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Structural changes induced in bovine serum albumin by covalent attachment of chlorogenic acid

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Abstract

Bovine serum albumin (BSA) was modified by covalent attachment of chlorogenic acid using different concentrations at pH 9. The derivatization was accompanied by a reduction of lysine, cysteine and tryptophan residues. The isoelectric points were shifted to lower pH values and formation of high molecular weight fractions was noted. The structural changes were studied using circular dichroism, differential scanning calorimetry (DSC), intrinsic fluorescence, and binding of anilinonaphthalenesulfonic acid. The results showed that the content of α-helix decreased with a parallel increase in unordered structures with higher degrees of derivatization. DSC revealed a decrease in both denaturation temperature and enthalpy. Surface hydrophobicity declined, indicating that hydrophilic regions were exposed on the molecular surface. Proteolytic digestion showed that, at a lower degree of derivatization, the tryptic degradation was most adversely effected, whereas the peptic digestion declined with increasing modification. A trypsin inhibitory effect of the breakdown products released from derivatized BSA was also observed.

Keywords: Chlorogenic acid; BSA; Food protein derivatization; Physicochemical characterization; Structure; Hydrophobicity; DSC; In-vitro proteolytic degradation

1. Introduction

Phenolic compounds represent the largest group of secondary plant metabolites and are widely distributed in plants. The occurrence, intake, physiological effects and chemical reactivity of phenolic and related compounds, as well as potential types of interactions of phenolic compounds and proteins, have been recently summarized (Rawel, Rohn, & Kroll, 2000). The most frequent form of phenolic compound found in plants is chlorogenic acid (5-caffeoyl-quinic acid, IUPAC numbering). As an ortho-diphenol, chlorogenic acid, at alkaline pH, is converted to the corresponding *o*-quinone, which can than covalently link with nucleophilic groups of proteins.

In our former attempt to characterize the reactions of food proteins with plant phenols (from a physicochemical and physiological viewpoint), we investigated different influencing factors. The food proteins tested included myoglobin, lysozyme, and whey proteins. A series of simple phenolic and related compounds (quinic-, ferulic-, caffeic-, chlorogenic-, and gallic acid, rescorcinol, pyrocatechol, hydroquinone representing *m*-, *o*-, *p*- dihydroxyphenols and finally *p*-quinone) were tested (Kroll & Rawel, 2001; Kroll, Rawel, & Seidelmann, 2000; Rawel, Kroll, & Hohl, 2001; Rawel, Kroll, & Rohn, 2001; Rawel, Kroll, & Riese, 2000). In these experiments it was observed that the low molecular weight proteins (myoglobin and lysozyme) formed derivatives, which were generally more or less insoluble. This was the main handicap in investigating structure–function changes induced by the covalent attachment of the phenolic compounds. Although whey proteins produced soluble derivatives, they were also unsuitable, being a mixture of different milk proteins. Preliminary experiments showed that a comparatively high molecular weight protein, such as bovine serum albumin (BSA), is suitable for such structure–function investigations since it produces soluble conjugates with chlorogenic acid.

The influence on the in vitro tryptic and chymotryptic digestion of the protein–phenol derivatives differed

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Nomenclature

ANS	1-anilino-8-naphthalensulfonate
BAEE	N_α-benzoyl-L-arg ethyl ester
BAPA	N_α-benzoyl-DL-arg-4-nitroanilide
BSA	bovine serum albumin
CD	circular dichroism
ΔH	enthalpy of denaturation
DSC	differential scanning calorimetry
IEF	isoelectric focussing
E:S	enzyme:substrate ratio
OD	optical density
RP-HPLC	reversed-phase high-performance liquid chromatography
SDS	Na-dodecylsulfate
TCA	trichloracetic acid
T_d	denaturation temperature
TNBS	trinitrobenzenesulphonic acid
UV	ultra-violet absorbtion.

from that documented in our former investigations. In the case of myoglobin (Kroll et al., 2000; Kroll & Rawel, 2001), it declined with increasing degree of derivatization, whereas in the cases of lysozyme and whey proteins it was promoted (Rawel, Kroll, & Riese, 2000; Rawel, Kroll, & Hohl, 2001). Preliminary experiments on in vitro tryptic digestion of BSA–chlorogenic acid derivatives gave different results comparable to the above-mentioned studies. The difference was with regard to the dependency on the degree of derivatization, where a lower derivatization of BSA caused the tryptic hydrolysis to become much slower than the corresponding higher derivatization. As a result, one of the objectives of the present study was to find any relation between structural parameters and the somewhat different proteolytic digestion. A further intention was to perform chemical tailoring by applying different concentrations of chlorogenic acid to investigate other possible structure-function relationships. To illustrate these relationships circular dichroism, differential scanning calorimetry, intrinsic fluorescence, binding of anilinonaphthalenesulfonic acid and limited proteolysis were applied.

2. Materials and methods

2.1. Materials

Bovine serum albumin (1 g, 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 95 ml). In each case, after addition of the chlorogenic acid solutions (in 5 ml ethanol; both from Fluka Chemie AG, Buchs, Switzerland), the pH was adjusted once more to 9. After 24 h reaction time under continuous stirring at room temperature (24 °C) with free exposure to air, the samples were dialyzed for 18–20 h against water and finally lyophilized. Molar ratios of BSA:chlorogenic acid present during the derivatization were approximately: 1:19, 1:27, 1:39, 1:64, 1:97 (equivalent to w/w ratios 10:1, 7:1, 5:1, 3:1 and 2:1). These were designated with the corresponding sample numbers 2–6. The non-derivatized protein (control) was prepared under the same conditions but without addition of phenolic compound (labelled as sample 1).

Trypsin was from porcine pancreas (EC 3.4.21.4, protein content 98%, SIGMA Chemicals Co., St. Louis, MO)—14,900 U/mg solid, one BAEE unit = ΔA_{253} of 0.001 per min with BAEE as substrate at pH 7.6 at 25 °C.

α-Chymotrypsin was 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 was from porcine stomach mucosa (EC 3.4.23.1, protein content ca. 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.

2.2. Physicochemical characterization

The protein content in the solutions was determined according to a modified LOWRY method (Lowry, Rosebrough, Farr, & Randall, 1951). Changes in the content of free amino groups according to Adler-Nissen (1972) were analyzed using trinitrobenzenesulfonic acid (TNBS) in a 1% Na-dodecylsulfate (SDS) solution of the samples. Tryptophan content was estimated (Jackman & Yada, 1989) in 8 M urea using a Jasco fluorescence detector FP 920 (Gross-Umstadt, Germany), as described by Rawel, Kroll, and Rohn (2001). The change in thiol content (cysteine) was determined by liquid chromatography of fluorescently labelled proteins with monobrombimane as described by O'Keefe (1994).

UV–vis spectra were measured at a concentration of 0.1 mg/ml (path length 10 mm) in 0.1 M sodium phosphate buffer pH 7.

Chlorogenic acid (9–138 mol/ml in 0.1 M sodium phosphate buffer pH 7) was used to calibrate the regression curve for absorbtion at 324 nm ($Y = 0.0183 \times X$, $R^2 = 0.998$) in order to calculate the amount attached to BSA.

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, total acrylamide concentration $T = 10\%$), as described by Rawel, Kroll, and Rohn (2001).

2.3. Structural characterization

Far-UV circular dichroism (CD) measurements of the samples were recorded in the range 178–260 nm in 0.1 M sodium phosphate buffer pH 7 at a protein concentration of 0.2 mg/ml, using a Jasco J 710 spectropolarimeter (Gross-Umstadt, Germany). A quartz cylindrical cell having 1 mm path length was used for the measurements. The following parameters were set: step resolution = 1 nm; speed = 20 nm/min; band width = 1 nm; response = 4 s and sensitivity = 200 mdeg. Mean ellipticity, using a mean residue molecular weight of 114 for BSA, was calculated from the amino acid sequence (NCBI Protein bank, Data bank source: pir: locus ABBOS; Accession: ABBOS for bovine serum albumin). The CD spectra were analyzed by a curve-fitting programme software CDPro using CONTIN, SELCON and CDSSTR methods, as described by Sreerama and Woody (2000) to obtain the secondary structural contents of the proteins. The estimation was performed using two different reference sets of proteins (a 22-protein reference set and a 43-protein reference set), each giving different assignment of the secondary structures according to Sreerama and Woody (2000).

