isomers and only trace amounts of oxidation products of ChA were present in *OX*-LMWF, we did not quantify quinic acid released after alkaline treatment in this fraction. Instead, we searched for "reacted" ChA in the *OX*-HMWF. We assumed that the oxidized caffeic acid was the

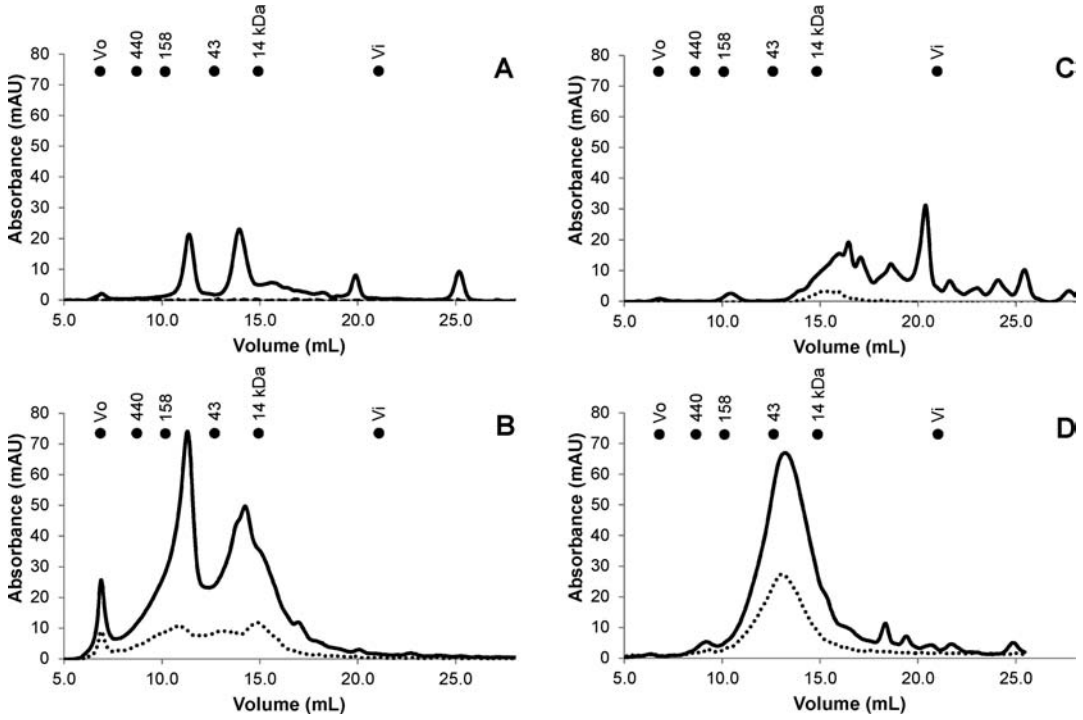

Figure 4. Molecular weight distribution of unhydrolyzed proteins from potato extracts prepared by using (A) sodium hydrogen sulfite (*S*) or (B) water and of protease-treated proteins from potato extracts prepared by using (C) sodium hydrogen sulfite or (D) water. All profiles were monitored at 280 nm (solid lines) and at 400 nm (dotted lines).

reactive moiety of ChA that was present in HMWF with the quinic acid moiety still unmodified. The release of quinic acid by alkaline treatment of the HMWF[21] was used to assess the quantity of ChA occurring in HMWF. The "reacted" ChA content in *OX*-HMWF was higher (1.87 ± 0.25%, w/w) than that in *S*-HMWF (0.68 ± 0.17%, w/w). As expected, proteins were found to be the major constituents of *S*-HMWF (85.4 ± 1.5%, w/w) and *OX*-HMWF (81.8 ± 2.4%, w/w).

Effect of Chlorogenic Acid on Molecular Weight Distribution and UV−vis Absorption of Proteins. The molecular weight distribution of unhydrolyzed *S*-HMWF obtained at 280 nm by size exclusion chromatography (SEC; Figure 4A) showed patatin isoforms (eluting as dimers of approximately 85 kDa) and protease inhibitors (approximately 20 kDa) as the most abundant fractions in *S*-HMWF, as judged from the obtained molecular masses, in agreement with previous reports.[17,18] Within the family of protease inhibitors, PSPI has been reported as the most abundant representative in potato juice.[27] Some aspecific binding to the column material was observed as judged from the peak (280 nm) eluting after the included volume. Compared to *S*-HMWF, SEC of the unhydrolyzed *OX*-HMWF revealed an increase of the absorption at 280 nm in the void volume, patatin fraction, and protease inhibitors fraction (Figure 4B), even though the difference in protein content in both solutions did not exceed 5% (w/w). Contrasting with the lack of absorption at 400 nm in the profile of *S*-HMWF, in *OX*-HMWF all protein fractions exhibited absorption at 400 nm. It is known that proteins modified with oxidized phenolics exhibit increase in the absorption in the UV−vis range compared to unmodified proteins.[7] Hence, the absorbance at 400 nm, together with the increase in absorbance at 280 nm in *OX*-HMWF, were assumed to be the result of the presence of oxidation products of ChA associated with proteins. SDS-PAGE (not shown) of *OX*-HMWF revealed a band that did not migrate into the gel, suggesting that the peak at the void volume observed for *OX*-HMWF was caused by, in addition to aggregation effects, cross-linking of proteins.

