Reactions of Chlorogenic Acid with Lysozyme: Physicochemical Characterization and Proteolytic Digestion of the Derivatives

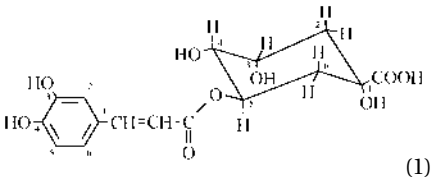
H.M. RAWEL, J. KROLL, AND B. RIESE

ABSTRACT: The lysozyme-chlorogenic acid-derivatives formed at different pH were characterized in terms of their physicochemical and digestion properties, whereby pH 10 led to the highest derivatization. The results showed reduction of lysine residues and a distinctive decrease of the tryptophan fluorescence with increasing lysozyme derivatization. The solubility decreased over a broad pH range with a parallel increase in hydrophobicity of the derivatives. The isoelectric points were shifted to lower pH values and high molecular fractions were formed. The influence of the protein derivatization on the in vitro digestibility was also demonstrated. The peptic digestion of the derivatized lysozyme was adversely affected, whereas the tryptic and chymotryptic hydrolysis seemed to be favored.

Key Words: Plant phenolic substances, chlorogenic acid, lysozyme, food protein derivatization, physicochemical characterization, in-vitro proteolytic degradation

Introduction

DIETARY PHENOLIC SUBSTANCES HAVE received much attention in recent years due to their biological activity. They have been shown to possess antimutagenic, anticancerogenic, antiglycemic, and antioxidative beneficial properties (Ho and others 1992; Maleveille and others 1996; Friedman 1997; Chung and others 1998). Some of these effects have also been reported for caffeic and chlorogenic acids as important plant phenolic substances by Kitts and others (1994) and Kono and others (1995). The oxidation products of phenolic compounds appear to be involved in the defense of plants against invading phytophatogens, including bacteria, fungi, and viruses (Friedman 1997). Polyphenols on the other hand can also display many possible detrimental effects, including inhibition of iron absorption (Mehansho and others 1987) and irreversible complexation of gut enzymes and dietary proteins (Mehansho and others 1987; Robbins and others 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 up to 1 g/day (Maleveille and others 1996) and that of chlorogenic acids (CA) and other cinnamates ranges from 25 mg up 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). The content of chlorogenic acid is relatively high with 6% to 10 % in green coffee beans, and a cup of coffee may contain up to 675 mg (Clifford 1999). Potatoes also contain relative high amounts of chlorogenic acid, which may range from 9.6 to 18.7 mg/100g fresh weight depending on its variety (Friedman 1997). Chlorogenic acid consists of caffeic and quinic acids (ester bonding); depending on the hydroxyl group of quinic acid involved, 4 isomers are possible. The most frequent form found in plants is the 3-O-(3,4-dihydroxycinnamic acid) with the following structure:



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The presence of phenolic compounds in foods and beverages produces a sensation of astringency, possibly arising from precipitation of oral proteins and mucopolysaccharides. They do not only precipitate oral proteins, but may also interact with dietary proteins and digestive enzymes in the gut, resulting in a variety of antinutritive and toxic effects (Baxter and others 1997). There are 4 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 stabilizing of the complexes formed, whereby phenolic compounds have a significant affinity for proteins that contain a high proportion of proline residues in their sequences (Muralidhara and Prakash 1995; Siebert and others 1996; Baxter and others 1997; Siebert 1999).

Chlorogenic acid may be oxidized with ease in the presence of a polyphenol oxidase or in an alkaline solution to its corresponding quinone (Hurrell and Finot 1984). The quinones represent a species of highly reactive substances which normally react further with other quinones to produce dark pigments. These result in deterioration of flavor, color, and nutritional quality of foods. The quinones 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 (1969 a,b) and Hurrell and others (1982). The possible reaction steps that may take place or the possible reaction products formed with lysine, cysteine, tryptophan, and methionine residues have been reviewed by Machholz and Lewerenz (1989). In these reports, no discrete isolation of derivatized proteins with regard to detailed changes in physicochemical properties is mentioned and the in-vitro digestion was not characterized.

The presented work is a continuation of our attempt to characterize the reac-

tions of food proteins with secondary plant metabolites from the physicochemical and physiological viewpoint. In these investigations, reactions of isothiocyanates (breakdown products of glucosinolates) with lysozyme and other food proteins were shown (Kroll and Rawel 1996; Hernandez-Triana and others 1996; Rawel and others 1998a,b,c). Hereby, it was observed that lysozyme serves well for model investigations, since its structure is well defined and characterized.

In the following study an attempt was made to show the effect of reactions of lysozyme with different amounts of chlorogenic acid at different pH conditions in a model system. The lysozyme derivatives with chlorogenic acid (with special attention to the role of covalent bonding) have been characterized in terms of changes in their physicochemical properties and with regard to influence on their digestion with the three physiologically main proteolytic enzymes (trypsin, a-chymotrypsin, and pepsin) of the gastrointestinal tract.

Materials and Methods

Materials

Lysozyme (0.3 g) from hen egg (Fluka chemie AG, Buchs, Switzerland) was dissolved in distilled water and the pH-value of the protein solution was adjusted to the required value (4, 7, or 10) using 0.5 M NaOH or 0.5 M HCl (final volume 27 mL). Under continuous stirring at room temperature chlorogenic acid (3-O-[3,4-dihydroxycinnamate, Fluka chemie AG, Buchs, Switzerland), dissolved in 10 mL ethanol, was added (in 2 different concentrations: 50 and 150 mg/g protein equivalent to 0.14 mM and 0.42 mM CA/g protein, respectively) and the pH adjusted once more to the required value (4, 7, or 10). After 24 h reaction time under continuous stirring at room temperature (24 °C) with free exposure to air, the samples were dialyzed for 18 to 20 h and finally lyophilized. The nonderivatized protein (control) was prepared under the sameconditions but without addition of chlorogenic acid.