Intrinsic fluorescence emission spectra between 300 and 800 nm were recorded with a Jasco fluorescence detector FP 920 using an excitation wavelength at 290 nm and quartz optical cells of 10 mm path length. Excitation and emission bandwidths were set at 18 and 40 nm, respectively. The protein concentrations were adjusted to 0.1 mg/ml (0.1 M sodium phosphate buffer pH 7). Corrections were made for the buffer blanks.

Differential scanning calorimetry of 20% protein solutions in distilled water (w/w) was performed using a SEIKO 120 DSC analyzer (measuring cell SSC 5200 H, Neu Isenburg, Germany), as outlined by Ismond, Murray, and Arntfield (1985). Sample solutions (40 μl) were analyzed in air tight aluminium capsules. Thermal curves were established at a heating rate of 5 °C/min over a temperature range of 20–150 °C. The thermal parameters T_d (denaturation temperature), ΔH (enthalpy of denaturation), onset and offset temperatures were determined from all thermal curves. All thermal parameters were measured using a minimum of three samples, means and standard deviations were calculated for each.

The surface hydrophobicity of the samples was determined using a hydrophobic fluorescence probe, 1-anilino-8-naphthalensulfonate (ANS), according to the method of Hayakawa and Nakai (1985). The protein concentrations applied were 0.125–0.5 mg/ml in 0.1 M sodium phosphate buffer pH 7. Fluorescence was recorded with a Jasco fluorescence detector FP 920 using excitation at 390 nm (slit 18 nm) and emission between 400 and 900 nm (slit 40 nm). The initial slope (So) of the fluorescence intensity vs. soluble protein concentration plot was used as an index of the protein surface hydrophobicity.

2.4. Proteolytic digestion

The samples were dissolved in 8 M urea, heated for 5 min at 100 °C to denature them, and the urea concentration was decreased after heating to 2 M by diluting the solutions with 0.1 M Tris–HCl pH 8 containing 0.02 M $CaCl_2$ (final concentration 6 mg/1.5 ml). Tryptic, and chymotryptic hydrolysis (100 μl enzyme, 1 mg/ml) of BSA, as well as its derivatized products were investigated by incubating at 35 °C (enzyme/substrate ratio—E:S = 1:60). The proteolytic hydrolysis was also performed without denaturation (without urea and the heating step) before incubating with the appropriate enzyme. After different durations of hydrolysis (5–120 min, 24 h), 300 μl were removed from the incubation mixture and the digestion was stopped by addition of 300 μl trifluoroacetic acid (0.5%). RP-HPLC of the 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, 324 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: 10–60% acetonitrile—40 min; 60% acetonitrile—3 min; 60–10% acetonitrile—2 min; 10% acetonitrile—15 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 enzymatic digestion.

Sample preparation for SDS-PAGE analysis of the peptides was performed by removing 100 μl from the incubation mixture and stopping the digestion after addition of denaturation buffer (100 μl 0.05 M Tris–HCl pH 6.8 containing 4% SDS, 5% Mercaptoethanol, 12% Glycerin and 0.01% Coomassie Brilliant blue R 250) and heating at 100 °C for 5 min. SDS-PAGE analysis was performed (separating gel having $T = 18\%$) according to the method of Laemmli (1970).

Peptic digestion (100 μl pepsin, 1 mg/ml in de-ionized water) of BSA, as well as its derivatized products (6 mg/1.5 ml; 2 M urea, 0.075 M HCl; E:S = 1:60), was investigated by incubating at 35 °C. The time dependent digestion was monitored by means of RP-HPLC after inactivation of the enzyme with trichloroacetic acid, by the procedure described above.

Table 1
Content of tryptophan, free amino and thiol groups in BSA–chlorogenic acid derivatives [mol/mol protein]

Sample	Tryptophan	Free amino groups	Thiol groups	Total amount of protein groups blocked	Amount of chlorogenic acid bound
BSA, theoretical values	2	60	35		
1 (BSA, control)	1.55±0.06	50.14±0.91	38.19±1.91	–	–
Amount of groups blocked					
2 (10:1)[a]	1.10±0.00	3.81±0.24	2.38±0.12	7.3	5.1
3 (7:1)[a]	1.25±0.01	4.59±0.40	3.05±0.15	8.9	7.1
4 (5:1)[a]	1.44±0.00	7.25±0.36	5.04±0.25	13.7	9.5
5 (3:1)[a]	1.46±0.00	10.38±0.33	8.19±0.33	20.0	13.9
6 (2:1)[a]	1.49±0.00	9.89±0.29	11.04±0.50	22.4	17.9

[a] BSA: chlorogenic acid ratios (w/w) corresponding to molar ratios 1:19, 1:27, 1:39, 1:64, and 1:97 as applied in the experiments during derivatization.

2.5. Inhibition of tryptic digestion

The inhibitory effects of BSA derivatives and/or that of products released by limited tryptic digestion were also evaluated. Limited tryptic hydrolysis (100 μl enzyme, 1 mg/ml in 0.05 mM HCl/ 0.01 mM $CaCl_2$) of BSA, as well as its derivatized products, was allowed to proceed for 5 min by incubating at room temperature (6 mg/1.5 ml BSA in 67 mM Tris–HCl pH 7.6; enzyme/ substrate ratio—E:S = 1:60) without prior denaturation. This mixture (0.2 ml) was then added to 3 ml of 0.25 mM N_{α}-benzoyl-DL-arginine-nitroanilid (BAPA) in deionized water, the solution mixed and the extinction measured at once at 410 nm against a blank without trypsin. The extinction was further noted after every min for a duration of 10 min. Total trypsin activity for BAPA was determined by repeating the analysis without BSA in the incubation mixture. The difference ΔE 410 nm/min (slope) was determined and set in the following equation to determine trypsin activity for BAPA:

Trypsin BAPA U/ml = $(\Delta E\,410/\text{min} \times 15)/(0.001 \times 0.2)$; where 15 is the dilution of trypsin applied, 0.2 the amount of the incubated trypsin/BSA mixture in ml and 0.001 a calculation factor.

Trypsin-inhibitor BAPA U/mg = (trypsin activity in U/ml-trypsin activity in presence of BSA in U/ml)/BSA in mg/ml

2.6. Statistical analysis

The results (Table 1) were repeated 10 times; Student's *t* test and ANOVA/POST-HOC test were performed. The digestions and other analyses were repeated at least three times and, where possible, evaluated by means and standard deviation. A maximum of ±5% standard deviation from the averaged values was generally tolerated (when not otherwise specified). The averaged values are documented in the respective figures.

3. Results and discussion

3.1. Characterization of physicochemical properties

Generally, phenolic substances may be readily oxidized in alkaline solutions, or in the presence of polyphenol oxidase, to the respective quinones, which in turn can react with nucleophiles such as, protein-bound tryptophan, lysine and cysteine residues. The reactions of the oxidized phenolic compounds with these groups have been discussed by Rawel, Kroll, and Rohn (2001) and in Rawel, Rohn, and Kroll (2000) and are dependent on different factors such as pH, concentration, protein, phenol structures. The experimentally determined mean values for tryptophan, lysine and cysteine amounted to 1.6, 50.1 and 38.2 mol/mol BSA as shown in Table 1. They were in reasonable agreement with those calculated from the sequence (Table 1, theoretical value). The amounts of these three reactive groups decreased with increasing amount of chlorogenic acid present during the derivatization and the corresponding amount blocked is given in Table 1. The application of student's t tests showed that the values obtained for the derivatives (samples 2–6, Table 1) were significantly different (<0.001) from that of the BSA control (sample 1, Table 1). The comparison of the three residues with regard to their reactivity with chlorogenic acid shows that tryptophan is much more reactive than lysine and cysteine, where already a low BSA:chlorogenic acid molar ratio of 1:19 (sample 2) causes a corresponding strong decrease of tryptophan of up to 71%. The reactivity of lysine and cysteine residues seems to be more or less similar, except at the higher molar ratio applied (1:64,1:97, samples 5–6 and Table 1), where the reactivity of cysteine appears to be more stronger. The combination of the experimentally determined values for these three residues allows an estimation of the total amount of protein side chains involved in the reaction with chlorogenic acid as indicated in Table 1. This estimation

