Physicochemical Properties and Susceptibility to Proteolytic Digestion of Myoglobin−Phenol Derivatives

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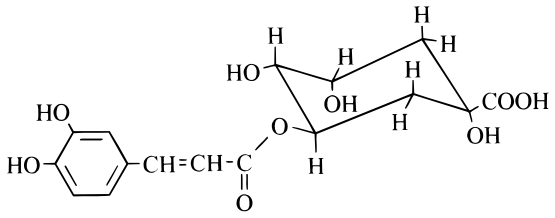
This paper deals with the interactions of chlorogenic, caffeic, and quinic acids and *p*-quinone with myoglobin. The myoglobin derivatives formed have been characterized in terms of physicochemical properties and susceptibility to proteolysis. The results show that the free amino group and tryptophan contents of the myoglobin−phenol derivatives decrease with the increasing extent to which the protein becomes derivatized. Furthermore, the solubility of myoglobin−phenol derivatives decreases in the pH range 3.5−6.5 as compared to solubility of the native protein. The reaction also influences the hydrophilic−hydrophobic character of the protein. The isoelectric point of the derivatized myoglobin is shifted to a lower pH value, and formation of high molecular fractions is also documented. This paper also demonstrates the influence of the protein derivatization with plant phenols on susceptibility to digestion by trypsin, α-chymotrypsin, and pepsin, determined in vitro. The enzymatic digestion of the derivatized proteins is adversely affected.

Keywords: *Plant phenolic substances; quinic, chlorogenic, caffeic acid; p-quinone; myoglobin; food protein derivatization; physicochemical characterization; RP-HPLC; IEF; MALDI-MS; in-vitro proteolytic degradation*

INTRODUCTION

Dietary phenolic substances have received in recent years much attention due to their biological activity. They have been attributed with positive properties such as having antimutagenic and anticarcinogenic effects as well as antioxidative effects—a possible mechanism by which dietary components protect the body from free radicals and reactive oxygen species (Huang et al., 1992; Maleveille et al., 1996; Friedman, 1997; Chung et al., 1998). These effects have been reported for plant phenolic substances such as caffeic and chlorogenic acid (Kitts et al., 1994; Kono et al., 1995). Polyphenols, on the other hand, can also display many possible detrimental effects, including inhibition of iron absorption (Mehansho et al., 1987) and irreversible complexation of gut enzymes and dietary proteins (Mehansho et al., 1987; Robbins et al., 1991; Scalbert, 1991), the consequences of which may result mostly in polyphenol-rich foods being nutritionally poor. The human dietary intake of flavonoids (a further class of plant phenols) is estimated to be up to 1 g/day (Maleveille et al., 1996) and that of chlorogenic acids and other cinnamates ranges from 25 mg to 1 g/day depending upon the dietary constitution (Clifford, 1999). Both caffeic and chlorogenic acids are widely distributed in the plant kingdom, and their content in many beverages, fruits, and vegetables has been recently reviewed by Clifford (1999). Caffeic acid attached to quinic acid forms chlorogenic acid with four possible isomers depending on the hydroxyl group of quinic acid involved in the ester bond. The most frequent form found in plants is 3-*O*-(3,4-dihydroxycinnamic acid) with the following structure:



There are four potential types of interactions of phenolics and proteins: hydrogen bonding, hydrophobic, ionic, and covalent (Hagerman, 1992). The phenolic hydroxyl group is an excellent hydrogen bond donor and forms strong hydrogen bonds with the amide carbonyl of the peptide backbone (Hagerman, 1992). Hydrophobic interactions are more important for the stabilization of the complexes formed, whereby proline residues seem to play a key role (Muralidhara and Prakash, 1995; Siebert et al., 1996; Baxter et al., 1997; Siebert, 1999).

Chlorogenic and caffeic acid may be oxidized with ease in the presence of a polyphenol oxidase or in an alkaline solution to their corresponding quinones (Hurrell and Finot, 1984). The quinones represent a species of highly reactive substances that normally react further with other quinones to produce colored compounds of high molecular weight. They may, however, also react with lysine, methionine, cysteine, and tryptophan residues in a protein chain (Hurrell and Finot, 1984). Studies showing the reaction of enzymatically generated caffeoquinone and chlorogenoquinone with amino acids and proteins have been reported by Pierpoint (1969a,b) and Hurrell et al. (1982). The possible reaction steps that may take place or the possible reaction products formed with lysine, cysteine, tryptophan, and methionine resi-

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dues have been revised by Machholz and Lewerenz (1989). In this report, the in-vitro digestion was not carried out.

The presented work is a continuation of previous attempts to characterize the reactions of food proteins with secondary plant metabolites from the physicochemical and physiological viewpoint. In earlier investigations we showed that isothiocyanates (breakdown products of glucosinolates) react with myoglobin and other food proteins (Kroll and Rawel, 1996; Hernandez-Triana et al., 1996; Rawel et al., 1998a−c). It was observed that myoglobin, the structure of which is well defined and characterized, serves well for model investigations.

In the present paper an attempt is made to show in a model system the effects of myoglobin reactions with different amounts of chlorogenic, caffeic, and quinic acids as well as with *p*-quinone. Muralidhara and Prakash (1995) have shown on the basis of microcalorimetric investigations that the interactions of these substances with human serum albumin take place and that the strength of binding follows the order caffeic acid ≫ chlorogenic acid ⋙ quinic acid. They showed that the effect of temperature on binding of chlorogenic acid to human serum albumin is dominated by hydrophobic and hydrogen bonding, whereby the role of covalent bonding was not discussed.

