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Food Chemistry 72 (2001) 59–71

Food Chemistry

www.elsevier.com/locate/foodchem

Reactions of phenolic substances with lysozyme — physicochemical characterisation and proteolytic digestion of the derivatives

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Received 5 April 2000; received in revised form 20 June 2000; accepted 20 June 2000

Abstract

Lysozyme was modified by covalent attachment of selected phenolic compounds (*m*-, *o*-, *p*-dihydroxybenzenes, ferulic and gallic acid) at pH 9. The derivatives formed were characterized in terms of their physicochemical and digestion properties. The derivatization was accompanied by a reduction of lysine and tryptophan residues. Moreover, the solubility of the derivatives decreased over a broad pH range and the derivatization increased the hydrophobicity. The isoelectric points were shifted to lower pH values and formation of high molecular weight fractions occurred. In vitro experiments showed that, the peptic digestion of the derivatized lysozyme was adversely affected, whereas the tryptic, chymotryptic and pancreatic hydrolysis seemed to be favoured. The lytic activity of all the resulting lysozyme derivatives was reduced. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Plant phenolic substances; Lysozyme; Food protein derivatization; Physicochemical characterization; *In-vitro* proteolytic degradation; Lytic activity

1. Introduction

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 (Friedman, 1996). Enzyme-catalyzed browning reactions of amino acids and proteins with oxidized plant phenols may cause deterioration of foods during storage and processing, leading to a loss in nutritional quality, which is especially serious in underprivileged countries. Plant phenols may also display many other possible detrimental effects, including inhibition of iron absorption (Mehansho, Butler & Carlson, 1987) and irreversible complexation of gut enzymes and dietary proteins (Mehansho et al.; Robbins, Hagerman, Austin, McArthur & Hanley, 1991; Scalbert, 1991), the consequences of which may result in polyphenol-rich foods being nutritionally poor, causing simultaneous protein deficiency. But the phenolic compounds have also been attributed with positive properties such as antimutagenic and anticarcinogenic effects, as well as being antioxidants — a possible mechanism by which dietary components protect the body from free radicals and reactive oxygen species (Friedman, 1997; Maleveille, Hautefeuille, Pignatelli, Talaska, Vineis & Bartsch, 1996).

Accurate data on population-wide intakes of phenolic compounds are not available. Recent studies show that the human dietary intake of chlorogenic acids and other cinnamates ranges from 25 mg up to 1 g/day, depending on the dietary constitution (Clifford, 1999). The occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods has been summarized by Herrmann (1989). Cinnamic acid conjugates (e.g. caffeic-, chlorogenic- and feruloylquinic acids) are widely distributed in the plant kingdom and their content in many beverages, fruits and vegetables

Abbreviations: BAEE, *N*α-benzoyl-L-arginine ethyl ester; DHB, Dihydroxybenzene; EGCG, (−)-epigallocatechin-3-gallate; E:S, enzyme:substrate ratio; MALDI-TOF-MS, matrix assisted laser desorption/ionization time of flight mass spectrometry; RP-HPLC, reverse phase-high performance liquid chromatography; SDS, Na-dodecylsulfate; TCA, trichloroacetic acid; TNBS, trinitrobenzenesulfonic acid.

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PII: S0308-8146(00)00206-5

has recently been reviewed by Clifford. The content of chlorogenic acid is relatively high with 6–10% in green coffee beans, and a cup of coffee may contain up to 675 mg (Clifford). The main source of gallic acid is tea, especially green tea, where it occurs as (−)-epigallocatechin-3-gallate (EGCG) and (−)-epigallocatechin. The consumption of EGCG was estimated for Japan to be as high as 1g/per day (Authors group, 1999). The human dietary intake of flavonoids (a further class of plant phenols) was estimated as up to 1 g/day (Maleveille et al.). A Dutch study showed, that fruit was the main dietary source of flavonoids and quercetin was the main aglycone (Robards, Prenzler, Tucker, Swatsitang & Glover, 1999). The absorption, metabolism and bioavailability of phenolic compounds is also controversial. Data on these aspects of phenolics are scarce and the limited data available have recently been reviewed by Robards et al. and by Chesson, Provan, Russel, Scobbie, Richardson and Stewart (1999).

The phenolic compounds are subject to both enzymatic and nonenzymatic oxidation in the presence of oxygen (Cilliers & Singleton, 1991; Friedman, 1997). Much attention has been devoted to determining the exact pathway by which enzymatic browning may occur. It is now generally accepted (Nicolas, Richard-Forget, Goupy, Amiot & Aubert, 1994) that two reaction steps are involved. The first reaction step consists of the hydroxylation of monophenols into *o*-diphenols and the oxidation of the *o*-diphenols into *o*-quinones (Nicolas et al.). These two reactions consume oxygen and are catalyzed by the enzymes monophenolase (or cresolase, EC 1.14.18.1) and *o*-diphenolase (or catecholase, EC 1.10.3.1). A third enzyme system involving laccases (EC 1.10.3.2), oxidizes *o*-diphenols as well as *p*-diphenols, forming their corresponding quinones. The unique ability to oxidize *p*-diphenols is used to distinguish laccase activity from that of the other polyphenoloxidases (Nicolas et al.). The quinones, as the primary products of enzymatic oxidation, represent a species of highly reactive substances which normally react further with other quinones to produce dark melanin pigments (Robards et al., 1999). These result in deterioration of flavor, color and nutritional quality of foods. The quinone, being a reactive electrophilic intermediate can also readily undergo attack by nucleophiles such as lysine, methionine, cysteine and tryptophan residues in a protein chain (Hurrell & Finot, 1984). The 2-position of the benzene ring, e.g. in the caffeic acid molecule, is the most electrophilic, and nucleophilic addition occurs preferentially here (Cheynier, Trousdale, Singleton, Salgues & Wylde, 1986). Upon further oxidation of this addition product, e.g. by laccase to form its quinone, a second addition occurs (Salgues, Cheynier, Gunata & Wylde, 1986), which may lead to formation of cross-linked protein polymers (Machholz & Lewerenz, 1989). The presence of phenolic compounds in foods and beverages produces a sensation of astringency, possibly arising from precipitation of oral proteins and mucopolysaccharides. They 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, Lilley, Haslam & Williamson, 1997).