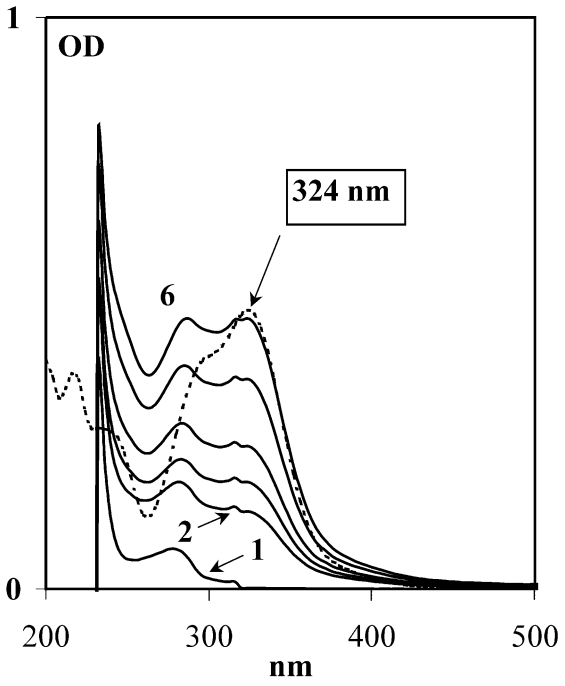


Fig. 1. UV–vis spectra of the BSA derivatives. Code: 1 = unmodified (control) BSA followed by samples 2–6 plotted in increasing order, sample 6 being the uppermost curve. (Samples 2–6 prepared with BSA: chlorogenic acid molar ratios of 1:19, 1:27, 1:39, 1:64, 1:97; corresponding w/w ratios were 10:1, 7:1, 5:1, 3:1, and 2:1); dotted line is the UV–vis spectrum of chlorogenic acid (27.5 nM/ml).

is incomplete since it has been theoretically and experimentally observed that further amino acid residues such as methionine, histidine tyrosine and N-terminal proline also have the potential to react with phenolic compounds as reported by Rawel, Rohn, and Kroll (2000).

Parallel to these observations, the amount of chlorogenic acid bound to BSA was also estimated. For this purpose, UV–vis spectra of chlorogenic acid and those of its derivatives with BSA were compared, as illustrated in Fig. 1. It can be observed that chlorogenic acid has an absorbtion maximum at 324 nm (dotted curve, Fig. 1), being absent in the BSA control (curve 1, Fig. 1), but present as a shoulder in UV–vis spectra of the BSA-chlorogenic acid derivatives (Fig. 1). The absorbtion at 324 nm increased from the second lowest curve (curve 2, Fig. 1), representing the lowest molar ratio of BSA-chlorogenic acid present during the derivatization (1:19) to the top curve (curve 6, Fig. 1) where the corresponding highest molar ratio (1:97) was applied. Since, under the conditions of derivatization, the excess of chlorogenic acid not bound to BSA was removed by exhaustive dialysis and there is a good correlation between chlorogenic acid concentration and absorbtion at 324 nm (9 – 138 nM/ml; $Y = 0.0183 \times X$, $R^2 = 0.998$), it is possible to evaluate the number of molecules of chlorogenic acid bound to one molecule of BSA, as documented in Table 1. This number increased with rising molar ratios of the reactants applied. An

Table 2
Assignment (%) of the secondary structures according to Sreerama and Woody (2000) by applying 43-protein reference set (190–240 nm)[a]

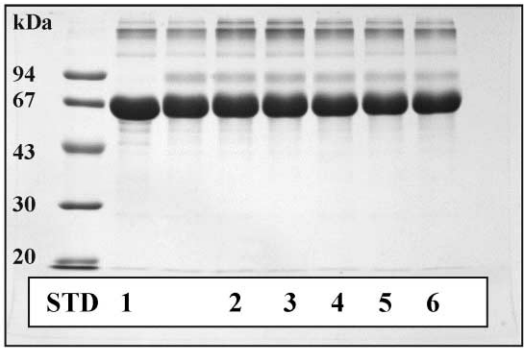
	H(r)	H(d)	S(r)	S(d)	H	S	Turn	Unrd
1	39	21	2	3	60	5	13	23
2	35	20	3	4	55	7	14	24
3	36	20	3	4	56	7	14	23
4	34	19	4	4	53	8	15	24
5	34	19	4	4	53	9	15	24
6	28	18	6	6	46	12	16	26

[a] H (r) = regular α-helix; H (d) = distorted helix; S (r) = regular β-strand; S (d) = distorted β-strand; H = total helix content; S = total β-strand content; Unrd = unordered. The standard deviation for all values was ±0–2%.

estimation of the effectiveness of the derivatization was also evaluated. The lowest molar ratio (1:19) present during the derivatization resulted in 26.8% of the chlorogenic acid being attached to BSA, decreasing over the other samples (3–5; 26.3%, 24.4%, 21.7%) to the highest molar ratio (1:97), where only 18.5% was bound to BSA. It is likely that, at higher concentration, the oxidized chlorogenic acid molecules tend to react with another, which may lead to non-enzymatic browning products (formation of melanin) in advanced stages, often observed during extraction of plant proteins.

The number of amino acid residues blocked in BSA is higher than the amount of chlorogenic acid bound (Table 1), underlining the fact that some protein groups must be involved in intra- and intermolecular cross-linking. The latter can be confirmed by SDS-PAGE analysis, as documented in Fig. 2. There is an increase in the amount of fractions with molecular weights above 125 kDa with a parallel decrease in the relative concentration of the main fraction (65–69 kDa), especially under conditions of derivatization applying low molar ratios (samples 2 and 3; Fig. 2). The molecular weight of the main fraction also increased from 65.8 kDa in the control to 66.1, 66.8, 67.1, 68.2 and 68.6 kDa in the corresponding samples 2–6. A new fraction, with a molecular weight between 85 and 89 kDa, was also noted (Fig. 2), arising from the intermolecular interactions with fractions of lower molecular weights present in the BSA control (45–60 kDa, 20 kDa).

As a result of blocking of ε-amino side chains of lysine on one side, and on the other side due to the introduction of the carboxylic groups, following the covalent attachment of the chlorogenic acid, a change in the net charge of the protein molecules is produced. This change in net charge can be documented by the corresponding change of the isoelectric point (Ip) of the BSA (Fig. 3). Two major fractions were detected in the BSA control (Ip = 5.8 and 5.9, curve 1, Fig. 3). As a result of derivatization with chlorogenic acid, a shift in isoelectric range was observed (Ip = 5.1–5.7; 5–6 fractions in samples 2–6, Fig. 3). Each of these fractions,



Sample	65 – 69 kDa	85 – 89 kDa	≥ 125 kDa
1	86.6 %	0.2 %	10.3 %
2	77.8 %	5.0 %	15.3 %
3	76.8 %	4.9 %	16.5 %
4	79.6 %	4.4 %	14.0 %
5	81.2 %	3.6 %	13.5 %
6	82.1 %	3.4 %	12.3 %

Fig. 2. SDS-PAGE of BSA derivatives. Code: 1 = unmodified (control) BSA; code: 2–6 = samples 2–6 prepared with BSA:chlorogenic acid molar ratios of 1:19, 1:27, 1:39, 1:64, 1:97 (corresponding w/w ratios were 10:1, 7:1, 5:1, 3:1, and 2:1).

representing different extents of derivatization, having in each case a different electrophoretical mobility and a correspondingly different isoelectric point. Generally, with higher degrees of derivatization, lower isoelectrical points for BSA can be observed; i.e. the protein becomes more acidic in nature. However, this change in isoelectric points does not influence the solubility of BSA over a broad pH range (results not shown). Experiments with low molecular weight proteins (myoglobin, lysozyme and whey proteins) showed a distinctly different solubility behaviour as a function of the level of derivatization (Kroll & Rawel, 2001; Kroll et al. 2000; Rawel, Kroll & Hohl, 2001; Rawel, Kroll, & Riese, 2000).