The purpose of the present work was to underscore the influence of derivatization with phenolic compounds on the physicochemical properties of myoglobin (with special attention to the role of covalent bonding) and its susceptibility to proteolytic digestion with the three physiologically main proteolytic enzymes (trypsin, α-chymotrypsin, and pepsin) of the gastrointestinal tract.

MATERIALS AND METHODS

Materials. Myoglobin (0.3 g) from horse heart (Fluka Chemie AG, Buchs, Switzerland) was dissolved in distilled water, and the pH value of the protein solution was adjusted to 9 using 0.5 M NaOH (final volume = 27 mL). Under continuous stirring at room temperature chlorogenic (3-*O*-[3,4-dihydroxycinnamate]), caffeic (3,4-dihydroxycinnamic acid), and quinic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid) as well as *p*-quinone (1,4-benzoquinone; all from Fluka Chemie AG) dissolved in 10 mL of ethanol were added. Each of these phenolic substances was added to a separate myoglobin solution in three different concentrations (0.14, 0.28, and 0.42 mM/g of protein), and the pH was adjusted once more to 9. After 24 h of reaction time under continuous stirring at room temperature with free exposure to air, the samples were dialyzed for 18−20 h and finally lyophilized. The nonderivatized protein (control) was prepared under the same conditions but without addition of the phenolic substances or *p*-quinone.

Trypsin from porcine pancreas (EC 3.4.21.4, protein content = 98%; 14900 units/mg of solid, 1 BAEE unit = ΔA_{253} of 0.001 per minute with BAEE as substrate at pH 7.6 at 25 °C) and pepsin from porcine stomach mucosa (EC 3.4.23.1, protein content ∼92%; 3100 units/mg of solid, 1 unit will produce a ΔA_{280} of 0.001 per minute at pH 2 at 37 °C, measured as trichloroacetic acid (TCA)-soluble products using hemoglobin as substrate) were purchased from Sigma Chemical Co. (St. Louis, MO), whereas α-chymotrypsin from bovine pancreas [EC 3.4.21.1, protein content = 96%; 53.1 units/mg, 1 unit will hydrolyze 1 μmol of Suc-(ala)2-Pro-Phe-4-NA/min at pH 7.8 and 25 °C] was from Fluka Chemie AG. All specific activities and definitions quoted here were given by the producers.

Physicochemical Characterization. The protein content in the solutions was determined according to the modified method of Lowry et al. (1951). The control myoglobin preparation was used to calibrate the Lowry regression curve ($Y = 0.06323 + 0.00552X$, $R^2 = 0.997$). The solubility profile of the lyophilized samples under varying pH conditions was determined in a 0.05 M sodium−phosphate buffer system by removing the insoluble material through centrifugation at 10700 rpm (9088*g*, 10 min, Megafuge 2.0R, Heraeus, Hanau, Germany). Changes in the content of free amino groups according to Adler-Nissen (1972) were analyzed using trinitrobenzenesulfonic acid (TNBS) in a 1% sdoium dodecyl sulfate (SDS) solution of the samples. Tryptophan fluorescence determination (Jackman and Yada, 1989) in 8 M urea using a Jasco fluorescence detector FP 920 (Gross-Umstadt, Germany) was carried out to learn possible changes taking place in the samples after derivatization with the phenolic substances. The sample containing 0.5 mg/mL substance was excited at 295 nm (slit = 18 nm) and emission recorded over the wavelength range of 300−900 nm (slit = 40 nm). The peak area under the emission curve from 300 to 360 nm was used to quantify the tryptophan content. The change in hydrophobic/hydrophilic character of the myoglobin−phenol derivatives was investigated by RP-HPLC. A Jasco chromatographic system with a MICRA-NPS-C18 column (33 × 4.6 mm, 1.5 μm, flow rate = 0.5 mL/min, UV detection at 220 nm) with a column temperature of 25 °C was used. A distilled water/acetonitrile (water acidified with 0.1% trifluoroacetic acid, v/v) gradient was applied under the following elution program: 100% water, 2 min; 0−70% acetonitrile, 10 min; 70% acetonitrile, 4 min; 0−100% water, 4 min; 100% water, 10 min (equilibration). The injection volume of the samples was 5 μL.

SDS−PAGE according to the method of Laemmli (1970) was applied for molecular weight determination. The change in the band intensity was estimated using densitometer scanning software (Bio-Rad, Fluor-S MultiImager, Hercules, CA).

Isoelectric focusing (IEF) of the samples was carried out in a pH range from 3 to 10 (PAGE, T = 12%) as described in Kroll and Rawel (1996). Sodium hydroxide (50 mM) and phosphoric acid (10 mM) were used as cathode and anode buffers, respectively. Pre-electrophoresis was done at 1000 V with 6 mA for 1 h. The main electrofocusing was conducted at 1000 V and 6 mA for 1−2 h. The proteins were fixed in gel with TCA, and Coomassie brilliant blue G 250 (Serva, Heidelberg, Germany) was used to dye the proteins.