The SEC profiles at 400 and 280 nm for the unhydrolyzed *OX*-HMWF coincided (Figure 4B). Furthermore, when analyzing *OX*-HMWF, before alkaline treatment, by RP-UHPLC-DAD-HESI-MS^n, no low molecular weight oxidation products of ChA were found (chromatogram not shown). These results suggested two possible scenarios for attachment of ChA to proteins: (i) Oxidized ChA was covalently attached to proteins. (ii) Oligomeric ChA, formed upon oxidation, was noncovalently attached to the potato proteins, with similar affinity to patatin and PSPI, and therefore with a elution pattern coinciding with that of the proteins. The first scenario seems to be most likely.

Effect of Protease Treatment on Molecular Weight Distribution of Proteins. To further determine whether "reacted" ChA in *OX*-HMWF was covalently linked to proteins or present as oligomeric ChA, enzymatic hydrolysis of the proteins was performed. The elution profiles of the protease treated samples were evaluated at 280 and 400 nm on SEC and compared with the *S*-HMWF profile of the unhydrolysed proteins. As expected, protease treatment of *S*-HMWF (Figure 4C) generated a wide distribution of products with lower molecular mass, e.g., as judged by the shift to higher elution volume in the absorption profile at 280 nm. The high amount of protease K required to achieve the hydrolysis of proteins in the *S*-HMWF might be the result of the presence of protease inhibitors as major components of potato protein preparations.[17] After hydrolysis, a small peak at an elution volume between 14 to 17 mL was observed at 400 nm. This signal might be the result of partial oxidation of aromatic amino acids

in the proteins induced by the pH (7.5) and temperature (40 °C) applied during the protease treatment.

The moderate shift in elution volume after enzymatic treatment of the *OX*-HMWF (Figure 4D) was indicative of less extensive hydrolysis of this sample compared to *S*-HMWF. The difficulty to degrade the oxidized proteins suggested that "reacted" ChA was strongly associated with proteins. After partial enzymatic hydrolysis, the chromatographic profiles at 280 and 400 nm coincided, suggesting that the oxidized ChA remained attached to the peptides produced. Considering that *in vitro* studies have shown that ChA is covalently attached to proteins, such as lysozyme and bovine serum albumin,[5,6] we speculated that oxidized ChA was attached to the potato proteins, similarly. Other studies with individual NH_2-blocked amino acids and oxidized ChA have shown that cysteine, lysine, tryptophan, histidine, and tyrosine are targets for covalent attachment of ChA.[4,28] Lysine, tryptophan, histidine, and tyrosine were indeed found to be present on the surface of patatin and PSPI (EMBL-EBI LION Bioscience AG, htpp://www.ebi.ac.uk/thornton-srv/databases). Therefore, one might speculate that oxidized ChA will be covalently attached to these solvent-exposed amino acid residues in potato proteins.

"Reacted" Chlorogenic Acid in Pellets. The amounts of pellet that were obtained in the three extractions were 68.4 ± 1.3, 79.1 ± 0.2, and 68.6 ± 1.0 g pellet/100 g potato DW in *OX*, *MeOH*, and *S*, respectively. As expected, the amount of pellet found in *MeOH* was higher, due to the lower solubility of, e.g., proteins and polysaccharides in methanol than in aqueous environment (*OX*, *S*). The amounts of protein present in the pellet of *OX* and *S* were approximately similar with 2.69 ± 0.15 and 2.27 ± 0.27 g protein/100 g potato DW, respectively. The amount of protein in the pellet of *MeOH* was 4.31 ± 0.27 g protein/100 g potato DW, consistent with poor solubility of proteins in methanol.

The amount of ("reacted") ChA was also analyzed in the pellet fractions. No free ChA was present, as expected after the exhaustive extraction procedure. The amount of "reacted" ChA found in the *MeOH*-pellet (0.029 ± 0.007 g "reacted" ChA/100 g pellet DW) was higher than that found in *S*-pellet (0.016 ± 0.002 g "reacted" ChA/100 g pellet DW) but similar to that found in *OX*-pellet (0.031 ± 0.005 g "reacted" ChA/100 g pellet DW). The presence of "reacted" ChA in the *MeOH*-pellet was surprising, as we considered oxidation of ChA by PPO unlikely during this control extraction. A possible explanation for this is that quinic acid might be part of other potato tuber constituents, such as the plant cell wall, although this has never been reported in the literature to our knowledge.