In summary, the physicochemical characterization underlines the nutritional consequence of these oxidative reactions of phenolic compounds in the food systems, leading to limited availability of the essential amino acids, lysine and tryptophan. The reaction proceeds to a further stage, where polymerization takes place. The isoelectric point of the protein is shifted to lower pH values, which in turn may effect its technofunctionality.

3.2. Structural characterization

A far-UV CD study was conducted to evaluate the influence of derivatization by chlorogenic acid on the secondary structure of BSA. The far-UV spectrum of the unmodified BSA (curve 1, Fig. 4) exhibited two

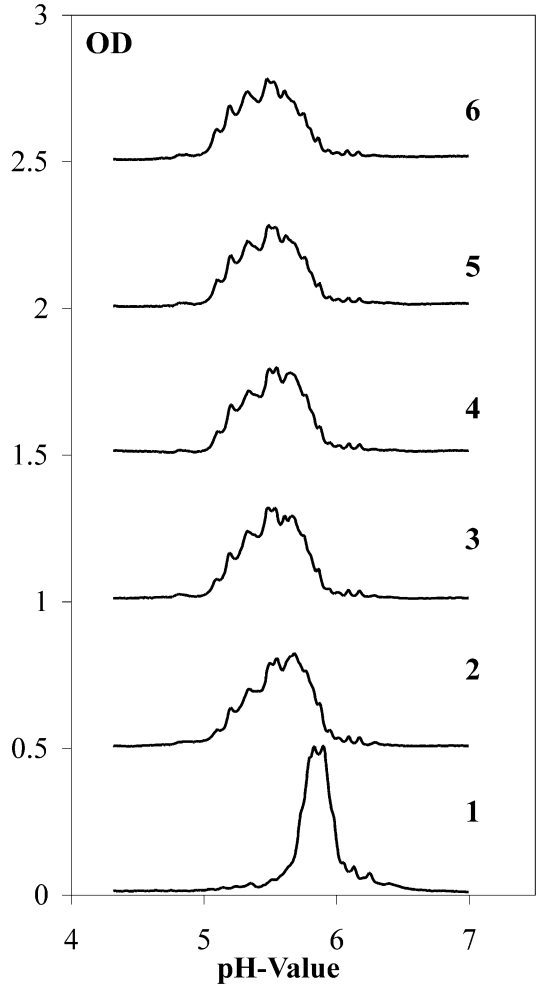


Fig. 3. Isoelectric focussing (IEF) of BSA derivatives. Code: 1 = unmodified (control) BSA; code: 2–6 = samples 2–6 prepared with BSA:chlorogenic acid molar ratios of 1:19, 1:27, 1: 39, 1:64, 1:97.

negative minima at 209 and 222 nm and a maximum at 190–195 nm, which is typical of the $\alpha+\beta$ class protein (Gerbanowski, Malabat, Rabiller, & Guéguen, 1999). The derivatization with chlorogenic acid caused only a decrease in band intensity at all wavelengths of the far-UV CD (curves 2 and 6 shown for the lowest and the highest degree of derivatization, Fig. 4) without any significant shift of the peaks. The CD spectra were analyzed by a curve-fitting programme software CDPro using CONTIN, SELCON and CDSSTR methods, as described by Sreerama and Woody (2000). The mean of the estimated values from the three methods was calculated and is presented for a 43-protein reference set (190–240 nm) in Table 2 and for a 22-protein reference set (178–260 nm) in Fig. 5, each giving a different assignment of the secondary structures (Sreerama & Woody, 2000). The application of the 43-protein reference set gives allocations for α-helix and β-strand, which are subdivided in regular and distorted structure groups. For simplicity, the values for this sub-groups are added and the resulting totals are also presented in

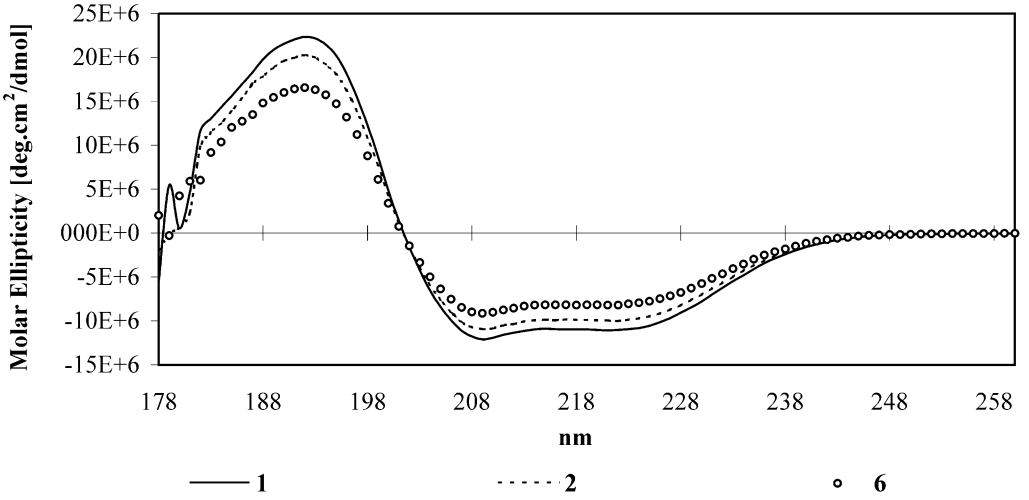


Fig. 4. CD spectra of BSA derivatives. Code: 1 = unmodified (control) BSA; samples 2 and 6 prepared with BSA:chlorogenic acid molar ratios of 1:19 and 1:97 (corresponding w/w ratios were 10:1 and 2:1).

Table 2. The unmodified control BSA contained, according to this allocation, 60% α-helix, 5% β-strand, 13% β-turn and 23% unordered structure (Table 2). The derivatization with chlorogenic acid caused a decrease in α-helix (especially the regular α-helix) with a parallel increase in the remaining structure, indicating the destructuring effect on BSA. The perturbation of the secondary structure became more significant with increasing degree of derivatization. Investigations on thermodynamics of interactions of caffeic acid and quinic acid with multisubunit proteins from sunflower seeds also suggested a marginal increase in β-sheet content, while α-helix and β-turn contents were marginally decreased (Suryaprakash, Kumar, & Prakash, 2000). Interaction of chlorogenic acid with human serum albumin was also shown to cause a decrease in α-helix structure, with other structures unaltered (Muralidhara & Prakash, 1995). These results are in agreement with our observations, except in our case the β-turn was not decreased, but marginally increased. Johnson (1999) first applied a reference set of 22 proteins and used the six secondary structure assignments (α-helix, 3/10 helix, β-sheet, turns, P2, and unordered) of King and Johnson (1999). With reference to Sreerama and Woody (2000), these results should not be combined or compared with those obtained from the other protein reference set (42 protein reference set, Table 2) because of the differences in the secondary structure assignments used. The main reason for applying this reference set to our far-UV CD measurements was that it also considers the CD absorbtion in the range 178–190 nm, due to allocation of P2 structure fraction, which results from poly(Pro) II structure segments. About 46% α-helix and 8% β-strand were documented for unmodified BSA (Fig. 5). The result also shows that there was a significant perturbation of the secondary structure with increasing degree of derivatization, causing a loss of the α-helix