MALDI-TOF-MS experiments of the proteins were performed by dissolving 1 mg of the protein sample in 0.7 mL of 0.1% trifluoroacetic acid v/v. Two microliters of this solution was brought on to the target and covered with 1 μL of sinapic acid as matrix. After crystallization of sample by air-drying, measurements were carried out on a Reflex II Bruker MALDI-TOF-MS instrument as described in detail by Rawel et al. (1998a).

Proteolytic Digestion. Tryptic and chymotryptic hydrolysis (100 μL of trypsin, 1 mg/mL) of myoglobin as well as its derivatized products (6 mg/1.5 mL; 2 M urea, 0.1 M Tris-HCl, pH 8, 0.02 M $CaCl_2$) was investigated by incubating enzyme with substrate (E/S = 1:60, w/w) at 35 °C. After different lengths of digestion 300 μL aliquots were removed from the incubation mixture and the reaction was stopped by the addition of 300 μL of 20% TCA. The reaction mixtures were allowed to stand for 10 min and then centrifuged (9088*g*, 10 min, Megafuge 2.0R, Heraeus, Hanau, Germany). RP-HPLC of the TCA-soluble peptides was performed on a MICRA-NPS-C18 column (33 × 4.6 mm, 1.5 μm, flow rate = 0.5 mL/min, UV detection at 220 nm) with a column temperature of 25 °C using a JASCO chromatographic system. A distilled water/acetonitrile (water acidified with 0.1% trifluoroacetic acid v/v) gradient was applied under following elution program: 100% water, 2 min; 0−70% acetonitrile, 10 min; 70% acetonitrile, 4 min; 0−100% water, 4 min; 100% water, 10 min (equilibration). The injection volume of the samples was 10 μL. The total peak area of the peptides was used to quantify the extent of tryptic digestion.

Similarly to the tryptic and chymotryptic reactions, peptic digestion was conducted with denatured protein solutions (6 mg/400 μL), which were prepared in 8 M urea and incubated at 100 °C for 3 min before use. After the addition of 1.15 mL of 0.1 M HCl, the solutions were mixed for 15 min; 50 μL of

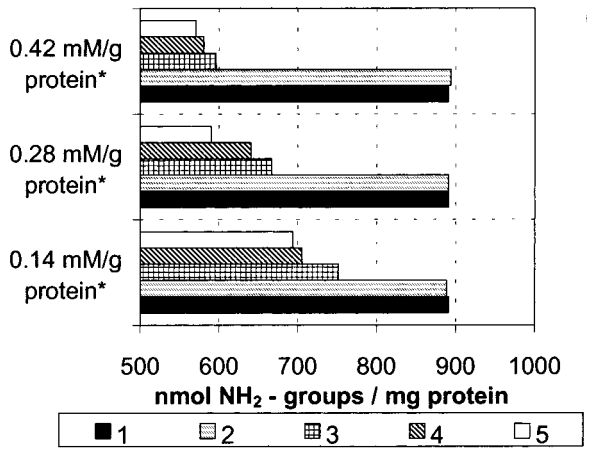

Figure 1. Content of free amino groups of myoglobin derivatives depending on derivatizing conditions: 1, myoglobin, control; 2, quinic acid derivative; 3, chlorogenic acid derivative; 4, caffeic acid derivative; 5, *p*-quinone derivative; *, concentration of the reaction partner during derivatization.

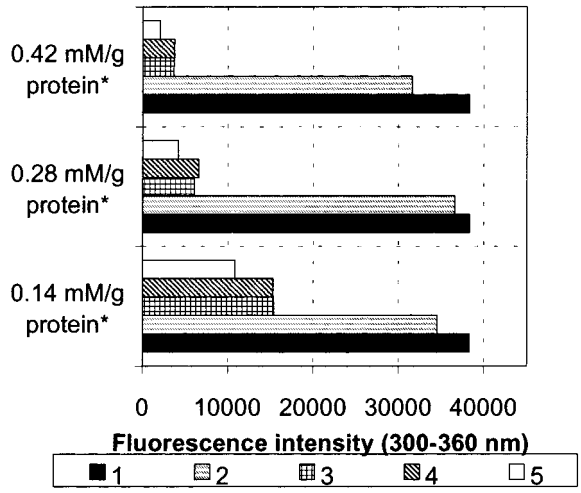

Figure 2. Change in tryptophan fluorescence of myoglobin derivatives depending on derivatizing conditions: 1−5, see Figure 1 caption.

pepsin (1 mg/ mL, E/S = 1:120, w/w) was added to this mixture, and hydrolysis was performed at 35 °C. Preliminary experiments showed that this E/S ratio was optimal with regard to high proteolytic activity of pepsin sample applied. The time course of digestion was monitored by means of RP-HPLC after inactivation of the enzyme with TCA similarly to the procedure described above.

Statistical Analysis. The digestions and other analyses were repeated three times. A maximum of ±5% from the averaged values was generally tolerated and the standard deviation was calculated. The averaged values are documented in the respective figures.