Tracking Chlorogenic Acid during Enzymatic Browning. To determine the quantitative fate of ChA during enzymatic browning, a mass balance was made (Figure 5). In all cases, independent of the fraction (LMWF, HMWF, pellet) or the sample analyzed (*MeOH*, *S*, *OX*), the quantities were reported as ChA equivalents. Thus, for example, concentrations of sulfo-ChA isomers and 2′-*S*-glutathionyl-ChA obtained in *S*-LMWF, as well as concentrations of "reacted" ChA in the pellets, were expressed as ChA equivalents. In the *S* experiment, the LMWF accounted for 78.4 ± 2.6 mg ChA/100 g potato DW, the HMWF accounted for 14.4 ± 1.5 mg ChA/100 g potato DW, and the pellet for 10.5 ± 1.1 mg ChA/100 g potato DW. Thus, a total amount of 103.3 ± 2.5 mg ChA/100 g potato DW was found, which matched very well with that obtained in *MeOH* (103.3 ± 6.2 mg/100 g potato DW).

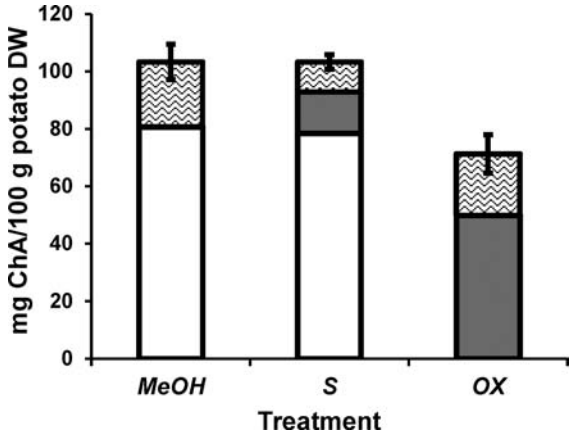

Figure 5. Total chlorogenic acid (ChA) equivalents in low molecular weight fraction (LWMF, white bars), high molecular weight fraction (HMWF, gray bars), and pellet (patterned bars). Error bars represent the standard deviation of the total amount. In all cases, independent of the fraction (LMWF, HMWF, pellet) or the sample analyzed (*MeOH*, *S*, *OX*), the quantities were calculated as ChA equivalents. The compounds that were summarized in the *MeOH* control were *neo*-ChA (**2**), ChA (**4**), and *crypto*-ChA (**5**) in LMWF and "reacted"ChA in pellet; in the *S* control were all sulfo-caffeoyl quinic acid isomers (**11**–**13**, **15**, **16**, **18**–**20**) and 2′-*S*-glutathionyl-ChA (**21**) in LMWF, and "reacted" ChA in both HMWF and pellet; and in the *OX* treatment were "reacted" ChA in both HMWF and pellet.

No ChA was found in the *OX*-LMWF, but 49.8 ± 7.1 mg ChA/100 g potato DW was found in *OX*-HMWF and 21.4 ± 3.5 mg ChA/100 g potato DW in the pellet. The total amount of ChA in the *OX* treatment (71.2 ± 6.7 mg ChA/100 g potato DW) represented only 70% of that obtained in *S* treatment. The 30% of ChA that was missing in the *OX* treatment might be the result of underestimation of ChA upon quantification by quinic acid release with alkaline treatment. Possibly, the quinic acid part of ChA participated in the later stages of the enzymatic browning reaction and participated in further reactions with proteins or was released as a low molecular weight oxidation product. It has been shown that oxidation of quinic acid produces compounds, such as citric acid, malic acid, and hydroquinone,[29,30] but no information on reactions in the presence of other components, such as proteins, is available.

Remarkably, the total amount of "reacted" ChA recovered (represented by the sum of the amounts of ChA in HMWF and pellet) was similar in the *MeOH* and the *S* extraction (Figure 5), but the contribution of ChA to the pellets differed. We speculate that all "reacted" ChA is precipitated, together with proteins and polysaccharides, in the methanolic environment of *MeOH*, whereas in the aqueous environment of *S*, part of the "reacted" ChA remained soluble and ended up in the HMWF. We do not have a good explanation for our observations that the pellets of *MeOH* and *OX* have similar amounts of "reacted" ChA and that the pellet of *OX* has a larger amount of "reacted" ChA than that of *S*, despite the fact that the aqueous extraction conditions of *OX* and *S* were similar. Perhaps the "reacted" ChA in *OX*-HMWF becomes part of the *OX*-pellet after further action of PPO, e.g., as a result of precipitation of proteins cross-linked by ChA. These ChA cross-linked proteins should be rich in ChA as the protein content of the *OX*- and *S*-pellet are similar. This might explain the observed discrepancy in the amount of "reacted" ChA in the pellets of *S* and *OX*. During further oxidation reactions the quinic acid moiety of ChA might participate and can subsequently not be analyzed as such

anymore. This might explain why the amount of "reacted" ChA is not higher in the *OX*-pellet, and the similar amounts of "reacted" ChA in the pellets of *MeOH* and *OX* might actually be a coincidence. In conclusion, our results show that the majority of the free ChA present in potato tubers is associated with (high molecular weight) proteins upon processing of potato juice in the absence of antibrowning agents.

■ ASSOCIATED CONTENT

Supporting Information

Fragmentation pattern of dopachrome in MS^2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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