Trypsin from porcine pancreas (EC 3.4.21.4, protein content 98%, SIGMA Chemicals Co., St. Louis, Mo., U.S.A.) – 14,900 U/mg solid, one N_a-benzoyl-L-arginine ethyl ester (BAEE) unit $\Delta = A_{253}$ of 0.001 per min with BAEE as substrate at pH 7.6 at 25 °C. δ-Chymotrypsin from bovine pancreas (EC 3.4.21.1, protein content 96%, Fluka Chemie AG, Buchs, Switzerland) – 53.1 U/mg, one unit will hydrolyze 1 μmol suc-$(ala)_2$-pro-phe-4-nitroanilide per min at pH 7.8 and 25 °C.Pepsin from porcine stomach mucosa (EC 3.4.23.1, protein content approximately 92%, SIGMA Chemicals Co., St. Louis, MO) – 3100 U/mg solid, one unit will produce a ΔA_{280} of 0.001 per min at pH 2 at 37 °C, measured as TCA-soluble products using hemoglobin as substrate. All specific activities and definitions quoted here were given by the producers.

Physicochemical characterization. The protein content in the solutions was determined according to a modified LOWRY method (Lowry and others 1951). Lysozyme was used to calibrate the regression curve ($Y = 0.1269 + 0.008195 \cdot X$, $R^2 = 0.995$) after determining its protein content by semi-micro Kjeldahl analysis (Kjeldatherm System KT 40, Gerhardt Laboratory instruments, Bonn, Germany). The solubility profile of the lyophilized samples under varying pH conditions was determined in a 0.05 M Na-phosphate/citric acid buffer system by removing the insoluble material through centrifugation at 9088 × g, 10 min, (Megafuge 2.0R, Heraeus, Hanau, Germany). Changes in the content of free amino groups according to Adler-Nissen (1972) were analysed using trinitrobenzenesulfonic acid (TNBS) in a 1% Na-dodecylsulfate (SDS) solution of the samples. Tryptophan fluorescence determination (Jackman and Yada 1989) in 8 M urea using Jasco fluorescence detector FP 920 (Gross-Umstadt, Germany; Tokyo, Japan) was carried out to give some idea of 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 to 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 was investigated by RP-HPLC, which was conducted with a JASCO (Gross-Umstadt, Germany; Tokyo, Japan) chromatographic system using a MICRA-

NPS-C18 column (33 mm × 4.6 mm, 1.5 μm, flow rate 0.5 mL/min, UV detection at 220 nm) with a column temperature of 25 °C. A distilled water/acetonitrile (water acidified with 0.1 % trifluoroacetic acid v/v) gradient was applied under the following conditions: 100% water, 2 min; 0% to 70% acetonitrile, 10 min; 70% acetonitrile, 4 min; 0% to 100% water, 4 min; 100% water, 10 min (regeneration/equilibration). The injection volume of the samples was 10 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, Calif., U.S.A.).

Isoelectric focusing (IEF) of the samples was carried out in a pH range from 3 to 10 (PAGE, total acrylamide concentration T = 10% to 12%) as described in Kroll and Rawel (1996) with the sample application on cationic side. Sodium hydroxide and phosphoric acid were used as cathode and anode buffers, respectively. Pre-electrophoresis was done at 1000 V with 6 mA for 1 h. The main electro-focusing was conducted at 1000 V and 6 mA for 45 min. The proteins were fixed in gel with trichloroacetic acid 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 0.1 % trifluoroacetic acid v/v. Two μL of this solution were brought on to the target and covered with 1 μL sinapic acid as matrix. After crystallization of the sample by air-drying, measurements were carried out on Reflex ™II Bruker MALDI-TOF-MS instrumentation as described in detail in Rawel and others (1998a).

Proteolytic digestion. Tryptic andchymotryptic hydrolysis (100 μL trypsin, 1 mg/mL) of lysozyme 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 at 35 °C. After different lengths of digestion (E:S = 1:60, where E:S is enzyme:substrate ratio), 300 μL were removed from the incubation mixture and the reaction was stopped by addition of 300 μL trichloroacetic acid (20% TCA). The reaction mixtures were allowed to stand for 10 min and then centrifuged at 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 mm × 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 (Labor und Datentechnik GmbH, Gross-Umstadt, Germany; Tokyo, Japan) chromatographic system. A distilled water/acetonitrile (water acidified with 0.1 % trifluoroacetic acid v/v) gradient was applied under the following conditions: 100% water, 2 min; 0% to 70% acetonitrile, 10 min; 70% acetonitrile, 4 min; 0% to 100% water, 4 min; 100% water, 10 min (regeneration/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. Analogue to the tryptic and chymotryptic analysis, peptic digestion was conducted with 400 ìl protein solutions (15 mg/mL) prepared in 8 M urea by incubating at 100 °C for 3 min. This step was necessary since lysozyme is known to have a compact globular structure and preliminary experiments showed that lysozyme was hardly digested even after 24 h without this denaturation. After addition of

1.15 mL of 0.1 M HCl, the solutions were mixed for 15 min. At this stage the final urea concentration was reduced to 2 M. Fifty μL pepsin (1 mg/ mL, enzyme/substrate ratio (E:S) = 1:120) were added to this mixture and the hydrolysis was performed at 35 °C. The time dependent digestion was monitored by means of RP-HPLC after inactivation of the enzyme with trichloroacetic acid analogue to the procedure described above.