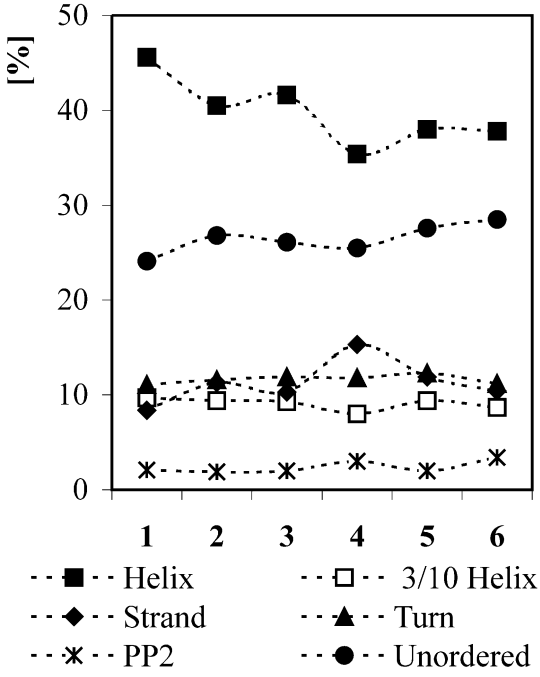


Fig. 5. Assignment (%) of the secondary structures according to Sreerama and Woody (2000) by applying a 22-protein reference set (178–260 nm). Code 1–6, Code: 1 = unmodified (control) BSA; code: 2–6 = samples 2–6 prepared with BSA:chlorogenic acid molar ratios of 1:19, 1:27, 1:39, 1:64, 1:97. Helix = α-helix, Strand = β-sheet, PP2 = poly(pro)II structure.

and a parallel increase in the content of unordered structure elements (Fig. 5). Further, two distinct structural transitions, especially in the content of α-helix and β-strand in samples 2 and 4, were also observed. In both cases the α-helix decreases, to increase again at the following higher level of derivatization. The fraction of β-turn segment increased only marginally.

A change in P2 (poly(Pro) II) structure segments was the second interesting aspect to be assessed. The

unmodified BSA results show a low P2 structure fraction (2.1%, Fig. 5). The content remains unchanged, except at higher degrees of derivatization (samples 4 and 6), where there seems to be a marginal increase of this fraction. The BSA sequence shows two proline-rich segments (134–143; 122–127), which may become involved in interaction with chlorogenic acid. This means that proline or other close residues (e.g lysine in segment 134–143) may be involved, such that more proline residues are brought into close proximity, causing the observed increase in P2 fraction.

Phenolic compounds are known to have a significant affinity for proteins that contain a high proportion of proline residues in their sequences (Baxter, Lilley, Haslam, & Williamson, 1997; Siebert, 1999; Siebert, Troukhanova, & Lynn, 1996). Proline residues seem to play a key role by undergoing hydrophobic interactions with phenolics, which are important for stabilizing the complexes formed. Among the first extensive studies, Mason and Peterson (1955) also followed the reaction of N-terminal proline in protamine with *o*-quinones. They proposed the possibility of formation of covalent binding and cross-linking of proteins as a result of advanced reaction.

In summary, CD studies and estimation of changes in secondary structures clearly demonstrate that the reaction of BSA with chlorogenic acid leads to a decrease in α-helix and an increase in the remaining structure. Chemical modification (acylation and sulfamidation) also produces a similar destructuring of proteins, as reported by Gerbanowski et al. (1999) and Schwenke, Knopfe, Seiffert, Görnitz, and Zirwer (2000).

An intrinsic fluorescence study was performed to evaluate changes in tertiary structure caused by reaction of BSA with chlorogenic acid. The maximum emission wavelength for unmodified BSA was 346 nm (curve 1, Fig. 6). The tryptophan residues are rather exposed on the protein surface, since the initial maximum of fluorescence emission appears at a wavelength close to that of exposed tryptophan model systems (Jackman & Yada, 1989). However, buried tryptophan groups in close proximity to polar residues may be capable of forming an excited state complex (exciplex), resulting in emission spectra characteristic of exposed residues with peaks close to 350 nm (Kronman & Robbins, 1970). Both tryptophan residues (Trp3, Trp158) present in the sequence of BSA are in close proximity to lysine residues, and the first one (Trp3) is located very near to the N-terminal. The resulting high exposure can be confirmed by the high reactivity of tryptophan, where already a low BSA:chlorogenic acid molar ratio of 1:19 (sample 2, Table 1) caused a corresponding strong decrease of tryptophan of up to 71%. The maximum emission also decreased in intensity as a function of the level of modification and a red shift of the maximum emission was observed (Fig. 6, samples 2–6). The

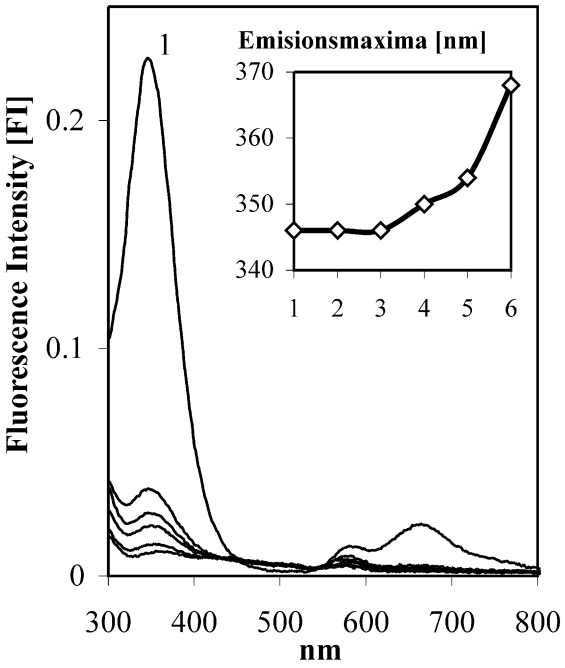


Fig. 6. Intrinsic fluorescence spectra of BSA derivatives. Uppermost curve is for sample 1, followed by those of sample 2, 3 etc., the bottom one being for sample 6. Code: 1 = unmodified (control) BSA; code: 2–6 = samples 2–6 prepared with BSA : Chlorogenic acid molar ratios of 1:19, 1:27, 1:39, 1:64, 1:97.

interaction of the aromatic ring of caffeic acid with aromatic residues, such as tyrosine and tryptophan of the protein, may be responsible for quenching of fluorescence intensity, based on tryptophan fluorescence experiments (Suryaprakash et al., 2000). Such interactions can be confirmed, as shown in Table 1 by the progressive decrease in the amount of free tryptophan. Further complementary experiments, conducted to determine the decrease in tryptophan content after alkali hydrolysis of the proteins derivatized with phenolic compounds, also confirmed these observations (unpublished data). From the progressive quenching and the red shift observed in the maximum fluorescence emission of derivatized BSA, it can be deduced that conformational changes induced by the modification lead to a further exposure of tryptophan residues to the polar solvent (Gerbanowski et al., 1999; Jackman & Yada, 1989).

Differential scanning calorimetry was applied to assess the response of BSA–chlorogenic acid derivatives from a thermal stability perspective. Complete denaturation as a result of derivatization, as assessed by the absence of an endotherm, was not observed. The thermal parameters, determined for unmodified BSA, included denaturation temperature (T_d) = 82.9 °C and enthalpy of denaturation (ΔH) = 8.1 mJ/mg protein, onset and offset temperatures being 62 and 89.6 °C respectively (Fig. 7). The thermal stability of BSA, as reflected by T_d, decreased significantly as a function of

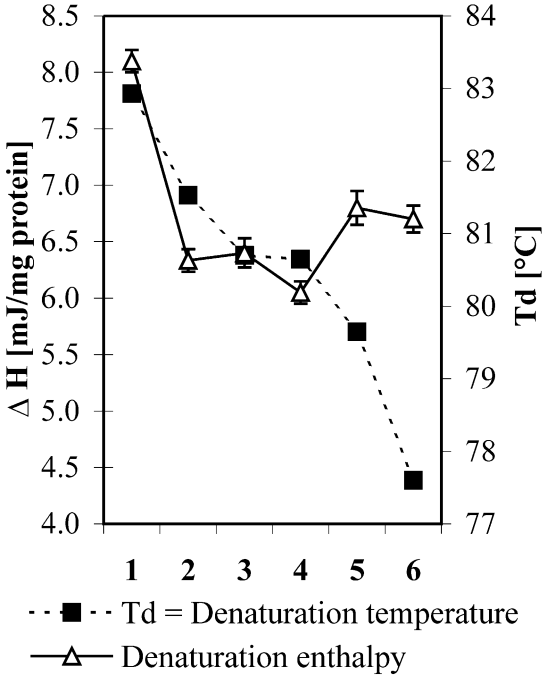


Fig. 7. Differential scanning calorimetry (DSC) of BSA derivatives. Code: 1 = unmodified (control) BSA; code: 2–6 = samples 2–6 prepared with BSA:chlorogenic acid molar ratios of 1:19, 1:27, 1:39, 1:64, 1:97; T_d = denaturation temperature, ΔH = enthalpy of denaturation.