The present study is a continuation of our attempt to characterize the nonenzymatic reactions of food proteins with secondary plant metabolites from the physicochemical and physiological viewpoint. In preliminary investigations, reactions of chlorogenic acid with lysozyme were characterized (Rawel, Kroll & Riese-Schneider, 2000). In the present study an attempt was made to show the effect of reactions of lysozyme with different simple mono-(ferulic acid), di-(*o*-, *m*-, *p*-dihydroxybenzene) and trihydroxy-(gallic acid) phenols in a model system. In this study it was intended to show the influence of the position and amount of the hydroxyl groups present in the respective phenols. Further, *p*-quinone representing the oxidation product of *p*-dihydroxybenzene was also included in the study (*o*-quinone was not applied due its relative instability). Lysozyme, as a food protein serves well for such model investigations, since its structure is well defined and characterized. The lysozyme derivatives (with special attention to the role of covalent bonding) have been characterized in terms of changes in selected physicochemical properties and with regard to influence on their digestion with the main physiological proteolytic enzymes (trypsin, α-chymotrypsin, pepsin and pancreatin) of the gastrointestinal tract. The effect of lysozyme derivatized with phenolic compounds) on its lytic activity against *Micrococcus lysodeikticus* cells has also been investigated.

2. Materials and methods

2.1. Materials

Lysozyme (3 g) from hen egg white (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 270 ml). Based on the amount of lysine present in lysozyme (486 nmol/mg protein), the derivatives were prepared with corresponding double amounts of the phenolic compounds as follows:

1. 27 ml of lysozyme solution + 3 ml distilled water — control, unmodified sample.
2. 27 ml of lysozyme solution + 33 mg of *m*-(resorcinol), *o*-(pyrocatechol) and *p*-(hydroquinone) dihydroxybenzene (DHB), each in 3 ml distilled water and each added to a separate lysozyme solution — *m*-, *o*- and *p*-DHB derivatives.

3. 27 ml of lysozyme solution + 32.4 mg *p*-(1,4-benzoquinone) quinone in 3 ml distilled water — *p*-quinone derivative.
4. 27 ml of lysozyme solution + 58.3 mg ferulic acid — ferulic acid derivative.
5. 27 ml of lysozyme solution + 56.4 mg gallic acid monohydrate — gallic acid derivative.

In each case, after addition of the respective phenolic compounds and *p*-quinone (all from Fluka Chemie AG, Buchs, Switzerland), the pH was adjusted once more to 9. After 24h reaction time under continuous stirring at room temperature (24°C) with free exposure to air, the samples were dialyzed for 18–20 h against distilled water and finally lyophilized. The non-derivatized protein (control) was prepared under the same conditions but without addition of phenolic compound.

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.

Pancreatin was from porcine pancreas (Sigma Chemicals Co., St. Louis, MO) — activity at least equivalent to 3 × USP specifications. All specific activities and definitions quoted here were given by the producers.

Micrococcus lysodeikticus, ATCC 4698 was as lyophilized cells (Sigma Chemicals Co., St. Louis, MO).

2.2. Physicochemical characterization

The protein content in the solutions was determined according to a modified LOWRY method (Lowry, Rosebrough, Farr & Randall, 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 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 analyzed using trinitrobenzenesulfonic acid (TNBS) in a 1% Na-dodecylsulfate (SDS) solution of the samples. Tryptophan fluorescence determination (Jackman & Yada, 1989) in 8 M urea using a 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 300–900 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 Shimadzu (Duisburg, Germany) chromatographic system using a Micra-NPS-C18 column (33 × 4.6 mm, 1.5 μm, flow rate 0.5 ml/min, UV detection at 220 nm) with a column temperature of 25°C. A distilled water/acetonitrile (water acidified with 0.1% trifluoroacetic acid v/v) gradient was applied under the following conditions: 20% acetonitrile–2 min; 20–40% acetonitrile — 16 min; 40–70% acetonitrile — 2 min; 70% acetonitrile — 2 min; 70–20% acetonitrile — 2 min; 20% acetonitrile — 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, 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 in Kroll and Rawel (1996). Sodium hydroxide and phosphoric acid were used as electrode buffers, respectively. Pre-electrophoresis was done at 1000 V with 6 mA for 1 h. The main electro-focussing 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 1 ml 0.1% trifluoroacetic acid/acetonitrile (50%, 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 Rawel, Kroll, Haebel and Peter (1998).

2.3. Proteolytic digestion

Tryptic, chymotryptic and pancreatic hydrolysis (100 μl enzyme, 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 [enzyme/substrate ratio (E:S) = 1:60]. After 60 min of digestion, 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 × 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; 0-70% acetonitrile — 10 min; 70% acetonitrile — 4 min; 70% acetonitrile–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 enzymatic digestion.

Peptic digestion (50 μl pepsin, 1 mg/ml) of lysozyme as well as its derivatized products (6 mg/1.5 ml; 2 M urea, 0.074 M HCl; E:S = 1:120) 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 analogue, by the procedure described above.

2.4. Lytic activity

The evaluation of the lytic activity against *Micrococcus lysodeikticus* was carried out according to turbidometric methods based on the decrease in turbidity of a cell suspension following the addition of lysozyme or its derivatives with the phenolic substances. The Sigma quality control test procedure for the enzymatic assay of lysozyme (Sigma Chemicals Co., St. Louis, MO) according to Shugar (1952) was used to determine the activity.

2.5. Statistical analysis

The digestions and other analyses were repeated thrice and evaluated by standard deviation. Student *t*-test and Anova / Post-hoc-test were performed where applicable (Tables 1 and 2). A maximum of ± 5% standard deviation from the averaged values was generally tolerated. The averaged values are documented in the respective figures.

3. Results and discussion

3.1. Characterization of physicochemical properties

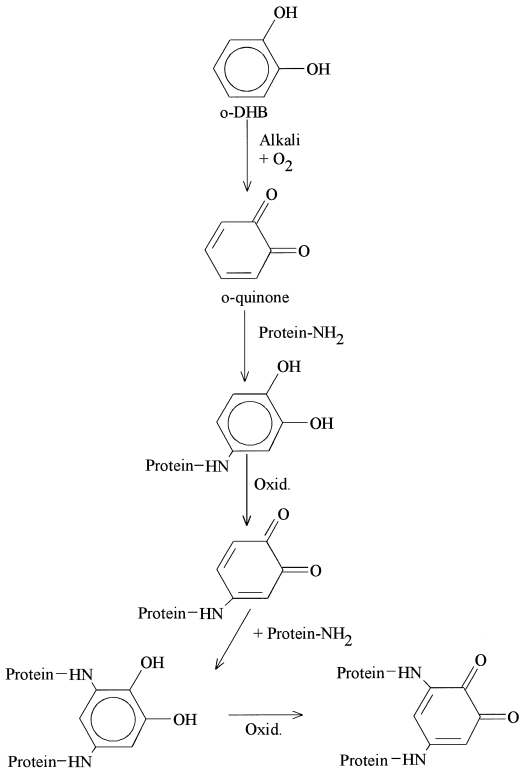
The reaction of lysozyme with *p*-quinone produced a derivative, which was insoluble and could not be characterized with the applied physicochemical methods. *p*-Quinone is not a phenolic compound, but represents the oxidation product of *p*-DHB.