Statistical analysis. The digestions and other analysis were repeated thrice and evaluated by standard deviation. Student t test and ANOVA/POST-HOC-test were performed for the results shown in Fig. 1 and 2. In both cases ANOVA significance of 0.00 was calculated. The level of replication for results documented in Fig. 1 to 3 and 8 to 10 showed a maximum deviation of 7% of the mean values. A maximum of ± 5% standard deviation from the averaged values was generally tolerated. The averaged values are documented in the respective figures.

General remarks

The nutritional value of food proteins is governed by amino acid composition, ratios of essential amino acids, susceptibility to hydrolysis during digestion, source, and the effects of processing. To optimize the biological utilization of proteins, especially of low-quality proteins in underdeveloped countries, a better understanding is needed of the various interrelated parameters that influence their nutritional value. Enzyme-catalyzed browning reactions of amino acid and proteins with oxidized plant phenols may cause deterioration of food during storage and processing leading to a loss in nutritional quality, which is especially serious in underprivileged countries (Friedman 1997). Some complex polyphenols, such as tannins, are present in many cereal foods, for example grain sorghum, the latter being widely used in many African countries. Further, alkaline processing of proteins may also be applied in technological treatments of foods and feeds by solubilization and purification, to destroy toxic contaminants, to obtain functional properties, including the formation of textured vegetable protein fibers (Provansal and others 1975). As already mentioned, dietary intake of chlorogenic acids and other cinnamates ranges from 25 mg up to 1 g/day, depending upon the dietary constitution (Clifford 1999). The content of chlorogenic acid is relatively high with 6% to 10% in green coffee beans, and a cup of coffee may contain up to 675 mg (Clifford 1999). Potatoes also contain relative high amounts of chlorogenic acid, which may range from 9.6 to 18.7 mg/100 g of fresh weight, depending on its variety (Friedman 1997). We applied 2 concentrations of chlorogenic acid (50 and 150 mg chlorogenic acid) in our model investigations. Depending on dietary composition, such intake could be awaited. The results of these preliminary model experiments document whether a reaction of chlorogenic acids with protein has occurred, which types of products are formed and finally, and which characteristic changes have taken place in the derivatives. These preliminary results should help to evaluate consequent nutritional and toxicological effects due to intake of such products, the latter describing our future objectives.

Characterization of physicochemical properties

Reaction at the free amino groups. The theoretical value for the amount of free

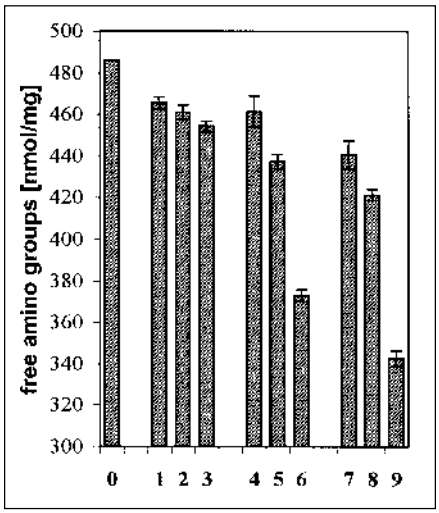

Fig. 1—Content of free amino groups of lysozyme derivatives depending on derivatizing conditions. Code: 0 = theoretical value; 1 to 3 = lysozyme-control without addition of CA at pH 4, 7, 10; 4 to 6 = derivatization at pH 4, 7,and 10 with 50 mg CA/g protein; 7 to 9 = derivatization at pH 4, 7, and 10 with 150 mg CA/g protein (statistical analysis - t test for derivatives 4 to 9 in comparison to their corresponding unmodified controls were 0.45; 0.00; 0.00; 0.01; 0.00 and 0.00 respectively; ANOVA - post-hoc-tests gave for the derivative 4 a value of 0.26; all other derivatives gave a high significance of 0.00).

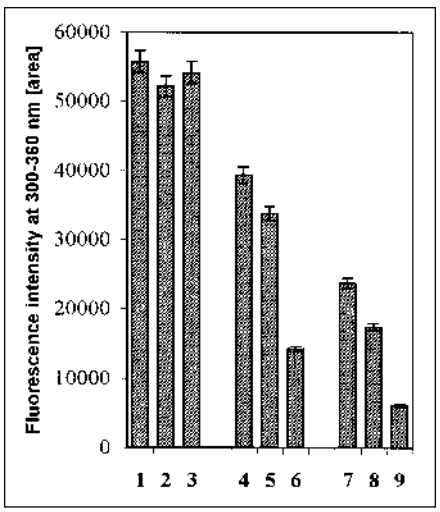

Fig. 2—Change in tryptophan fluorescence of lysozyme derivatives depending on derivatizing conditions. Code: 1 to 3 = lysozyme-control without addition of CA at pH 4, 7, 10; 4 to 6 = derivatization at pH 4, 7,and 10 with 50 mg CA/g protein; 7 to 9 = derivatization at pH 4, 7, and 10 with 150 mg CA/g protein (statistical analysis - t test and ANOVA - post-hoc-tests for derivatives 4 to 9 in comparison to their corresponding unmodified controls were of a high significance of 0.00).