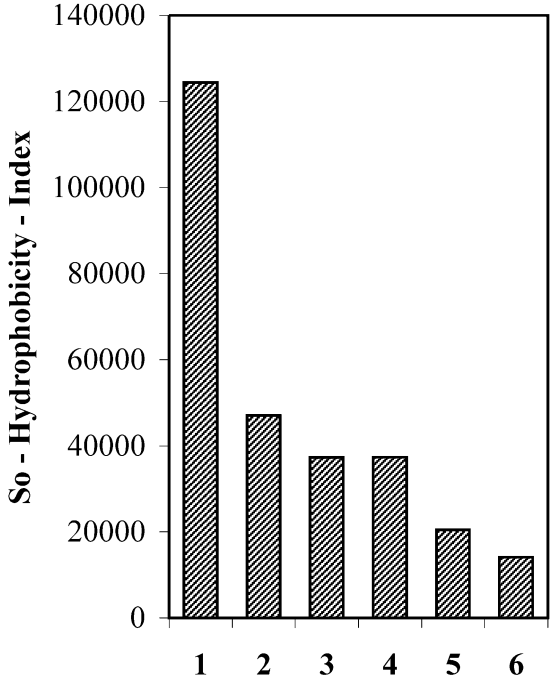


Fig. 8. Surface hydrophobicity of BSA derivatives as determined with 1-anilino-8-napthalensulfonate (ANS). Code: 1 = unmodified (control) BSA; code : 2–6 = samples 2–6 prepared with BSA:chlorogenic acid molar ratios of 1:19, 1:27, 1:39, 1:64, 1:97.

the level of derivatization, reaching 77.6 °C for the highest BSA:chlorogenic acid ratio applied (1:97, sample 6, Fig. 7). Generally the ΔH of derivatized BSA was also lowered. This decrease was not continuous, making conformational changes apparent. The corresponding onset temperatures increased with increasing derivatization of BSA (up to 66 °C), whereas the offset temperatures decreased (down to 87 °C). The end-product of such a denaturation is generally a partly random coiled protein; this result is caused primarily by the disruption of non-covalent interactions (Ismond, Murray, & Arntfield, 1988). The increase in the random coiled or unordered structure has been documented by CD analysis as described above (Table 2, Fig. 5). Conformational disturbances were evident in samples 2 and 4, in terms of a decreased enthalpy of denaturation, which increased marginally on a further higher level of derivatization of BSA with chlorogenic acid. A similar change was also observed in the change of α-helix structure components for both of these samples (Fig. 6). The apparent increase in ΔH may be due to formation of new non-covalent forces by the introduction of the carboxylic groups (following the covalent attachment of the chlorogenic acid), which have a potential to undergo electrostatic interactions.

The influence on surface hydrophobicity of BSA molecules was documented by binding of hydrophobic fluorescence probe, 1-anilino-8-naphthalensulfonate (ANS). ANS is widely used to monitor conformational changes in proteins and to characterize surface exposure of hydrophobic sites (Gerbanowski et al., 1999; Ismond et al., 1985, 1988; Hayakawa & Nakai, 1985). The surface hydrophobicity decreased with increasing degree of derivatization of BSA, as shown by the low initial slopes (So—hydrophobicity index), calculated from the corresponding fluorescence intensity vs. protein concentration plots (Fig. 8). This means that the surface of BSA becomes hydrophilic. This also explains the better solubility of the BSA–chlorogenic acid derivatives than e.g. myoglobin–phenol derivatives, which exhibited increased hydrophobicity (Kroll et al., 2000). According to Hayakawa and Nakai (1985), the decrease in solubility generally correlates well with an increase in surface hydrophobicity, as measured by the hydrophobic fluorescence probe ANS. The observed decrease in the surface hydrophobicity can be explained as combined effect of a conformational change of the protein that increased the exposure of some additional hydrophilic regions which were previously buried, the covalent blocking of exposed hydrophobic residues, such as tryptophan (Table 1), and that the chlorogenic molecules bound to BSA are exposed, being readily accessed. The exposure of the bound chlorogenic acid, would also mean exposure of the carboxylic groups, opening possibilities for electrostatic interactions with polar solvents and thereby improving the solubility. Confirmation of the first explanation with regard to conformational changes can be given by the results of CD analysis where an

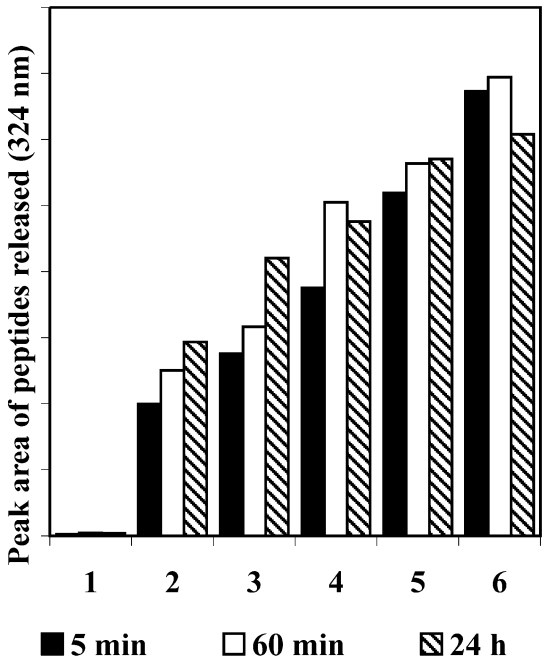


Fig. 9. Tryptic digestion of BSA derivatives (HPLC analysis—peptides detected at 324 nm). Code: 1 = unmodified (control) BSA; code : 2–6 = samples 2–6 prepared with BSA:chlorogenic acid molar ratios of 1:19, 1:27, 1:39, 1:64, 1:97.

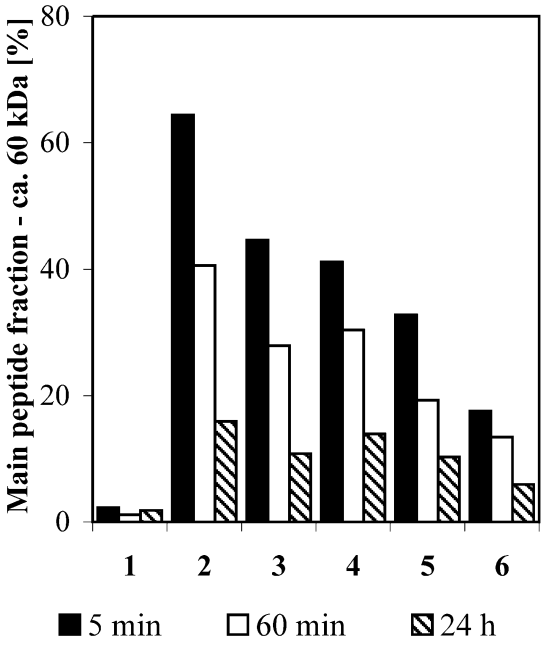


Fig. 10. Tryptic digestion of BSA derivatives—SDS-PAGE analysis of the breakdown products, change in the main product (60 kDa) released. Code: 1 = unmodified (control) BSA; code : 2–6 = samples 2–6 prepared with BSA:chlorogenic acid molar ratios of 1:19, 1:27, 1:39, 1:64, 1:97.

increase in unordered structure elements was observed (Fig. 5), by the red shift observed in the intrinsic fluorescence (Fig. 6), and by the decrease in T_d and ΔH, as documented in Fig. 7. These results can be attributed to the expansion of the protein resulting from the unfolding of the protein. Confirmation that exposure of the bound chlorogenic acid on the BSA surface, is readily accessed, can be seen by monitoring the release of tryptic peptides (HPLC) at 324 nm (Fig. 9). As mentioned above, chlorogenic acid is responsible for the absorbtion at 324 nm and Fig. 9 shows, that most of the peptides are released in the first 5 min of tryptic digestion. Their content increases only slightly on further tryptic hydrolysis, as illustrated for 60 min and 24 h (Fig. 9). Further experiments on proteolytic digestion (see following results) show, by contrast, that most of BSA molecules in the derivatized BSA (samples 2–6) still have relatively high molecular weights (60 kDa, Fig. 10) after 5 min of tryptic digestion. In comparison, in the unmodified BSA, this fraction was more or less completely digested (sample 1, Fig. 10).