RESULTS AND DISCUSSION

Characterization of Physicochemical Properties. *Reaction at the Free Amino Groups.* Chlorogenic and caffeic acid and *p*-quinone react with the free amino groups of myoglobin. The corresponding decrease in the amount of free amino groups is documented in Figure 1. The reactivity of the phenolic substances and *p*-quinone against myoglobin increased in the order chlorogenic acid, caffeic acid, and *p*-quinone. At 0.42 mM phenol or quinone/g of myoglobin, 22.2, 33.8, and 35.9% of the free amino groups of protein reacted with chlorogenic acid, caffeic acid, and *p*-quinone, respectively. The reaction depended on the concentration of the phenols or quinone, whereby an increased degree of derivatization resulted from the higher concentration of chlorogenic acid, caffeic acid, and *p*-quinone applied. Quinic acid as a simple cyclic compound did not react with the free amino groups.

Changes in Tryptophan Fluorescence. A possibility of reaction of oxidized phenolic compound at the heterocyclic N-atom of tryptophan has also been discussed by Macholz and Lewerenz (1989). Therefore, we investigated the change of the tryptophan fluorescence of myoglobin depending on the reactions with different concentrations of chlorogenic acid, caffeic acid, and *p*-quinone. The control myoglobin and its derivatives were dissolved in 8 M urea to provide complete solubilization and denaturation in solution and gave an emission maximum between 344 and 356 nm. A decrease in the relative fluorescence intensity of the derivatives with increasing amounts of reacting quinic, chlorogenic, and caffeic acid and *p*-quinone was generally observed; the latter depended on the concentration of the reaction partner applied (Figure 2). The differences in fluorescence intensity of quinic acid derivatives and controls were comparatively smaller than those of the other derivatives. The following decreases of fluorescence intensity were observed for the highest concentration (0.42 mM) of the derivatizing substance applied: 17.5, 90.4, 90.2, and 94.6% for quinic, chlorogenic, and caffeic acid and *p*-quinone, respectively. These results document further that the indole structure of tryptophan in the derivatives is most likely to be involved in the reaction of myoglobin with the phenolic substances. The reaction of quinic acid with tryptophan side chains in myoglobin, although to a small extent, also took place. To our knowledge, such reactions of quinic acid have not been cited in the literature.

Changes in Solubility. Because the reaction of myoglobin with the derivatizing substances is accompanied by a corresponding blocking of the hydrophilic amino groups, a parallel change in solubility should be expected. In general, a decrease in the solubility of the derivatives over a broad range of pH was noted, whereby the isoelectric point was shifted to an acidic range. The solubilities of myoglobin derivatives with the different reaction partners at a derivatizing concentration of 0.28 mM/g of protein are illustrated in Figure 3. This change in solubility was also dependent on the concentration of the phenolic substance and quinone applied (Supporting Information, pages 1 and 2). Quinic acid only slightly affected the solubility of myoglobin. The reaction of myoglobin with chlorogenic and caffeic acid leads to a much stronger decrease in solubility in the pH range 3.5−6, especially emphasizing the distinct shift in p*I* of native myoglobin to the pH range 4.5−5. In this context, even the solubility of the *p*-quinone derivative was similarly affected, but this effect was comparatively smaller than that of chlorogenic and caffeic acid, which is due to the changed number of charged groups present in the individual derivatives. The carboxyl groups of the chlorogenic and caffeic acid may play an important role as potentially negatively charged groups decreasing the solubilities of their corresponding derivatized myoglobin samples.

Changes in Hydrophilic/Hydrophobic Character. Another molecular property of myoglobin that can be affected by the reaction with chlorogenic and caffeic acid

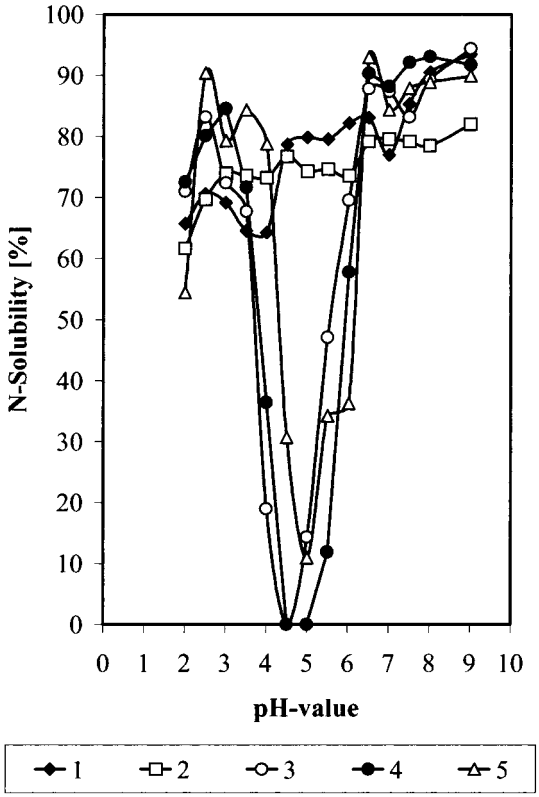

Figure 3. Solubility profiles of myoglobin derivatives depending on reaction partners: 1−5, see Figure 1 caption; concentration of the reaction partner during derivatization was 0.28 mM/g of protein.