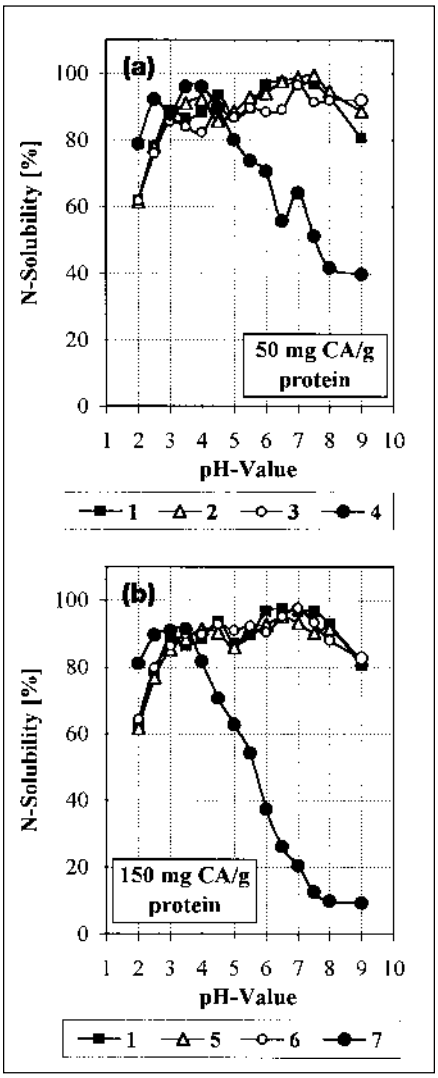

Fig. 3—Solubility profiles of lysozyme derivatives depending on derivatizing conditions. A - Code: 1 = lysozyme-control without addition of CA; 2 to 4 = derivatization at pH 4, 7, and 10 with 50 mg CA/g protein; B - Code: 1 = lysozyme-control without addition of CA; 5 to 7 = derivatization at pH 4, 7, and 10 with 150 mg CA/g protein.

amino groups in lysozyme amounts to 486 nmol/mg protein (calculation based on molecular weight of 14400 Da, 1 mmol lysozyme represents accordingly 14400 mg protein). The experimentally determined values were slightly less ranging from 455 to 465 nmol/mg protein. As a consequence of the reaction of chlorogenic acid with lysozyme, a corresponding decrease in the amount of free amino groups as determined using trinitrobenzenesulfonic acid (TNBS) reagent can be documented (Fig. 1). The reactivity increased parallel to the increasing pH value of the reaction medium. For example, at a reacting concentration of 150 mg chlorogenic acid/g protein, 5.4%, 8.7% and 24.7% of the free amino groups reacted at the corresponding pH values of 4, 7, and 10. The decrease in the amount of free amino groups is also dependent on the concentration of the chlorogenic acid applied as shown in Fig. 1. As cited by Hurell and Finot (1982), the reactivity may also be influenced by the extent of oxidation of the chlorogenic acid to the corresponding chlorogenoquinone. As known phenolic substances may be readily oxidized in alkaline solutions or in the presence of polyphenol oxidase to respective quinones, which in turn can react with free amino groups of proteins. Such reactions have been discussed by Hurell and Finot (1984) as well as by Pierpoint (1969 a,b). As discussed by Macholz and Lewerenz (1989), the advanced reaction between free lysine side chains and quinone may result in polymerization of protein molecules, leading to formation of complex products.

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 lysozyme depending on the reactions with different concentrations of chlorogenic acid at different pH conditions. The quenching of the fluorescence intensity as an indicator of tryptophan changes was studied after activation at 295 nm and measurement of the emission between 300 to 360 nm (area under the curve illustrated as columns, Fig. 2). The control lysozyme and its derivatives were dissolved in 8 M urea to provide complete solubilization and denaturation in solution and gave an emission maximum between 346 to 348 nm. A decrease in the relative fluorescence intensity of the derivatives with increasing amounts of reacting chlorogenic acid as well as with increasing pH value during derivatization was generally observed (Fig. 2). These differences in fluorescence intensity indicate in the 1st instance possible changes in the structural properties of lysozyme. The results also document further that the indol structure of tryptophan in the derivatives is most likely to be involved in the reaction of lysozyme with the phenolic substances. In this context, further complementary experiments will have to be undertaken to determine the decrease in tryptophan content after alkali hydrolysis of the derivatized samples. These measurements are planned and will be reported appropriately.

Changes in solubility. Since the reaction of lysozyme with the derivatizing substances is accompanied by a corresponding blocking of the hydrophilic, positively charged amino groups, a parallel change in solubility should be awaited. The experimental and calculated isoelectric points of lysozyme (hen egg) lies between pI 10.5 to 11.3 at 25 °C (Patrickios and Yamasaki 1995). Due to lack of appropriate buffer and comparable conditions, the solubility profile only in the of range pH 2 to 9 was investigated. As a result, the characteristic pI of lysozyme of approximately 11 was not documented. In general, a decrease in the solubility of the derivatives prepared at pH 10 over a broad range of pH was noted (Fig. 3, curve 4 and 7). This change in solubility was also dependent on the concentration of the chlorogenic acid applied (compare Fig. 3a with 3b, curve 4 and 7).

Changes in hydrophilic/hydrophobic character. A further molecular property of lysozyme thatcan be affected by the reaction with chlorogenic acid is its hydrophilic-hydrophobic character, which can be illustrated by reverse phase-high performance liquid chromatography (RP-HPLC) (Fig. 4). This is documented in a rise of the retention times of the main peak. The peak formation seemed to be adversely affected, possible due to denaturation (structural changes) and molecular interactions. With regard to reaction of chlorogenic acid at pH 4 and 7, no change in chromatogram pattern was noted (curves 2, 3, 5, and 6 in Fig. 4). An increase in the hydrophobicity of the lysozyme derivatives was observed with increasing degree of derivatization as shown at pH 10 for curves 4 and 7 for corresponding 50 mg chlorogenic acid (CA) and 150 mg CA/g protein (Fig. 4), which confirms the results obtained for the solubility profiles (Fig. 3a,b). According to Hayakawa and others (1985), the decrease in solubility correlates generally with an increase in hydrophobicity, similar to the results obtained for lysozyme derivatives as documented above.