3.2. Reaction at the free amino groups

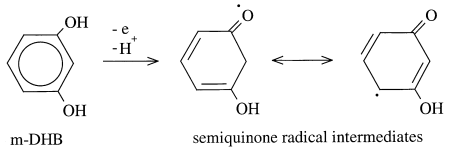
The calculated theoretical value for the amount of free amino groups in lysozyme amounts to 486 nmol/mg protein (based on molecular weight of 14,400 Da). The experimentally determined mean value was slightly less, amounting to 447 nmol/mg protein. A reaction of lysozyme with a phenolic compound produces a corresponding decrease in the amount of free amino groups, as determined using TNBS reagent (Table 1). All values obtained for the derivatives are significantly different from the control lysozyme, as shown by applying *t*-test and ANOVA post hoc tests (Table 1). The highest reactivity was noted for the gallic acid derivative, where up to 37% of the free amino groups were blocked. The reactivity of the phenolic compounds is influenced by the number and the position of the hydroxyl groups. As a result, the reactivity against free amino groups, and the strength of binding, follows the order: gallic acid > *o*-DHB > *p*-DHB > ferulic acid > *m*-DHB. Since the amount of free amino groups was determined in the presence of 1% SDS (a well known agent, which destroys non-covalent protein interactions), we can assume, that the derivatization has occurred through covalent binding. Generally, 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. The stepwise addition of protein bound amino groups e.g. to oxidized *o*-DHB, can proceed according to Macholz and Lewerenz (1989) as follows.

3.2.1. Reaction (1)

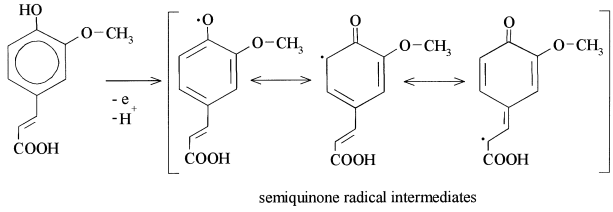
The rate of oxidation is pH-dependent, as documented for caffeic acid (Cilliers & Singleton, 1991); therefore the further reaction with nucleophilic amino groups was correspondingly influenced as observed for chlorogenic acid (Rawel et al., 2000). The formation of phenolate ions is believed to be necessary for the formation of an intermediate semiquinone which will then undergo further reaction (Cilliers & Singleton) *p*-DHB and gallic acid react in a similar way. Such reactions have been reported by Hurrell and Finot (1984) as well as by Pierpoint (1969a,1969b). 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 [reaction (1)]. Similarly, no reaction of protein-bound amino groups can be expected for *m*-DHB and ferulic acid, since both these phenolic compounds cannot be oxidized to corresponding quinone derivatives. Table 1 shows that the significance *t*-test a



Reaction (1).



Reaction (2).



Reaction (3).

possible reaction of *m*-DHB and ferulic acid with nucleophilic amino groups. One possible explanation is the formation of a semiquinone radical, which in turn can attack a nucleophilic reaction partner, as proposed in the following reaction steps [reaction (2) for *m*-DHB and reaction (3) for ferulic acid].

3.2.2. Reaction (2) + reaction (3)

Similar reaction mechanisms, proceeding with phenolate ion formation, which may be followed by reaction

Table 1
Free amino group content of lysozyme phenol derivatives

Sample	Free amino groups			
	(nmol/mg protein)	(%[a])	*t*-test[b]	Anova post-hoc tests[b]
Lysozyme, control	447±20.4	100.0±4.5	–	–
m-DHB derivative	407±18.2	91.0±4.5	0.01	0.00
o-DHB derivative	327±7.08	73.1±2.2	0.00	0.00
p-DHB derivative	354±5.98	79.3±1.7	0.00	0.00
Ferulic acid derivative	385±18.4	86.1±4.8	0.00	0.00
Gallic acid derivative	280±12.7	62.8±4.5	0.00	0.00

[a] In relation to 447 nmol/mg protein set as 100% for unmodified lysozyme.
[b] In comparison to unmodified, control lysozyme.

Table 2
Tryptophan content of lysozyme phenol derivatives

Sample	Tryptophan content			
	(nmol/mg)	(%[a])	*t*-test[b]	Anova post-hoc tests[b]
Lysozyme control	28.8±0.07	100±0.2	–	–
m-DHB derivative	24.9±0.13	86.3±0.5	0.00	0.00
o-DHB derivative	12.1±0.04	42.0±0.3	0.00	0.00
p-DHB derivative	7.34±0.04	25.5±0.5	0.00	0.00
Ferulic acid derivative	28.3±1.27	98.2±4.5	0.53	0.24
Gallic acid derivative	4.71±0.05	16.4±1.1	0.00	0.00

[a] In relation to 28.78 nmol/mg set as 100% for unmodified lysozyme.
[b] In comparison to unmodified, control lysozyme.

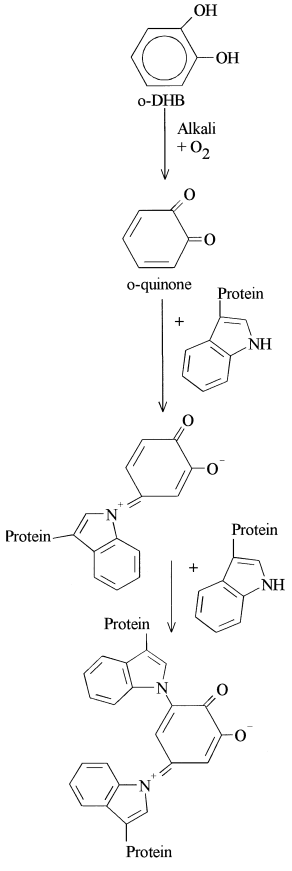
with oxygen to produce semiquinones, have also been postulated for oxidation reactions of caffeic acid by Cilliers and Singleton (1991). The semiquinones thus formed react further with ϵ-amino groups of lysine. The nutritional consequence of these oxidative reactions of phenolic compounds is the limited availability of the essential amino acid lysine.

3.3. 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), as the following reaction shows.

3.3.1. Reaction (4)

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 and 360 nm. The reactivity of the phenolic compounds increased with higher number of hydroxyl substituents and depends on the position of the hydroxyl groups (Table 2). As a result the reactivity and the strength of binding follows the order: gallic acid > *p*-DHB > *o*-DHB > *m*-DHB > ferulic acid. Since the tryptophan fluorescence was measured in the presence



Reaction (4).