In summary the intrinsic fluorescence, DSC measurements and ANS binding experiments clearly indicate significant changes in the conformation of BSA derivatized with chlorogenic acid.

3.3. Influence on proteolytic digestion/inhibitory effects

The tryptic hydrolysis was monitored by HPLC and SDS-PAGE, both giving similar results. The results obtained by means of SDS-Page are reported in Fig. 10. A relatively stable intermediary product, having a molecular weight of 60 kDa, was released and its decrease was monitored. The control BSA (Sample 1, Figs. 10 and 11) was more or less extensively digested after 5 min, whereas the BSA-chlorogenic acid derivatives (samples 2–6, Fig. 10) were only partly digested even after 24 h exposure to trypsin. About 6–16% of the intermediary product (60 kDa) was still seen after 24 h hydrolysis. An interesting observation with regard to the behaviour of the BSA–chlorogenic acid derivatives was also documented, where the low derivatized BSA (sample 2, Fig. 10) proved to be most difficult to digest by means of trypsin. The tryptic digestion improved with increasing level of chlorogenic acid attached, but still remained difficult in comparison to that of the control unmodified BSA (Fig. 10). As observed above, the release of peptides showing absorbtion at 324 nm (Fig. 9), together with the parallel release of a relatively stable high molecular weight intermediary product (60 kDa, Fig. 10), implies that the chlorogenic acid (E_{max}—324 nm) must be bound to the surface of the BSA molecules. An increased level of derivatization of BSA with chlorogenic acid may cause the protein molecules to expand due to progressive unfolding, facilitating a better tryptic attack and explaining the trend observed in Fig. 10. Principally, a similar process of unfolding may also be induced by prolonged incubation at 35 °C during tryptic degradation promoting destructuring of the protein molecules (Fig. 10). Confirmation of such a process

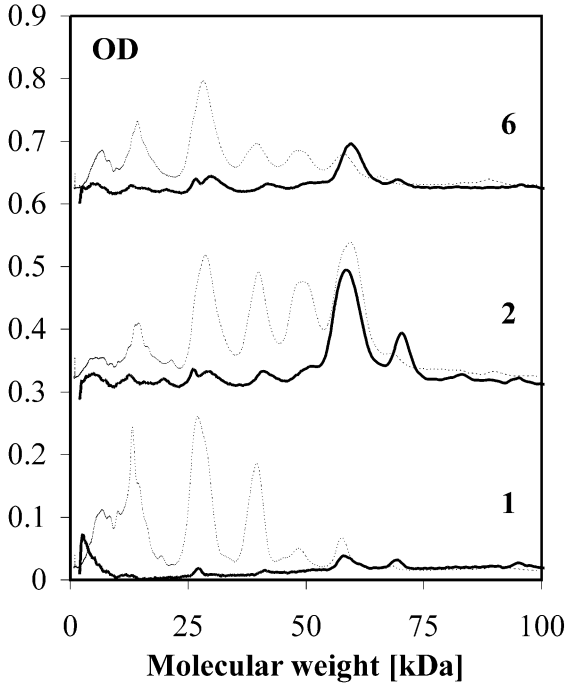


Fig. 11. Tryptic digestion with and without prior denaturation of the samples (SDS-PAGE analysis of the breakdown products). Code:1 = unmodified (control) BSA; samples 2 and 6 prepared with BSA:chlorogenic acid molar ratios of 1:19 and 1:97 (corresponding w/w ratios were 10:1 and 2:1); ..... without prior denaturation; —— denaturation with urea and heating.

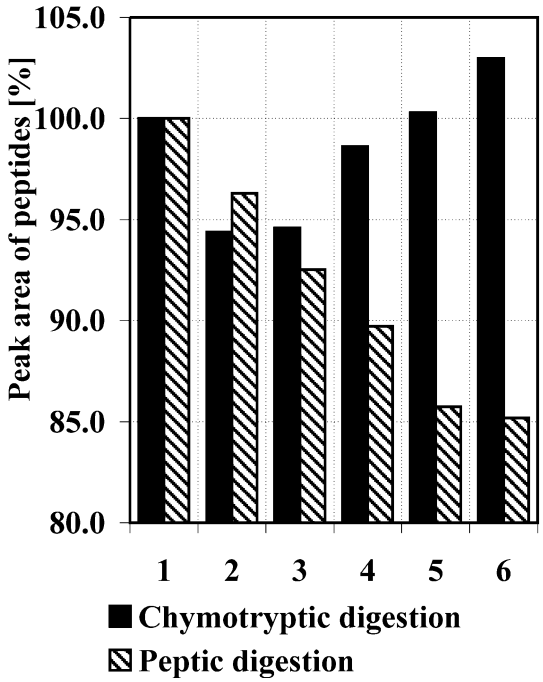


Fig. 12. Chymotryptic and peptic digestion of BSA derivatives (HPLC analysis of peptides detected at 220 nm after 60 min digestion, E:S = 1:60). Code: 1 = unmodified (control) BSA; code : 2–6 = samples 2–6 prepared with BSA:chlorogenic acid molar ratios of 1:19, 1:27, 1:39, 1:64, 1:97.

shown in Fig. 11 where the tryptic degradation was also conducted without prior denaturation as described in the methods section. The dotted lines represent the tryptic digestion without prior denaturation of the samples. Under these conditions, it is apparent that the low derivatized BSA (sample 2, Fig. 11) proves even more difficult to digest. All these observations of limited proteolysis suggest that a low derivatization of BSA with chlorogenic acid produces a structure, which withstands the tryptic attack better. With increasing modification, a progressive loss of this structural advantage occurs and the increasing unfolding of the protein leads to loss of this ability to withstand tryptic attack. The same reasoning also applies to chymotryptic digestion, illustrated in Fig. 12.

Chymotrypsin is known to have primary specificity for those peptide bonds which contain aromatic amino acid residues, such as tryptophan, tyrosine and phenylalanine (Bond, 1989). The tryptophan residues are rather exposed on the protein surface, since the initial maximum of fluorescence emission appears at a wavelength close to that of exposed tryptophan model systems, as shown by intrinsic fluorescence (Fig. 6). Both tryptophan residues (Trp3, Trp158) present in the sequence of BSA are in close proximity to lysine residues, and the first one (Trp3) is located very near to the N-terminal. This, combined with the fact that even a low BSA:chlorogenic acid molar ratio of 1:19 (sample 2, Table 1) caused a corresponding strong decrease of tryptophan of up to 71%, leads only to a slight decrease in chymotryptic degradation. The increasing degree of derivatization, accompanied by the progressive unfolding, facilitates a better access for chymotryptic attack, as shown in Fig. 12.

Pepsin is a non-specific 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 BSA derivatization by chlorogenic acid on the peptic hydrolysis, depending on the concentration tested (Fig. 12). These results are in agreement with those achieved with other proteins and phenolic compounds (Kroll & Rawel, 2001; Kroll et al., 2000; Rawel, Kroll, & Hohl, 2001; Rawel, Kroll, & Riese, 2000). Inhibitory effects of the derivatized products on peptic digestion may be attributed in this case to their resulting conformation and structural changes, especially with regard to reaction at tryptophan side chains (Table 1) and polymerization of the reaction products (Fig. 2).