Isoelectric focusing. The derivatization changes the amount of charged groups as documented exemplary for free amino groups (Fig. 1). Generally, a loss of charged groups due to addition of the oxidized phenolic moiety for example to the ε-amino groups of lysine is accompanied with a

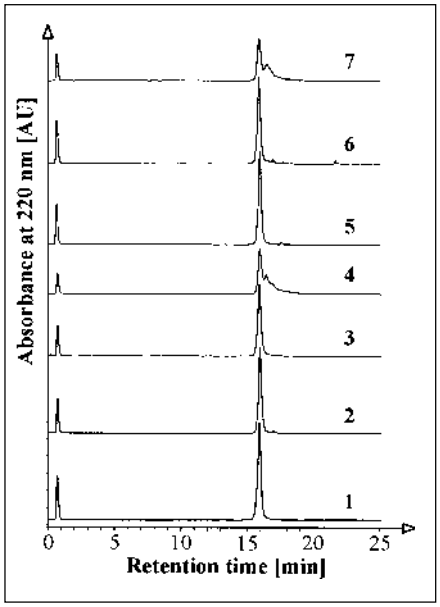

Fig. 4—RP-HPLC of lysozyme derivatives depending on derivatizing conditions. Code: 1 = lysozyme-control without addition of CA; 2 to 4 = derivatization at pH 4, 7, and 10 with 50 mg CA/g protein; 5 to 7 = derivatization at pH 4, 7, and 10 with 150 mg CA/g protein.

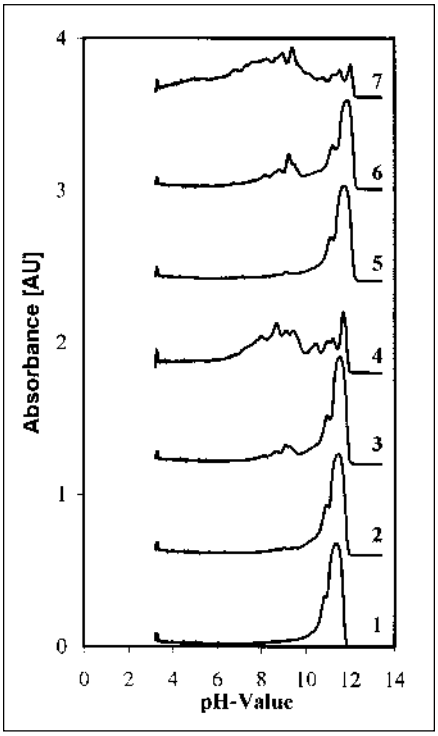

Fig. 5—IEF of lysozyme derivatives depending on derivatizing conditions. Code: 1 = lysozyme-control without addition of CA; 2 to 4 = derivatization at pH 4, 7, and 10 with 50 mg CA/g protein; 5to 7 = derivatization at pH 4, 7, and 10 with 150 mg CA/g protein.

change of the isoelectric point of the proteins. The experimental and calculated isoelectric points of lysozyme (hen egg) lies between pI 10.5 to 11.3 at 25 °C (Patrickios and Yamasaki 1995). As shown in Fig. 5, these correspond to the peaks in the unmodified control sample with pI values between 10.0 to 11.8. A subsequent reaction of 50 and 150 mg chlorogenic acid with 1 g lysozyme shifts the isoelectric range from pI 10.0 to 11.8 in the control to pI 4.0 to 10.0 for the majority of the reaction products (Fig. 5). A shift of the isoelectric range to a lower pH, was also dependent on the degree of derivatization at the 2 different concentrations tested, as illustrated by curves 4 and 7 at pH 10 in Fig. 5. Slight changes in the isoelectric focusing electropherograms (curves 3 and 6, Fig. 5) were also observed for samples derivatized at pH 7. No changes were recorded, when lysozyme was derivatized at pH 4 (curves 2 and 5, Fig. 5).

SDS-PAGE analysis. SDS-PAGE of the control lysozyme showed the main fraction with a molecular weight of 14,840 Da (Fig. 6, curve 1), similar to values cited in literature (14,400 Da). The derivatization leads to the formation of a dimer with a molecular weight of approximately 30,000 Da, the relative concentration of which increases with the pH value present during derivatization process. The maximum relative concentration of approximately 25% of the dimer was achieved at pH 10. Moreover at pH 10, the formation of higher molecular weight fractions with 42,500, 55,000, and 67,500 Da was also recorded (Fig. 6, curves 4 and 7). This formation of high molecular weight fractions was not influenced much by the degree of derivatization as shown in Fig. 6 for derivatization at pH 10 (curve 4 and 7). Hurell and Finot (1984), Pierpoint (1969 a,b) as well as Macholz and Lewerenz (1989) also reported on the polymerization of protein molecules as a possible subsequent reaction of different proteins with phenolic substances. Chlorogenic acid may be oxidized with ease in an alkaline solution to its corresponding quinone (Hurrell and Finot 1984). The quinone being a reactive electrophilic intermediate can readily undergo attack by nucleophiles, such lysine, methionine, cysteine, and tryptophan residues, in a protein chain (Hurrell and Finot 1984). The 2-position of the benzene ring of the caffeic acid moiety in chlorogenoquinone molecule is the most electrophilic, and nucleophilic addition occurs preferentially here (Cheynier and others 1986). Upon further oxidation of this addition product to form its quinone, a 2nd addition occurs in the 6-position, which leads