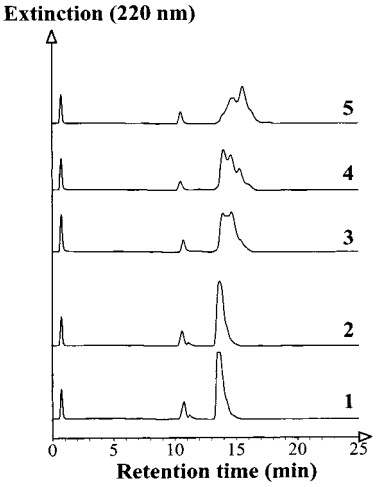

Figure 4. RP-HPLC of myoglobin derivatives depending on reaction partners: 1−5, see Figure 1 caption; concentration of the reaction partner during derivatization was 0.28 mM/g of protein.

and *p*-quinone is its hydrophilic−hydrophobic character, which can be illustrated by RP-HPLC (Figure 4). The increase in the hydrophobicity of the phenol-derivatized myoglobin is documented in a rise of the retention times of the main peak. The peak formation seemed to be adversely affected, indicating possible denaturation and molecular interactions (Figure 4). With regard to the reaction of quinic acid, no change in chromatogram pattern was noted. In agreement with these results, an increase in the hydrophobicity of the myoglobin derivatives was also produced with increasing concentration of chlorogenic and caffeic acid and *p*-quinone applied (Supporting Information, pages 3 and 4). This confirms

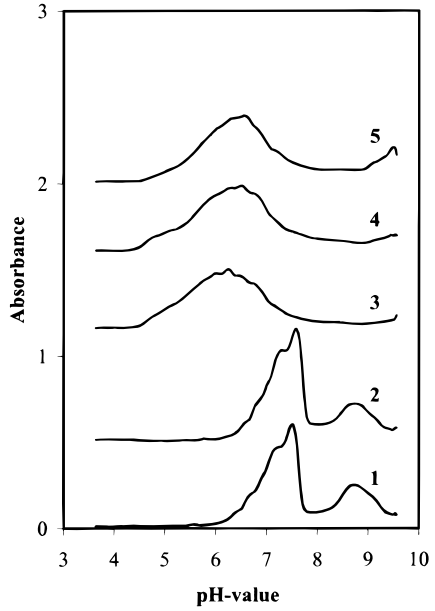

Figure 5. IEF of myoglobin derivatives depending on reaction partners: 1−5, see Figure 1 caption; concentration of the reaction partner during derivatization was 0.28 mM/g of protein.

the results obtained for the solubility profiles (Figure 3; Supporting Information, pages 1 and 2). According to Hayakawa et al. (1985) the decrease in solubility correlates generally with an increase in surface hydrophobicity, as measured by a hydrophobic fluorescence probe, 8-anilinonaphthalene-1-sulfonate (ANS). However, in the case of myoglobin derivatives, our efforts to measure the surface hydrophobicity (results not shown) did not reveal any distinct trend. This may be due to structural changes taking place in the derivatized myoglobin, whereby an uneven distribution of hydrophobic groups on the surface of and inside the molecule takes place. Obviously the ANS method is not suitable for such characterization of hydrophobic changes in protein derivatives.

Isoelectric Focusing. The derivatization changed the amount of charged groups as documented exemplary for free amino groups (Figure 1). Generally, a loss of charged groups due to nucleophilic addition of the oxidized phenolic moiety, for example, to the ϵ-amino groups of lysine, is accompanied with a change of the isoelectric point of the proteins. The experimental and calculated isoelectric points of myoglobin (horse muscle) lie at pH 7.33 and 7.45, respectively, at 25 °C (Patrickios and Yamasaki 1995). As shown in Figure 5, these correspond to the peaks in the unmodified control sample with p*I* values between 7 and 7.5. A subsequent reaction of 0.28 mM of chlorogenic and caffeic acid and *p*-quinone with 1 g of myoglobin shifts the isoelectric range from p*I* 7.0−7.5 in the control to p*I* 4.5−7 for the majority of the reaction products (Figure 5). A shift of the isoelectric range to a lower pH was also dependent on the concentration of caffeic and chlorogenic acid and *p*-quinone applied (Supporting Information, pages 5 and 6), reflecting the trend obtained for the determination of the solubility behavior as already documented above. Quinic acid does not react with myoglobin as confirmed by the unchanged electrophoretic pattern with regard to that of the control myoglobin (Figure 5).

SDS−PAGE Analysis. SDS−PAGE investigations of the control myoglobin showed the main fraction with a

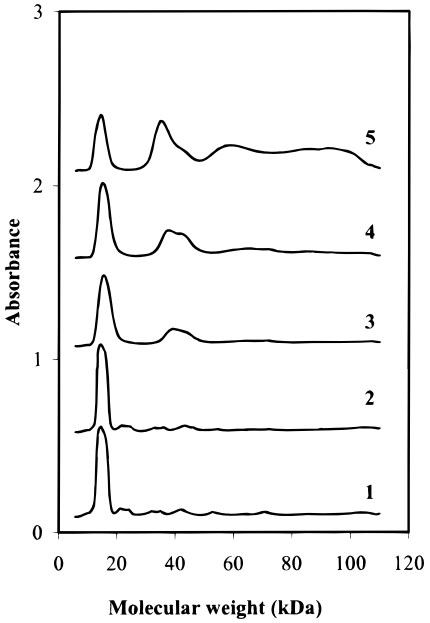

Figure 6. SDS−PAGE of myoglobin derivatives depending on reaction partners: 1−5, see Figure 1 caption; concentration of the reaction partner during derivatization was 0.28 mM/g of protein.