Preliminary experiments were also conducted to characterize the inhibitory effect of the breakdown products released by limited tryptic digestion (5 min, E:S = 1:60; room temperature) on BAPA (N_{α}-benzoyl-DL-arg-4-nitroanilide) hydrolysis. The trypsin inhibitor activity in BAPA U/mg BSA or BSA-derivative were

calculated as described in the methods section. The results showed that unmodified BSA (control) reduced trypsin activity by 58 BAPA U/mg, whereas the samples 2 and 6 gave almost similar values of 97.5 and 104 BAPA U/mg. These observations demonstrate the inhibitory effects of the breakdown products released from BSA–chlorogenic acid derivatives. But, there is no clear dependence on the level of modification.

4. Conclusions

This, and former results, confirm that chlorogenic acid reacts with proteins, consequently blocking their lysine, tryptophan and cysteine residues. The reaction proceeds to a further stage, where polymerization takes place. The isoelectric point of the BSA was shifted to lower pH values. Significant structural changes were observed after these reactions. CD studies, intrinsic fluorescence and DSC measurements, as well as ANS binding experiments, indicated distinct changes in the BSA conformation. The protein became unfolded and hydrophilic, opening possibilities for electrostatic interactions with polar solvents and thereby influencing the solubility of the derivatives. The enzymatic digestion of these derivatives by trypsin and pepsin was adversely effected. Inhibitory effects of the breakdown products released by the tryptic digestion of such derivatives were also observed. These results underline the possible nutritional consequence of such reactions in food systems, resulting in limited digestibility of proteins as well as limited availability of the essential amino acids, lysine and tryptophan. Further, after intake of food materials containing phenolic compounds and after their corresponding absorption, these free ligands may interact with many transport proteins and enzymes, as shown by the in vitro experiments of caffeic acid with human serum albumin (Muralidhara & Prakash, 1995). The nutritional and toxicological significance of consuming such protein-phenol derivatives is largely unknown. These preliminary results are significant with regard to further in vivo experiments, planned for assessing physiological and toxicological effects of proteins derivatized with phenolic compounds.

Possible effects on techno-functionality of proteins, opening good strategies and possibilities for chemical tailoring of proteins, especially in the non-food sector, can also be recognized. The hydrophobic–hydrophilic balance has also been modified by reactions of BSA with chlorogenic acid, which in turn may influence the functional properties (solubility, emulsion, and foam properties) of the derivatives. On the other hand, to optimize the biological utilization of proteins, a better understanding is needed of the various interrelated parameters that influence their nutritional value. Such interactions of plant phenols with indigenous proteins, especially of those contained in oil plant seeds (e.g. sunflower, soy beans and rapeseeds) should be considered when optimizing harvest, storage and oil processing conditions. Another important field, where such interactions also play an important role is that of protein extraction, which generally takes place under alkaline conditions. The utilization of plant proteins for nutritional purposes may as a result be limited by their denaturation, particularly when their utilization does not involve indigenous phenolic compounds, phytic acid and other secondary plant metabolites, which might undergo interactions with such proteins.

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References

Adler-Nissen, J. (1972). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural & Food Chemistry*, *27*, 1256–1262.

Baxter, N. J., Lilley, T. H., Haslam, E., & Williamson, M. P. (1997). Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry*, *36*, 5566–5577.

Bond, J. S. (1989). Commercially available proteases. In R. J. Beynon, & J. S. Bond (Eds.), *Proteolytic enzymes* (pp. 232–240). Oxford: IRL Press.

Gerbanowski, A., Malabat, C., Rabiller, C., & Guéguen, J. (1999). Grafting of aliphatic and aromatic probes on rapeseed 2S and 12S proteins: Influence on their structural and physicochemical properties. *Journal of Agricultural & Food Chemistry*, *47*, 5218–5226.

Hayakawa, S., & Nakai, S. (1985). Solubility of milk and soy proteins. *Journal of Food Science*, *50*, 486–491.

Ismond, M. A. H., Murray, E. D., & Arntfield, S. D. (1985). Stability of vicilin, a legume seed storage protein, with step-wise electrostatic modification. *Int. J. Peptide Protein Res.*, *26*, 584–590.

Ismond, M. A. H., Murray, E. D., & Arntfield, S. D. (1988). The role of noncovalent forces in micelle formation by vicillin from *Vicia faba*. III. The effect of urea, Guanidine Hydrochloride and sucrose on protein interactions. *Food Chemistry*, *29*, 189–198.

Jackman, J. L., & Yada, R. Y. (1989). Ultraviolet absorption and fluorescence properties of whey-potato and whey-pea protein composites. *Can. Inst. Food Sci. Technol. J.*, *22*, 252–259.

Johnson, W. C. Jr. (1999). Analyzing protein circular dichroism spectra for accurate secondary structures. *Proteins: Structure & Function Genetetics*, *35*, 307–312.

King, S. M., & Johnson, W. C. Jr. (1999). Assigning secondary structure from protein coordinate data. *Proteins: Structure Function & Genetetics*, *35*, 313–320.

Kroll, J., & Rawel, H. (2001). Reactions of plant phenols with myoglobin: Influence of chemical structure of the phenolic compounds. *Journal of Food Science*, *66*(1), 48–58.

Kroll, J., Rawel, H., & Seidelmann, N. (2000). Physicochemical properties and susceptibility to proteolytic digestion of myoglobin-phenol

derivatives. *Journal of Agricultural & Food Chemistry*, *48*(5), 1580–1587.

Kronman, M. J., & Robbins, F. M. (1970). Buried and exposed groups in proteins. In G. D. Fasman, & S. N. Timasheff (Eds.), *Fine structure of proteins and nucleic acids* (pp. 271). New York, NY: Marcel Dekker.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T. *Nature*, *277*, 680–685.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, J. (1951). Protein measurement with folin phenol reagent. *Journal of Biological Chemistry*, *193*, 265–275.

Mason, H. S., & Peterson, E. W. (1955). The reaction of quinones with protamine and nucleoprotamine: N-terminal proline. *Journal of Biological Chemistry*, *212*, 485–493.

Muralidhara, B. K., & Prakash, V. (1995). Interaction of 3′-O-caffeoyl D-quinic acid with human serum albumin. *Int. J. Peptide Protein Res.*, *46*, 1–8.

O'Keefe, D. O. (1994). Quantitative electrophoretic analysis of proteins labeled with monobrimane. *Analytical Biochemistry*, *222*, 86–94.

Rawel, H., Kroll, J., & Hohl, U. C. (2001). Model studies on reactions of plant phenols with whey proteins. *Nahrung/Food*, *45*(2), 72–81.

Rawel, H., Kroll, J., & Riese, B. (2000). Reactions of chlorogenic acid with lysozyme- physicochemical characterisation and proteolytic digestion of the derivatives. *Journal of Food Science*, *65*(6), 1091–1098.

Rawel, H., Kroll, J., & Rohn, S. (2001). Reactions of phenolic substances with lysozyme-physicochemical characterisation and proteolytic digestion of the derivatives. *Food Chemistry*, *72*(1), 59–71.

Rawel, H., Rohn, S., & Kroll, J. (2000). Reactions of selected secondary plant metabolites (glucosinolates and phenols) with food proteins and enzymes—influence on physico-chemical protein properties, enzyme activity and proteolytic degradation. In *Recent research developments in phytochemistry*, Vol. 4 (pp. 115–142). India: Research Signpost.

Schwenke, K. D., Knopfe, C., Seiffert, A., Görnitz, E., & Zirwer, D. (2000). Acetylation of faba been legumin: conformational changes and aggregation. *Journal of the Science of Food Agriculture*, *81*, 126–134.

Siebert, K. J. (1999). Effects of protein-polyphenol interactions on beverage haze, stabilization, and analysis. *Journal of Agricultural & Food Chemistry*, *47*, 353–362.

Siebert, K. J., Troukhanova, N. V., & Lynn, P. Y. (1996). Nature of polyphenol-protein interactions. *Journal of Agricultural & Food Chemistry*, *44*, 80–85.

Sreerama, N., & Woody, R. W. (2000). Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON and CDSSTR methods with an expanded reference set. *Analytical Biochemistry*, *287*, 252–260.

Suryaprakash, P., Kumar, R. P., & Prakash, V. (2000). Thermodynamics of interaction of caffeic acid and quinic acid with multisubunit proteins. *International Journal of Biological Macromolecules*, *27*, 219–228.