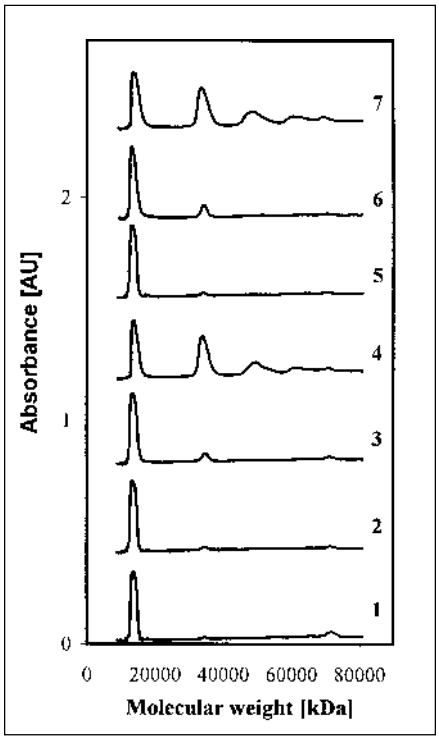

Fig. 6—SDS-PAGE of lysozyme derivatives depending on derivatizing conditions. Code: 1 = lysozyme-control without addition of CA; 2 to 4 = derivatization at pH 4, 7, and 10 with 50 mg CA/g protein; 5 to 7 = derivatization at pH 4, 7, and 10 with 150 mg CA/g protein.

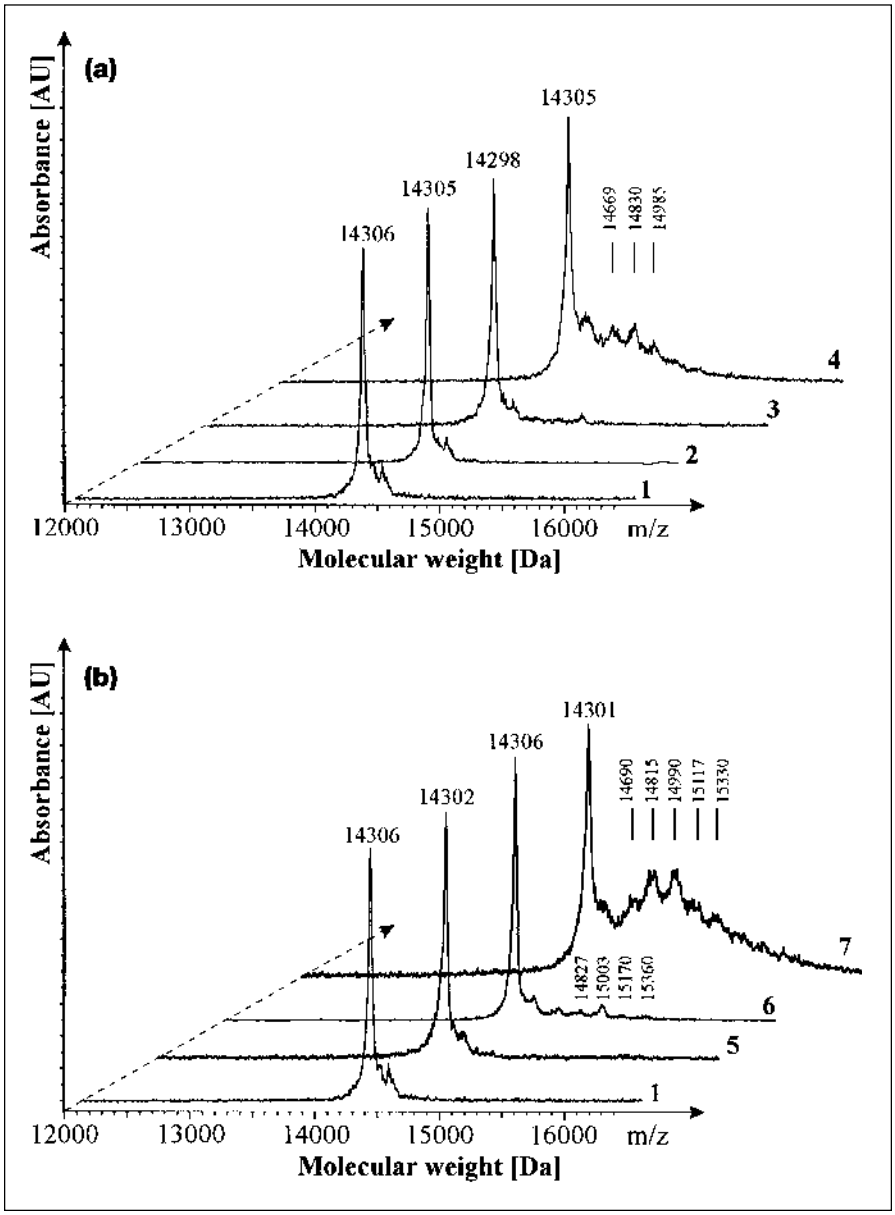

Fig. 7—MALDI-TOF-MS of lysozyme derivatives depending on derivatizing conditions. A - Code: 1 = lysozyme-control without addition of CA; 2 to 4 = derivatization at pH 4, 7, and 10with 50 mg CA/g protein; B - Code: 1 = lysozyme-control without addition of CA; 5 to 7 = derivatization at pH 4, 7, and 10 with 150 mg CA/g protein.

to formation of cross-linked protein polymers (Machholz and Lewerenz 1989).

The possibility of such consequent covalent cross linking as a result of reactions at the free amino groups and tryptophan discussed above and can be confirmed by SDS-PAGE of lysozymederivatives. Exemplary for lysozyme derivatives prepared with 150 mg CA/g protein, a correlation of $R^2 = 0.989$ (y = 0,4293x - 1,0161) with increasing pH value (4, 7, and 10) with regard to the increase in the amount of high molecular fractions (%, y-axis) plotted against the amount of free amino groups blocked (nmol/mg protein, x-axis) could be obtained. Similarly, with the corresponding decrease of tryptophan fluorescence (FI, x-axis), a correlation of $R^2 = 0.978$ (y = 0,0014x + 6,0644) was determined.

Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). This method of analysis was applied to evaluate detailed changes in molecular weights in derivatized monomer lysozyme molecules. The MALDI mass spectra of the samples obtained from the reaction of 50 and 150 mg CA/g lysozyme are presented in Fig. 7a and 7b, respectively. The molecular weight of lysozyme determined with this method was 14,306 Da, agreeing with data calculated from its sequence (GenBank Protein sequences, Accession No. 231220 for lysozyme from hen egg white). Each spectrum shows a mixture of products with different degrees of derivatization. An increase in molecular weight was observed with increasing amount of the reacting CA present during the derivatization process (Fig. 7a and 7b). Similar observation was also made, when the pH value during derivatization was increased over pH 7 (curves 3,4, 6, and 7, Fig. 7a and 7b). Generally, the mass spectra show peaks, which are approximately separated by an increment of the molecular weight of the reacting molecules of CA. The reaction of lysozyme with chlorogenic acid (352.3 Da) at a concentration of 150 mg/g protein delivered the highest molecular

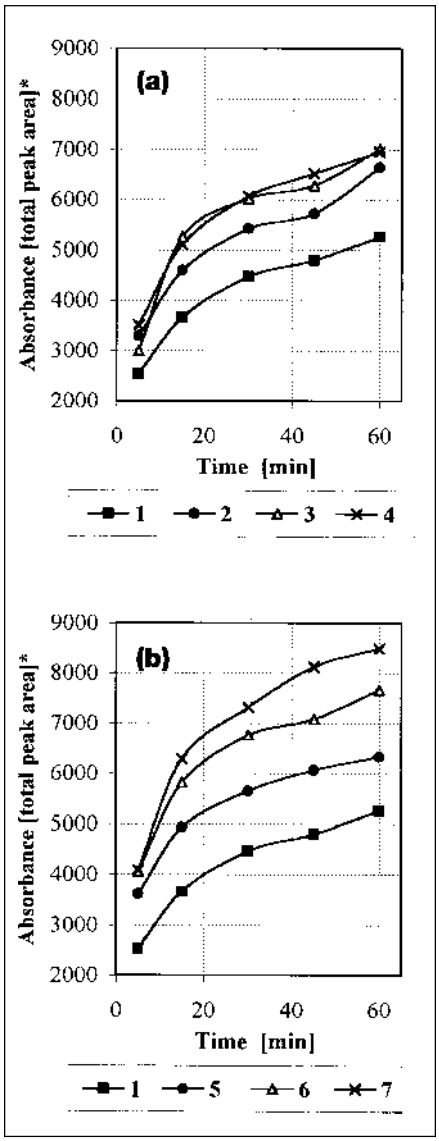

Fig. 8—Tryptic digestion of lysozyme derivatives depending on derivatizing conditions. A - Code: 1 = lysozyme-control without addition of CA; 2 to 4 = derivatization at pH 4, 7, and 10 with 50 mg CA/g protein; B - Code: 1 = lysozyme-control without addition of CA; 5 to 7 = derivatization at pH 4, 7, and 10 with 150 mg CA/g protein; * TCE soluble peptides.

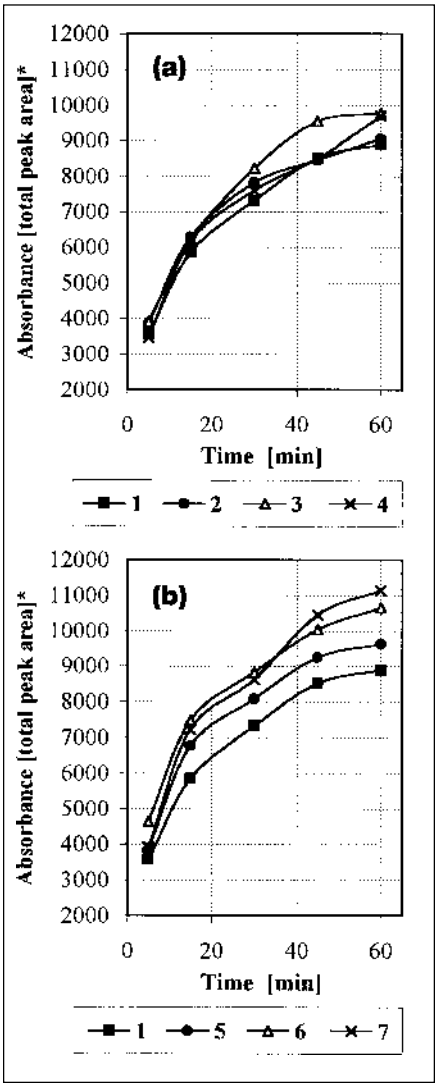

Fig. 9—Chymotryptic digestion of lysozyme derivatives depending on derivatizing conditions. A - Code: 1 = lysozyme-control without addition of CA; 2 to 4 = derivatization at pH 4, 7, and 10 with 50 mg CA/g protein; B - Code: 1 = lysozyme-control without addition of CA; 5 to 7 = derivatization at pH 4, 7, and 10 with 150 mg CA/g protein; * TCE soluble peptides.