relative protein concentration of 96% (14−16 kDa, Figure 6, curve 1). Because the control protein was also held at pH 9 for 24 h during the derivatization, a dimer (40 kDa, relative concentration of the dimer = 4%) was likely produced as a result of possible isopeptide binding. Reaction with quinic acid led to no distinct changes in the electropherogram pattern as documented in Figure 6, curve 2. On the other hand, the dimer fraction content increased markedly in the order of chlorogenic and caffeic acid and *p*-quinone (Figure 6). This formation of high molecular weight fractions was also influenced by the concentration of chlorogenic and caffeic acid and *p*-quinone applied (Supporting Information, pages 7 and 8). Moreover, especially for *p*-quinone, subfractions with even higher molecular weights, particularly those around 55−65 and 85−95 kDa, were also observed (Figure 6). In summary, the chlorogenic acid derivative at a reacting concentration of 0.28 mM/g of protein contained 18.3%, the caffeic acid derivative, 31.2%, and the *p*-quinone derivative, 61% of the high molecular fractions with corresponding molecular weights of about 40, 55−65, and 85−95 kDa, respectively. In the case of *p*-quinone derivatives the content of these high molecular weight fractions increased from 51.3% at 0.14 mM/g of protein to 61% at 0.28 mM/g of protein, finally reaching 67.8% at 0.42 mM/g of protein (Figure 6; Supporting Information, pages 7 and 8). There was also a distinct shift of the distribution of the high molecular weight fractions toward the higher 85−95 kDa range, increasing from 8.1 to 15 and 24.2% at the three concentrations tested. Hurell and Finot (1984), Pierpoint (1969a,b), and Macholz and Lewerenz (1989) also reported on the polymerization of protein molecules as a possible subsequent reaction of different proteins with phenolic substances. For the first time, the experimental proof of such polymerization using SDS−PAGE can be given for the myoglobin−phenol derivatives. The percentage of increases in amounts of high molecular weight fractions correlated well ($R^2 = 0.975$) with the decrease in the amount of free amino groups at the different concentrations of *p*-quinone that reacted with

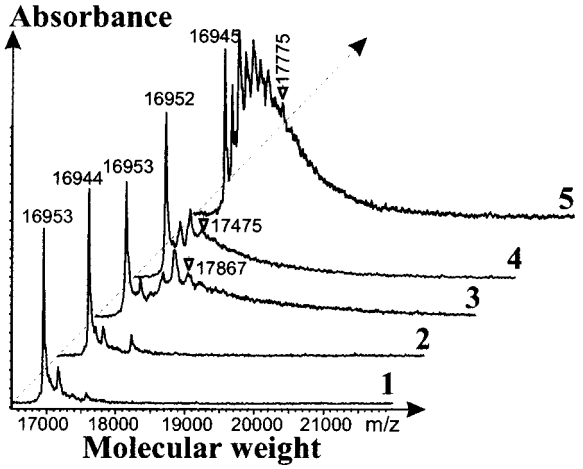

Figure 7. MALDI-TOF-MS of myoglobin derivatives depending on reaction partners: 1−5, see Figure 1 caption; concentration of the reaction partner during derivatization was 0.28 mM/g of protein.

myoglobin. A similarly strong relationship ($R^2 = 0.998$) was found between the percentage of increases in the amounts of high molecular weight fractions and the decrease in the fluorescence of tryptophan.

MALDI-TOF-MS. This method of analysis was applied to evaluate detailed changes in molecular weights in derivatized monomer myoglobin molecules. The MALDI mass spectra of the samples obtained from the reaction of 0.28 mM quinic, chlorogenic, and caffeic acid as well as *p*-quinone per gram of protein are presented in Figure 7. The molecular weight of myoglobin determined with this method was 16953 Da, agreeing with data calculated from its sequence (GenBank Protein sequences, Accession No. 2506462). Generally, an increase in molecular weight was also observed with increasing amount of the reacting substance present during the derivatization process (Supporting Information, pages 9 and 10). Quinic acid was again an exception as it did not cause any relevant changes in mass spectra (Figure 7; Supporting Information, pages 9 and 10). Generally the mass spectra show peaks, which are separated by an increment of the molecular weight of the reacting molecules (chlorogenic and caffeic acid, *p*-quinone). The reaction of myoglobin with chlorogenic acid (352.3 Da) at a concentration of 0.28 mM/g of protein delivered the highest molecular mass of 17867 Da, which in turn could account for the addition of at least two to three molecules of chlorogenoquinone to one myoglobin molecule (Figure 7). Similarly for caffeic acid (180.2 Da), a mass of 17475 Da could be documented, accounting for a possible addition of three caffeioquinone molecules. In the case of *p*-quinone (108.1 Da), the following high molecular weight substances were determined: 17561, 17775, and 18117 Da at 0.14, 0.28, and 0.42 mM quinone/g of protein, respectively. This accounts for a corresponding addition of 6, 8, and 11 molecules of *p*-quinone in each case (Figure 7; Supporting Information, pages 9 and 10). These findings underscore the highest reactivity for *p*-quinone compared to the other tested substances. Because the method does not allow identification of the position of the reacting site, a peak of a specific molecular mass certainly represents several isomers. Further experiments coupled with enzymatic hydrolysis are necessary for identification of these reaction sites. On the other hand, these results of MALDI-MS allow the documentation of the first primary addition of a reacting substance, for