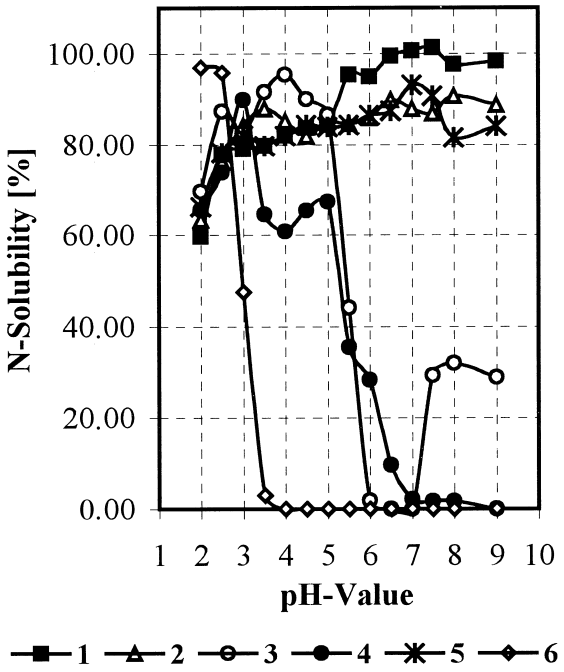


Fig. 1. Solubility profiles of lysozyme derivatives. Code: 1 = unmodified lysozyme control; 2 = *m*-DHB derivative; 3 = *o*-DHB derivative; 4 = *p*-DHB derivative; 5 = ferulic acid derivative; 6 = gallic acid derivative.

of 8 M urea (destroying of non-covalent protein interactions), it can be assumed that covalent binding has occurred as illustrated in reaction (4). All values obtained for the derivatives, except for the ferulic acid derivative, are significantly different from the control lysozyme as shown by applying *t*-test (Table 2). This means that *m*-DHB has also reacted to a considerable extent as illustrated in Table 2. The reaction of *m*-DHB may proceed with phenolate ion formation, which is followed by reaction with oxygen to produce semiquinones intermediates [compare reaction (2). The semiquinones thus formed may react further with the heterocyclic N-atom of tryptophan. The results document, that the indole structure of tryptophan in the lysozyme derivatives is most likely to be involved in the reaction with the phenolic substances. The nutritional consequence of these oxidative reactions is the limited availability of the essential amino acid tryptophan.

Besides the above illustrated influence on lysine and tryptophan concentrations of lysozyme, the content of the essential methionine may also be adversely affected by undergoing similar reactions with oxidized phenolic compounds (Macholz & Lewerenz, 1989).

3.4. Changes in solubility

The reaction of lysozyme with phenolic substances is well illustrated by the solubility profile of the N-containing substances as shown in Fig. 1. The control

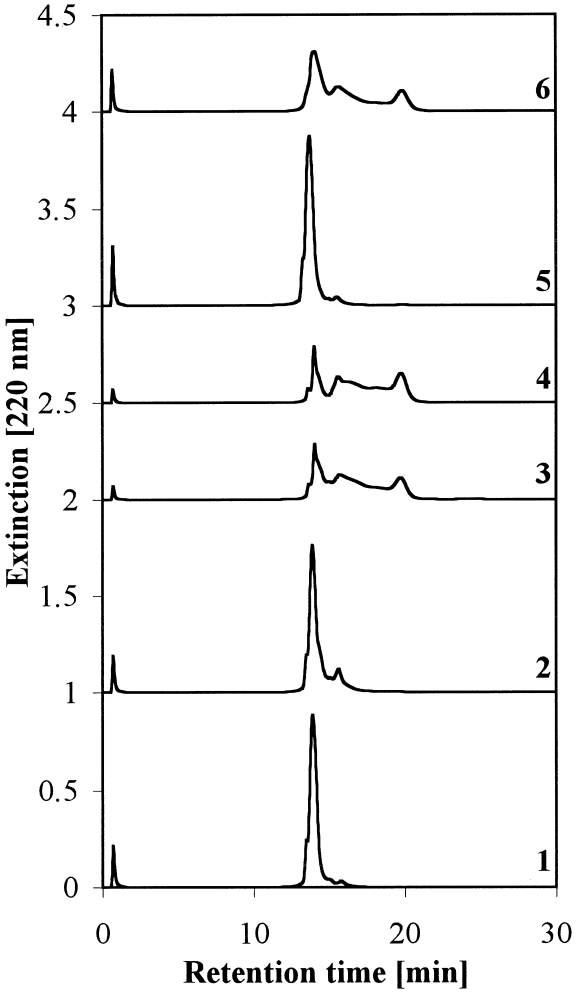


Fig. 2. RP-HPLC of lysozyme derivatives. Code:1 = unmodified lysozyme control; 2 = *m*-DHB derivative; 3 = *o*-DHB derivative; 4 = *p*-DHB derivative; 5 = ferulic acid derivative; 6 = gallic acid derivative.

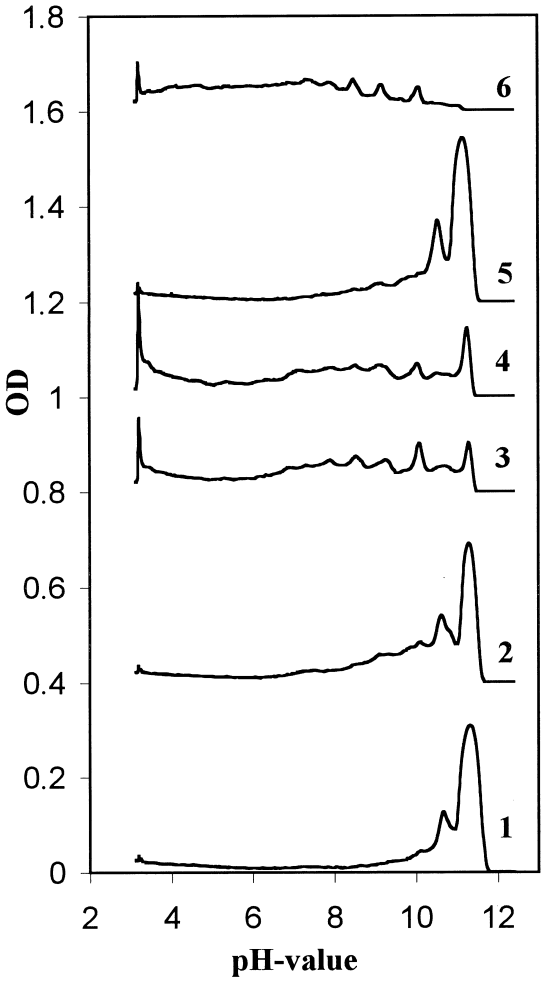


Fig. 3. IEF of lysozyme derivatives. Code: 1 = unmodified lysozyme control; 2 = *m*-DHB derivative; 3 = *o*-DHB derivative; 4 = *p*-DHB derivative; 5 = ferulic acid derivative; 6 = gallic acid derivative.