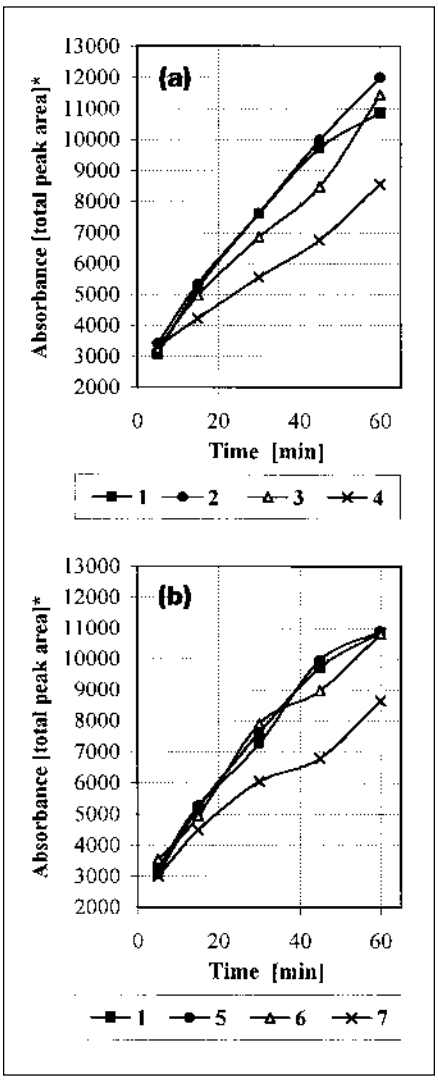

Fig. 10—Peptic digestion of lysozyme derivatives depending on derivatizing conditions. A - Code: 1 = lysozyme-control without addition of CA; 2 to 4 = derivatization at pH 4, 7, and 10 with 50 mg CA/g protein; B - Code: 1 = lysozyme-control without addition of CA; 5 to 7 = derivatization at pH 4, 7, and 10 with 150 mg CA/g protein; * TCE soluble peptides.

mass of 15330 Da, which in turn could account for addition of about 3 chlorogenoquinone molecules to one lysozyme molecule (curve 7, Fig. 7b). Since 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 1st primary addition of a reacting substance at, for example, epsilon amino groups of lysine side chains. The reaction products thus formed may react further with other lysozyme molecules resulting in polymerized products as identified by SDS-PAGE.

Effect of the derivatization on the proteolytic hydrolysis

The effect of lysozyme derivatization with chlorogenic acid on the in-vitro proteolytic digestion by the 3 main enzymes (trypsin, a-chymotrypsin and pepsin) of the gastrointestinal tract was tested to give preliminary indications for its physiological and nutritional consequence. For this purpose it was necessary to partly denature lysozyme with urea due to its compact globular structure. Generally, proteins taken with diet are also partly denaturated as a result of processing and cooking.

The results of tryptic digestion of lysozyme derivatives at a reacting concentration of 50 and 150 mg CA/g protein are shown in Fig. 8a and 8b, respectively. In comparison to the control unmodified lysozyme, the influence of derivatization causes the tryptic hydrolysis to become faster. This does not represent or reflect the trend generally observed by the physicochemical characterization especially with regard to the decrease in the amount of tryptophan and free amino groups. Depending on pH conditions applied (4, 7, and 10), there was also a corresponding higher amount of trichloroacetic acid soluble peptides (compared to the results of unmodified lysozyme hydrolysis) liberated after 1 h tryptic digestion (Fig. 8b). Trypsin splits preferentially those peptide linkages, which contain either lysine or arginine as amino side chains (Bond 1989). Since it has already been shown that phenolic substances react with _ - amino groups of lysine side chains (Fig. 1), we can expect that their derivatization by a phenolic substances shouldprevent or at least make the tryptic degradation difficult. Further as indicated by SDS-PAGE analysis (Fig. 6), the resulting polymerization may also further prevent the access of those peptide linkages, which can be split by trypsin due to resulting conformational changes. On the other hand, lysozyme is known to have a compact globular structure, and in this case it appears that the derivatization favors the tryptic digestion, which can be perhaps explained by preferred splitting of those peptide bonds which contain arginine. Moreover, the conformational structure must be changed, such that the access to relevant peptide bonds is easier. Further, the peptides split may also contain chlorogenic acid residues, which may increase absorbance in UV range (220 nm) used for the detection of the peptide bonds. To explain this phenomenon, further experiments should be followed up. The effect on the chymotryptic hydrolysis of lysozyme derivatives, depending on concentration applied, is illustrated in Fig. 9a and b. Generally, the chymotryptic digestion was not effected by derivatization in presence of 50 mg CA/g protein and slightly effected in presence of 150 mg CA/g protein. The chymotryptic digestion similar to tryptic hydrolysis was faster, whereby the effect increased with the rising degree of derivatization and with the respective higher derivatization pH value (Fig. 9b). Chymotrypsin is known to have primary specificity for those peptide bonds which contain aromatic amino acid residues like tryptophan, tyrosine and phenylalanine (Bond 1989). As shown in Fig. 2, the tryptophan fluorescence decreased with the degree of derivatization. This does not correspond with regard to the results obtained for the chymotryptic digestion, the explanation for this being similar as that for tryptic hydrolysis. A possible reason could also be the bulkiness of a chlorogenic acid adduct, which facilitates the access of chymotrypsin much more easily due to the resulting conformational structure.

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 derivatization, depending on the derivatization pH (Fig. 10a and b). Further the influence of derivatization is of more detrimental nature than that observed for chymotryptic and tryptic hydrolysis (Fig. 8 and 9). Inhibitory effects of the derivatized products on peptic digestion may be attributed in this case to their resulting conformation and structural changes.

Conclusion

THE RESULTS SHOW THAT PLANT PHENOLIC substances (in this case chlorogenic acid) reacts with proteins, influencing their physicochemical and in-vitro enzymatic degradation. An increase in the amount of the chlorogenic acid applied, led to a corresponding higher degree of derivatization. These results are important with regard to further experiments planned, involving physiological and toxicological effects of proteins derivatized with phenolic compounds.

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