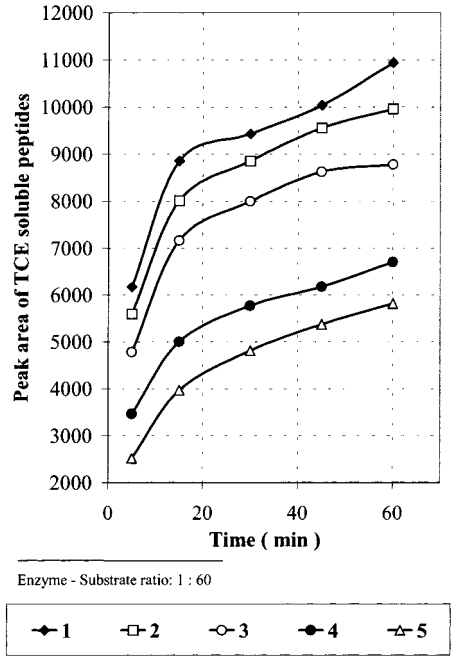

Figure 8. Tryptic digestion of myoglobin derivatives depending on reaction partners: 1−5, see Figure 1 caption; concentration of the reaction partner during derivatization was 0.28 mM/ of protein.

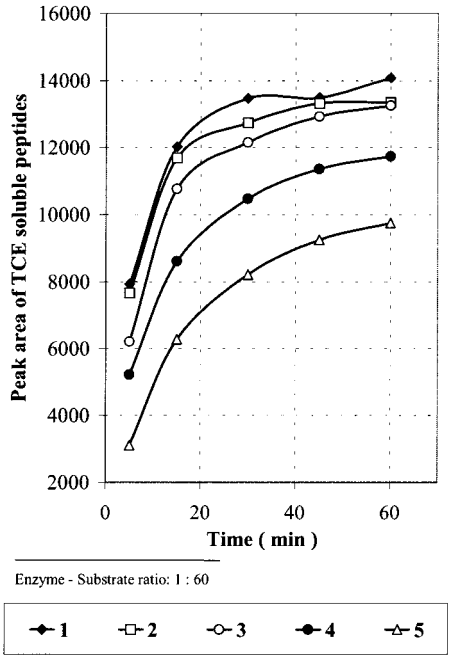

Figure 9. Chymotryptic digestion of myoglobin derivatives depending on reaction partners: 1−5, see Figure 1 caption; concentration of the reaction partner during derivatization was 0.28 mM/g of protein.

example, to ϵ-amino groups of lysine side chains of myoglobin. The reaction products thus formed may react further with other myoglobin molecules, resulting in polymerized products as identified by SDS−PAGE.

Effect of the Derivatization on the Proteolytic Hydrolysis. Principally our results showed that the myoglobin derivatization with phenols led to a corresponding detrimental effect on the in-vitro proteolytic digestion by the three enzymes (trypsin, α-chymotrypsin, and pepsin) of the gastrointestinal tract.

The results of tryptic digestion of myoglobin derivatives at a reacting concentration of 0.28 mM/g of protein are shown in Figure 8. In comparison to the control unmodified myoglobin, all four substances tested influence the tryptic hydrolysis negatively, whereby the effect increases in the following sequence: quinic acid < chlorogenic acid < caffeic acid < *p*-quinone. This represents and reflects the trend generally observed by the physicochemical characterization. Depending on the concentration of, for example, *p*-quinone applied (0.14, 0.28, and 0.42 mM/g of protein), there was also a corresponding lower amount of TCA-soluble peptides (70.3, 53.2, and 34.5% less than during hydrolysis of unmodified myoglobin) liberated after 1 h of tryptic digestion (Figure 8; Supporting Information, pages 11 and 12). Trypsin splits preferentially those peptide linkages that contain either lysine or arginine as amino side chains (Bond, 1989). Because it has already been shown that phenolic substances react with ϵ-amino groups of lysine side chains (Figure 1), we can conclude that their derivatization by phenolic substances prevents or at least makes difficult tryptic degradation. A high correlation ($R^2 = 0.94$, $y = -0.1835x + 102.12$) between the amount of free amino groups blocked (nanomoles per milligram of protein, *x*-axis) and the amount of the peptides liberated (percent of the control sample, *y*-axis) during the tryptic hydrolysis of myoglobin derivatives with different concentrations of *p*-quinone applied was obtained. The resulting polymerization products (SDS−PAGE analysis, Figure 6), with conformational changes in their structure, may also prevent access of trypsin to altered peptide linkages.

Effects of myoglobin complexes with phenolic substances on the chymotryptic hydrolysis are shown in Figure 9. Generally, the chymotryptic digestion was not affected by derivatization in the presence of quinic acid and only slightly affected by the presence of chlorogenic acid, and it did not depend on the concentration of acid (Figure 9; Supporting Information, pages 13 and 14). The chymotryptic digestion of caffeic acid and *p*-quinone myoglobin derivatives declined with increasing concentration of the myoglobin modifying agent (Figure 9; Supporting Information, pages 13 and 14). Chymotrypsin is known to have primary specificity for those peptide bonds that contain aromatic amino acid residues such as tryptophan, tyrosine, and phenylalanine (Bond, 1989). As shown in Figure 2, the tryptophan fluorescence decreased with the reactivity of the substance tested. This corresponds to the results obtained for the chymotryptic digestion of caffeic acid and *p*-quinone derivatives, but not with those for chlorogenic acid derivatives. It may be explained by the comparatively small amount of polymerized components found in the chlorogenic acid derivatives of myoglobin (Figure 6).