unmodified lysozyme had a good solubility in buffer over the investigated pH range (curve 1, Fig. 1). The experimental and calculated isoelectric point of lysozyme (hen egg white) lies between pI 10.5 and 11.3 at 25°C (Patrickios & Yamasaki, 1995). Under the investigated conditions the alternation in solubility around the isoelectric points could not be seen due to lack of an appropriate buffer. Since the reaction of lysozyme with the derivatizing substances is accompanied by a corresponding blocking of the hydrophilic amino groups, a parallel change in solubility should be expected. Both the lysozyme derivatives, with m-DHB and ferulic acid, showed a slight change in solubility in the pH range 5–9 (curves 2 and 5, Fig. 1), which decreased in comparison with the unmodified control lysozyme (curve 1, Fig. 1). Similarly, the solubility of the *o*-DHB lysozyme derivative decreased even further, in agreement with the comparatively higher amounts of free amino groups and tryptophan blocked (see Tables 1 and 2), and of minimum solubility in the pH range 6–7 was noted (curve 3, Fig. 1). A further shift of the isoelectric range of lysozyme to a lower pH range (3.5–9.0) was registered for the reaction product with *p*-DHB (curve 4, Fig. 1). The highest effect on the solubility was observed for the gallic acid derivative (curve 6, Fig. 1). In this case the decrease in solubility was extended further to the pH range 3–9, and the reaction product was completely insoluble in the pH range 3.5–9. These changes in solubility reflect, not only the respective changes in the amount of charged groups as shown for free amino groups (Table 1), but also the introduction of carboxylic groups following the covalent attachment of the phenolic acids. From nutritional and technological-functional points of view, such decrease in solubility may adversely affect the technological protein extraction from raw materials with relative high content of phenolic compounds (e.g. sorghum, sunflower, rape seed).

3.5. Changes in hydrophilic/hydrophobic character

A further molecular property of lysozyme that can be affected by the reaction with phenolic substances is its hydrophilic–hydrophobic character, which can be illustrated by RP-HPLC (Fig. 2). An increase in hydrophobicity was observed, as illustrated by the corresponding rise of the retention times of the main peak. In agreement with the above discussed results, the chromatographic patterns for unmodified control lysozyme and the ferulic acid derivative showed no apparent changes (curves 1 and 5, Fig. 2). The chromatogram for the *m*-DHB derivative showed the beginning of a second peak besides the main peak having increased retention time and exhibiting a corresponding change in hydrophobicity. The formation of new peaks with higher retention times in the respective chromatograms of the *o*-, *p*-DHB and gallic acid derivatives of lysozyme reflects the respective parallel increase in the hydrophobicity (curves 3, 4 and 6, Fig. 2). The peak formation was adversely affected, possibly due to denaturation (structural changes) and molecular interactions. Further, there seems to be a close relationship between the increasing insolubility of the samples and the increasing hydrophobicity. A good correlation between insolubility and hydrophobicity has also been reported for different proteins, e.g. milk and soy proteins (Hayakawa & Nakai, 1985).

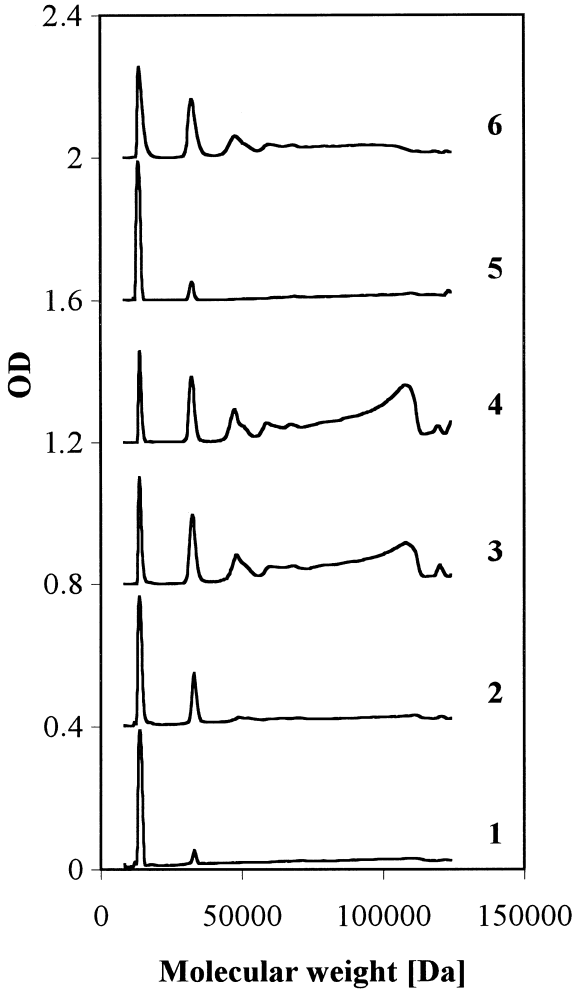


Fig. 4. SDS-PAGE of lysozyme derivatives. Code: 1 = unmodified lysozyme control; 2 = *m*-DHB derivative; 3 = *o*-DHB derivative; 4 = *p*-DHB derivative; 5 = ferulic acid derivative; 6 = gallic acid derivative.

3.6. Isoelectric focussing

The derivatization changes the amount of charged groups, as shown for free amino groups (Table 1). Generally, a loss of charged groups, due to addition of the oxidized phenolic moiety, for example to the ϵ-amino groups of lysine side chains but also the introduction of carboxylic groups following the covalent attachment of the phenolic acids, is accompanied by a change of the isoelectric point of the proteins. The experimental and calculated isoelectric point of lysozyme (hen egg white) lies between pI 10.5 and 11.3 at 25°C (Patrickios & Yamasaki, 1995). As shown in Fig. 3, this corresponds to the peaks in the unmodified control sample with pI values between 10.0 and 11.8. In conjunction with the decrease in the amount of the positively charged free amino groups of lysozyme (Table 1) and the introduction of carboxylic groups following the covalent attachment of the phenolic acids, the net negative charge of the molecules may increase depending on the degree to which they have been derivatized by the oxidized phenolic compounds. Consequently, a loss of the charged groups, due to addition of the oxidized phenolic moiety to proteins, is accompanied by a change of their isoelectric points. The reaction of lysozyme with *m*-DHB and ferulic acid produce, in agreement with the other results, only slight changes in the pI range of 8.5–10.2 (curves 2 and 5, Fig. 3). A subsequent reaction of the other phenolic substances (*o*-, *p*-DHB and gallic acid), with lysozyme, shifts the isoelectric range from pI 10.0 to 11.8 in the control to pI 6.5–10.2 for the majority of the reaction products (curves 3, 4 and 6, Fig. 3). The area under the main peak (pI 11.3) decreased and was not detected in the corresponding electrophortogram of the gallic acid derivative of lysozyme. As a result, we observe many new sub-fractions, each representing different extents of derivatization, having in each case a different electrophoretical mobility and, correspondingly, a different isoelectrical point. Five new distinct peaks with pI 10, 9.2, 8.5, 7.8 and 7.1 were shown for the derivatives. Generally, we can summarize the following: with higher degrees of derivatization, lower isoelectrical points for sub-fractions can be observed; i.e. the proteins become more acidic in nature.