Pepsin is a nonspecific protease but prefers hydrolysis of peptic bonds near aromatic and other hydrophobic amino acid residues, especially phenylalanine and leucine (Bond, 1989). Even in this case, there is a measurable influence of the protein derivatization by phenols on the peptic hydrolysis of myoglobin, depending both on the reactivity and on the concentration of the substance tested (Figure 10; Supporting Information, pages 15 and 16). Furthermore, the influence of derivatization on peptic hydrolysis of myoglobin−phenol derivatives seems to be more profound and distinct than that observed for chymotryptic hydrolysis (Figure 9). As in the case of tryptic digestion, the effect of derivatization on peptic proteolysis is in the same sequence:

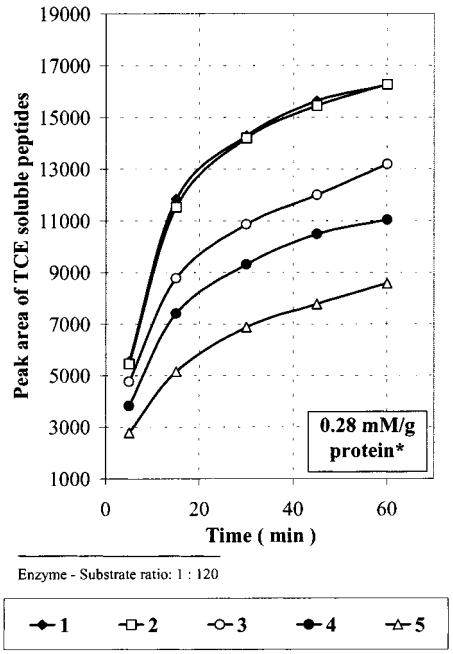

Figure 10. Peptic digestion of myoglobin derivatives depending on reaction partners: 1−5, see Figure 1 caption; concentration of the reaction partner during derivatization was 0.28 mM/g of protein.

chlorogenic acid < caffeic acid < *p*-quinone. Quinic acid did not influence the peptic hydrolysis. Inhibitory effects of the derivatized products on peptic digestion may be attributed in this case to their resulting conformation and structural changes.

In consideration of all the results presented with regard to the influence of myoglobin derivatization by phenols on the resulting changes in physicochemical properties and susceptibility to proteolytic hydrolysis, the following reaction possibilities can be explained and discussed. The reactions of amino acids, peptides, and proteins with *o*-quinones produced by enzymatic oxidation (e.g., by polyphenol oxidase) of chlorogenic and caffeic acid have been discussed by Hurell and Finot (1984) as well as by Pierpoint (1969a,b). The 2-position of the benzene ring in caffeic acid is the most electrophilic, and nucleophilic addition occurs preferentially here (Cheynier et al., 1986). Upon further oxidation of this addition product by laccase to form its quinone, a second addition occurs at the benzene ring in caffeic acid (Cilliers and Singleton, 1991). As discussed by Macholz and Lewerenz (1989), this advanced addition reaction between free lysine side chains and quinone results in polymerization of protein molecules, leading to formation of complex products.

The rate of oxidation of caffeic acid is very pH dependent in a nonenzymic autoxidative reaction system (Cilliers and Singleton, 1991). The effect of phenolate ion concentration on the first rate constant of caffeic acid oxidation at different temperatures indicated the involvement of phenolate ions in this oxidation. After phenolate ion formation takes place, reaction with oxygen produces a semiquinone, which will then undergo further reaction (Cilliers and Singleton, 1991). The semiquinones can couple to form different structural isomers or can undergo further addition reactions with nucleophilic side chains of proteins as described above. According to Cilliers and Singleton (1991) five semiquinones with different electrophilic reaction sites are possible. As a result, different mechanisms for the formation of reaction products with proteins can also be postulated. In the present paper, the reaction products thus formed (protein derivatives) have been characterized for the first time with regard to detailed molecular and structural changes. Another aspect reported in this paper is the experimental proof of the adverse affect on the susceptibility to proteolytic digestion of the myoglobin−phenol derivatives.

In conclusion, the results show that plant phenolic substances (in this case chlorogenic and caffeic acid) and *p*-quinone react with proteins, influencing their physicochemical properties and in-vitro enzymatic degradation. Furthermore, a clear trend was observed with regard to the reactivity of the substances tested. Quinic acid (a nonaromatic substance) only slightly reacts with protein. Stronger reactivity was observed with chlorogenic and caffeic acid and *p*-quinone. Changes in concentrations of phenolic compounds that modified the myoglobin structure were related to the physicochemical properties of the derivatives and to their susceptibility to proteolysis. These results seem to be important with regard to further experiments planned, which involve the physiological and toxicological effects of proteins derivatized with phenolic compounds.

ABBREVIATIONS USED

BAEE, N_α-benzoyl-L-arg ethyl ester; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TNBS, trinitrobenzenesulfonic acid.

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Supporting Information Available: Solubility profiles, RP-HPLC results, IEF results, SDS−PAGE results, MALDI-TOF-MS results; results of tryptic digestion; chymotryptic hydrolysis; and peptic digestion, in each case for 0.14 and 0.42 mM phenolic substance/g of protein.

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