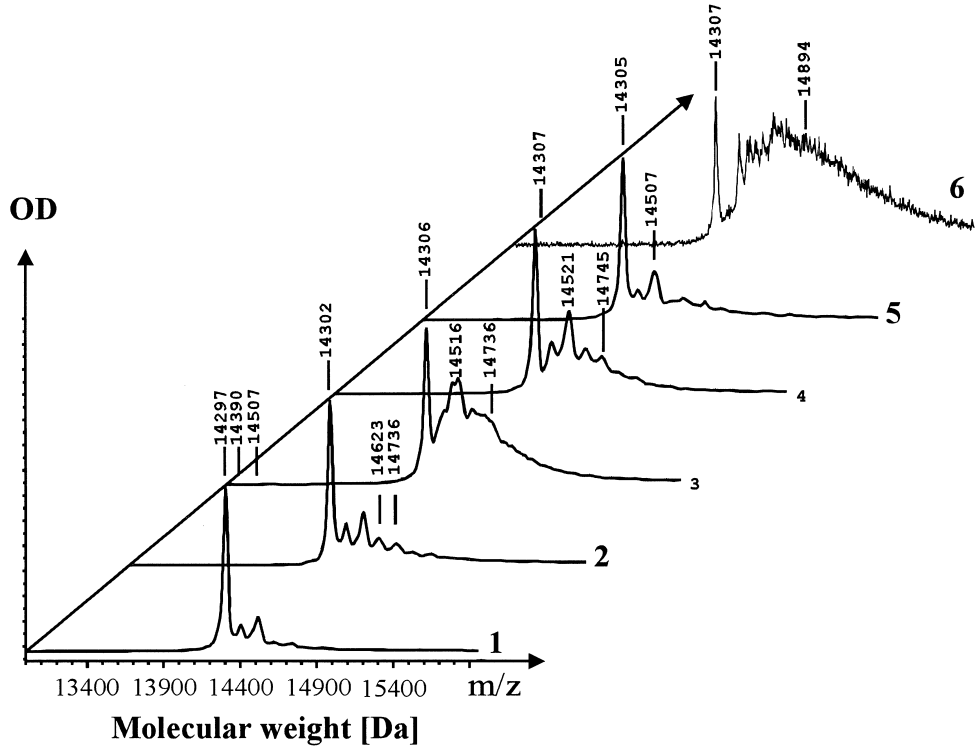


Fig. 5. MALDI-TOF-MS of lysozyme derivatives. Code: 1 = unmodified lysozyme control; 2 = *m*-DHB derivative; 3 = *o*-DHB derivative; 4 = *p*-DHB derivative; 5 = ferulic acid derivative; 6 = gallic acid derivative.

3.7. SDS-PAGE analysis

SDS-PAGE investigations of the control lysozyme showed a main fraction with a molecular weight of ca. 14,000 Da (Fig. 4, curve 1), similar to values cited in the literature (14,400 Da) and as determined using MALDI-TOF-MS (14,297 Da, curve 1, Fig. 5). The ferulic acid derivative showed (in comparison to unmodified lysozyme), no significant changes in molecular composition (curves 1 and 5, Fig. 4). The derivatization leads to the formation of a dimer with a molecular weight of ca. 32,000 Da, the relative concentration of which increased with the extent to which the protein became derivatized. Moreover, the formation of higher molecular weight fractions with ca. 47,000, 58,000 and 67,000 Da was also recorded (curves 3, 4 and 6, Fig. 4). Much of the derivatized protein had fractions of even higher molecular weights, which remained at the beginning of the separating gel or did not move into the gel at all (curves 3 and 4, Fig. 4). These high molecular complexes, were not spliced by use of SDS and mercaptoethanol. The latter reagent is generally used to split disulfide bonds during sample preparation. This is a further indication that the bonds formed between the molecules must be of a covalent nature. The formation of polymerized products, as a result of the reactions of phenolic compounds with lysozyme, follows the order:*p*-DHB > *o*-DHB > gallic acid > *m*-DHB > ferulic acid. Although gallic acid showed the highest reactivity against free amino groups and tryptophan of lysozyme (Tables 1 and 2), the formation of polymerized products seems to proceed here to a lesser extent than that observed with *o*- and *p*-DHB (compare curves 3, 4 and 6, Fig. 4). Hurrell and Finot (1984), Pierpoint (1969a,1969b) 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. Phenols may be oxidized with ease, in an alkaline solution, to their corresponding quinones (Hurrell & Finot). 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 & Finot). After this first nucleophilic addition and further oxidation of this product to form its quinone, a second addition occurs, which leads to formation of cross-linked protein polymers as shown in reaction (1) and (4). The possibility of such consequent covalent cross-linking, as a result of reactions at the free amino groups and tryptophan discussed above, can be confirmed by SDS-PAGE of lysozyme derivatives.

3.8. 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 are presented in Fig. 5. The molecular weight of lysozyme, determined by this method was 14,297 Da, agreeing with data calculated from its sequence (GenBank protein sequences, accession no. 231220 for lysozyme from hen egg white). An increase in molecular weight was observed for the *o*-, *p*-DHB and gallic acid derivatives of lysozyme (curves 3, 4 and 6, Fig. 5). Furthermore, *m*-DHB also produced two new peaks in the corresponding mass spectra (molecular weight ca. 14,623 or 14,736 Da, curve 2, Fig. 5). The reaction of ferulic acid with lysozyme produced, in comparison with unmodified control lysozyme, no apparent changes in the respective mass spectra (curves 1 and 5, Fig. 5). Generally, the mass spectra show peaks, which are approximately separated by a multiple increment of the molecular weight of the reacting molecules. The reaction of lysozyme with *o*-DHB (ca. 110 Da) delivered the highest molecular mass of 14,736 Da (molecular weight at the indicated peak maximum), which in turn could account for addition of about four molecules to one lysozyme molecule ($14297 + [4 \times 110] = 14737$ Da; curve 3, Fig. 5). Similarly, addition of approximately four molecules of *o*-DHB and ca. three gallic acid molecules can be derived for the corresponding derivative peaks shown in Fig. 5. 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. These results of MALDI-MS illustrate the first primary addition of a reacting substance at, e.g. 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 or with other oxidized phenolic compounds to give brown melanin pigments.

3.9. Effect of the derivatization on the proteolytic hydrolysis

The effect of lysozyme derivatization, with phenolic compounds, on the in-vitro proteolytic digestion by the main enzymes (trypsin, α-chymotrypsin, pepsin and pancreatin) of the gastrointestinal tract was tested to give preliminary indications of 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 the diet are also partly denatured as a result of processing and cooking. The results of the digestion of lysozyme derivatives are shown in Fig. 6. In comparison with the unmodified control lysozyme, the influence of derivatization causes the tryptic, chymotryptic and pancreatic hydrolysis to become faster. There was a corresponding higher amount of trichloroacetic-acid soluble peptides (compared to the results of unmodified lysozyme hydrolysis) liberated after 1 h digestion (Fig. 6). 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. The tryptic and chymotryptic hydrolysis of lysozyme derivatives (with *m*-, *o*-, *p*-DHB and ferulic acid) in each case liberated similar amounts of trichloroacetic acid-soluble peptides. The digestion of the gallic acid derivative of lysozyme produced (with both enzymes) the highest amount of peptides (Fig. 6).

Trypsin preferentially splits 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 ϵ- amino groups of lysine side chains (Table 1), we can expect that their derivatization by a phenolic substance should prevent or at least make the tryptic degradation difficult. Furthermore as indicated by SDS-PAGE analysis (Fig. 4), the resulting polymerization may also hinder the access to 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 perhaps be explained by the preferred splitting of those peptide bonds which contain arginine and, moreover, the conformational structure must be changed such that the access to relevant peptide bonds is easier.

The chymotryptic digestion, similarly to tryptic hydrolysis, was faster (Fig. 6). 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). As shown in Table 2, the tryptophan fluorescence decreased with the increasing extent to which the proteins became derivatized. This does not correspond with the results of chymotryptic digestion, the explanation for this being similar to that for tryptic hydrolysis.

Pancreatin (according to the manufacturer) is a mixture of many enzymes, containing besides protease, amylase, ribonuclease and lipase. The proteases in pancreatin include, besides trypsin, other unspecific protein splitting enzymes. The effect of derivatization with phenolic compounds here was faster and similar to tryptic hydrolysis (Fig. 6).

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

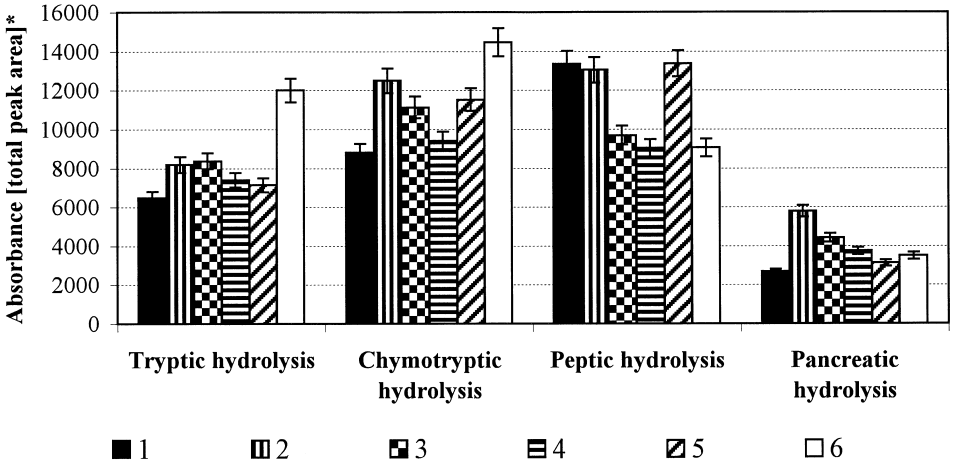


Fig. 6. Proteolytic digestion of lysozyme derivatives. Code: 1 = unmodified lysozyme-control; 2 = *m*-DHB derivative; 3 = *o*-DHB derivative; 4 = *p*-DHB derivative; 5 = ferulic acid derivative; 6 = gallic acid derivative; * TCA soluble peptides.

measurable influence of the derivatization (Fig. 6). Furthermore the influence of derivatization is more detrimental than that observed for chymotryptic, tryptic and pancreatic hydrolysis (Fig. 6). Inhibitory effects of the derivatized products on peptic digestion may be attributed in this case to their resulting conformation and structural changes. Corresponding to the above-discussed slight changes observed by the physicochemical characterization of *m*-DHB and ferulic acid derivatives of lysozyme, there is apparently no affect on their respective peptic hydrolysis. The effect on peptic hydrolysis of *o*-, *p*-DHB and gallic acid derivatives of lysozyme, on the other hand, is detrimental.

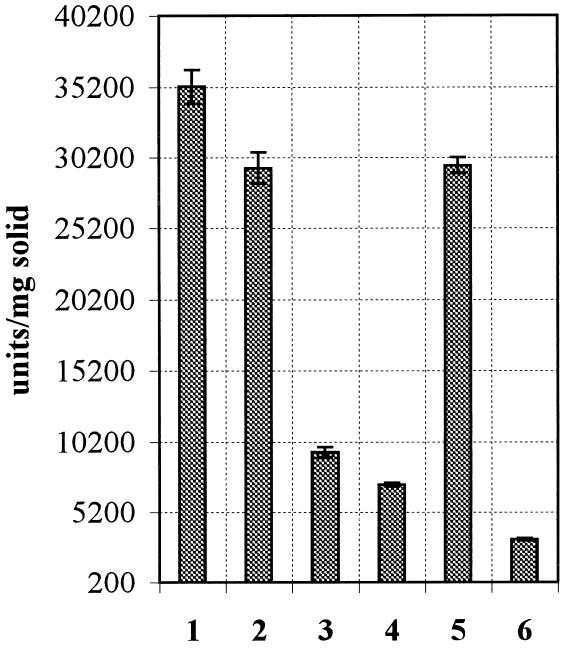


Fig. 7. Lytic activity of lysozyme derivatives against *Micrococcus lysodeikticus*. Code:1 = unmodified lysozyme control; 2 = *m*-DHB derivative; 3 = *o*-DHB derivative; 4 = *p*-DHB derivative; 5 = ferulic acid derivative; 6 = gallic acid derivative.

3.10. The evaluation of the lytic activity against Micrococcus lysodeikticus

The lytic activity of lysozyme derivatives with the different phenolic compounds is illustrated in Fig. 7. Due to covalent attachment of the phenolic substances, the lytic activity of lysozyme was significantly reduced. The effect of reaction of *m*-DHB and ferulic acid with lysozyme, in accordance with the decrease in the amounts of free amino groups and tryptophan (Tables 1 and 2) was not so high as obtained with the other investigated phenolic compounds. As a result, the reactivity and the effect on the lytic activity follows the order: gallic acid > *p*-DHB > *o*-DHB > *m*-DHB > ferulic acid. With regard to the decrease in the lytic activity a good correlation was obtained with the amount of free amino groups blocked and with the corresponding decrease of tryptophan content.

This decrease in activity of all the tested lysozyme derivatives accords with earlier investigations of Bernkop-Schnürch, Krist, Vehabovic and Valenta (1998), showing the same effect for lysozyme-caffeic acid conjugates and lysozyme-cinnamic acid conjugates. In their case, lysozyme was modified by covalent attachment of caffeic acid and cinnamic acid, respectively. The linkage of these acids was achieved by the constitution of amide bonds between the carboxyl group of ligands and the primary amino groups of lysozyme, mediated by a carbodiimide.

4. Conclusion

These and former results (Rawel et al., 2000) show that plant phenolic substances react with proteins, influencing their physicochemical and in-vitro enzymatic degradation. The reaction occurs at lysine side chains and at the indole ring of the tryptophan residues of the lysozyme. The reaction proceeds to a further stage, where polymerization to complex structures takes place. The enzymatic digestion of the lysozyme derivatives by trypsin, chymotrypsin, and pancreatin becomes faster. The proteolytic splitting of the lysozyme derivatives by pepsin was adversely affected. The lytic activity of all the resulting lysozyme derivatives was reduced. These results are significant with regard to further experiments planned, involving physiological and toxicological effects of proteins derivatized with phenolic compounds.

Acknowledgements

The authors wish to thank Dr. Sophie Haebel from University of Potsdam, Center of Biopolymers, D-14469 Potsdam, Am Neuen Palais 10, Germany, for performing the MALDI-TOF-MS analysis. This work was financially sponsored by the "Deutsche Forschungsge meinschaft" (DFG, INK research project 26).

References

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

Authors group. (1999). (−)-epigallocatechin-3-gallate (EGCG) and (−)-epigallocatechin (EGG). In F. M. Clydesdale, Special issue: ILSI North America food components reports. *CRC Critical Revision Food Science and Nutrition*, 39, 215–226.

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.

Bernkop-Schnürch, A., Krist, S., Vehabovic, M., & Valenta, C. (1998). Synthesis and evaluation of lysozyme derivatives exhibiting an enhanced antimicrobial action. *European Journal of Pharmaceutical Sciences, 6*, 301–306.

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

Chesson, A, Provan, G. J., Russel, W. R., Scobbie, L., Richardson, A. J., & Stewart, C. (1999). Hydroxycinnamic acids in the digestive tract of livestock and humans. *Journal of the Science of Food and Agriculture, 79*, 373–378.

Cheynier, V. F., Trousdale, E. K., Singleton, V. L., Salgues, J., & Wylde, R. (1986). Characterization of 2-S-glutathionylcaftaric acid and its hydrolysis in relation to grape wines. *Journal of Agricultural and Food Chemistry, 34*, 217–221.

Cilliers, J. J. L., & Singleton, V. L. (1991). Characterization of the products of nonenzymatic autoxidative phenol reactions in a caffeic acid model system. *Journal of Agricultural Food Chemistry, 39*, 1298–1303.

Clifford, M. N. (1999). Review: Chlorogenic acids and other cinnamates — nature, occurrence and dietary burden. *Journal of Science and Food Agriculture, 79*, 362–372.

Friedman, M. (1996). Nutritional value of proteins from different food sources. *A review, 44*, 6–29.

Friedman, M. (1997). Chemistry, biochemistry, and dietary role of potato polyphenols. A review. *Journal of Agricultural Food Chemistry, 45*, 1523–1540.

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

Herrmann, K. (1989). Occurence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. *Critical Reviews in Food Science and Nutrition, 28*, 315–347.

Hurrell, R. F., & Finot, P. A. (1984). Nutritional consequences of the reactions between proteins and oxidised polyphenolic acids. *Advances in Experimental and Medical Biology, 177*, 423–435.

Jackman, J. L., & Yada, R. Y. (1989). Ultraviolet absorption and fluorescence properties of whey-potato and whey-pea protein composites. *Canadian Institute of Food Science Technology Journal, 22*, 252–259.

Kroll, J., & Rawel, H. (1996). Reactions of benzyl isothiocyanate with myoglobin. *Journal of the Science of Food and Agriculture, 72*, 376–384.

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.

Machholz, R., & Lewerenz, H. J. (1989). Lebensmitteltoxikologie (pp. 226–231). Berlin: Springer-Verlag.

Maleveille, C., Hautefeuille, A., Pignatelli, B., Talaska, G., Vineis, P., & Bartsch, H. (1996). Dietary phenolics as anti-mutagens and inhibitors of tobacco-related DNA adduction in the urothelium of smokers. *Carcinogenesis, 17*, 2193–2200.

Mehansho, H., Butler, L. G., & Carlson, D. M. (1987). Dietary tannins and salivary proline rich proteins: interactions, induction, and defence mechanisms. *Annual Review of Nutrition, 7*, 423–440.

Nicolas, J. J., Richard-Forget, F. C., Goupy, P. C., Amiot, M.-J., & Aubert, S. Y. (1994). Enzymatic browning reactions in apple and apple products. *Critical Reviews in Food Science and Nutrition, 34*, 109–157.

Patrickios, C. S., & Yamasaki, E. N. (1995). Polypeptide amino acid composition and isoelectric point–II. Comparison between experiment and theory. *Analytical Biochemistry, 231*, 82–91.

Pierpoint, W. S. (1969a). *O*-Quinones formed in plant extracts — their reactions with amino acids and peptides. *Biochemical Journal, 112*, 609–617.

Pierpoint, W. S. (1969b). *O*-Quinones formed in plant extracts — their reactions with bovine serum albumin. *Biochemical Journal, 112*, 619–629.

Rawel, H., Kroll, J., Haebel, S., & Peter, M. G. (1998). Reactions of a glucosinolate breakdown product (benzyl isothiocyanate) with myoglobin. *Phytochemistry, 48*, 1305–1311.

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

Robards, K., Prenzler, P. D., Tucker, G., Swatsitang, P., & Glover, W. (1999). Phenolic compounds and their role in oxidative processes in fruits. *Food Chemistry, 66*, 401–436.

Robbins, C. T., Hagerman, A. E., Austin, P. J., McArthur, C., & Hanley, T. A. (1991). Variation in mammalian physiological responses to a condensed tannin and its ecological implications. *Journal of Mammology, 72*, 480–486.

Salgues, M., Cheynier, V., Gunata, Z., & Wylde, R. (1986). Oxidation of grape juice 2-S-glutathiolyl caffeoyl tartaric acid by *Botrytis cinerea*

laccase and characterization of a new substance: 2,5-di-S-glutathionyl caffeoyl tartaric acid. *Journal of Food Science, 51*, 1191–1194.
Scalbert, A. (1991). Review article number 63; antimicrobial properties of tannins. *Phytochemistry, 30*, 3875–3883.
Shugar, D. (1952). The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. *Biochemica et Biophysica Acta, 8*, 302–308.