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Inhibitory Effects of Plant Phenols on the Activity of Selected Enzymes

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Selected enzymes (α-amylase, trypsin, and lysozyme) were allowed to react with some simple phenolic and related compounds (caffeic acid, chlorogenic acid, ferulic acid, gallic acid, *m*-, *o*-, and *p*-dihydroxybenzenes, quinic acid, and *p*-benzoquinone). The derivatized enzymes obtained were characterized in terms of their activity. In vitro experiments showed that the enzymatic activity of the derivatives was adversely affected. This enzyme inhibition depended on the reactivity of the phenolic and related substances tested as well as on the kind of substrate applied. The decrease in the activity was accompanied by a reduction in the amount of free amino and thiol groups, as well as tryptophan residues, which resulted from the covalent attachment of the phenolic and related compounds to these reactive nucleophilic sites in the enzymes. The enzyme inhibition correlates well with the blocking of the mentioned amino acid side chains.

KEYWORDS: Enzyme activity; plant phenolic substances; α-amylase; trypsin; lysozyme

INTRODUCTION

Enzymes are applied in many technological processes in the food and feed industry. Hydrolases (such as proteases and carbohydrases), oxidoreductases, and isomerases play an important role in these procedures. For example, in the fruit and vegetable industry, pectic enzymes are used for the production of juice (*1*). In the baking industry, there are different applications for amylases and proteinases (*2*, *3*). Also, lysozyme is widely applied as a food preservative enzyme because of its ability to lyse bacteria (*4*, *5*).

In an organism, the first contact of ingested food components is made with the hydrolases of the digestive system. These enzymes, contained in the salivary and the pancreatic juices of the gastrointestinal tract, are responsible for the degradation of the main food components, namely carbohydrates, proteins, and lipids. The ability to catalyze reactions is of course the main task for enzymes, but they may also be involved in reactions with other components in food and feed systems. Also, under physiological conditions such reactions between enzymes and other substances cannot be excluded. The main reactive residues of the enzymes/proteins are the nucleophilic side chains such as lysin, tryptophan, and cystein (*6*). Further, it is known from the literature that other nucleophilic amino acids such as methionine have the potential to undergo reactions similar to those described (*6*). They can react with other food components such as secondary plant metabolites, which are becoming more and more interesting due to their physiological effects on humans (*7*, *8*). The largest class of the secondary plant metabolites is the phenolic compounds, which are widely distributed in the plant kingdom, and their content in many plant-based beverages, fruits, and vegetables is relatively high (*9*). Depending on their structure they can react with proteins/enzymes and alter various properties of these biopolymers such as their molecular weight, solubility, and in vitro digestibility (*10*−*12*).

In a recent work we have shown the effect of the reactions between α-chymotrypsin and phenolic substances, showing changes in selected physicochemical properties and the influence of the derivatization on the activity (*13*).

Now the objective is to increase the knowledge of such reactions by studying other enzymes, and as a result underlining their general significance. The special aim of the present study is to demonstrate the consequences of derivatization of selected enzymes (α-amylase, trypsin, and lysozyme) by various natural phenolic (caffeic acid, chlorogenic acid, ferulic acid, gallic acid, and *m*-, *o*-, and *p*-dihydroxybenzene) and related substances (*p*-benzoquinone and quinic acid) on their enzyme activity under various derivatization parameters (concentration of the phenolic and related substances, and pH value). The physiologically relevant enzymes α-amylase and trypsin were chosen because of their different substrate specificities and their occurrence in different parts of the gastrointestinal tract. The study of lysozyme was extended to include the modification with other phenolic compounds under varying derivatization parameters. Further, these enzymes were also chosen because of their well-known structures and well-characterized enzymatic properties. The mentioned plant phenolic and related substances were applied in order to illustrate the influence of the number and position of the hydroxyl groups present in the respective substances on the degree of enzyme derivatization and with regard to the consequent effects on their activity.

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MATERIALS AND METHODS

Materials. α-Amylase from porcine pancreas (EC 3.2.1.1, protein content 53%) was purchased from Fluka Chemie AG, Buchs, Switzerland (55 units/mg solid, one unit will hydrolyze 1 μmol maltose from starch per min at pH 6.9 and 25 °C). Trypsin from porcine pancreas (EC 3.4.21.4, protein content 100%) was obtained from Sigma Chemicals Co., St. Louis, MO (16700 units BAEE /mg protein, one BAEE unit = ΔA_{253} of 0.001 per min with BAEE as substrate at pH 7.6 and 25 °C). Lysozyme from hen egg white (EC 3.2.1.17, protein content 98%) was from Fluka Chemie AG (69490 units/mg solid, one unit = ΔA_{450} of 0.001 per min with *Micrococcus lysodeikticus* as substrate at pH 6.24 and 25 °C). The specific activities and definitions quoted here were given by the producers.

A 300-mg aliquot of enzyme per derivative was dissolved in distilled water and the pH was adjusted to 9 using 0.5 M NaOH (final volume 27 mL). For α-amylase the additional pH values 5 and 7 (for lysozyme pH 4, 7, and 10) were chosen and prepared according to the same procedure. On the basis of an assumed intake of phenolic substances of 100 mg/day (equivalent to 0.28 mmol chlorogenic acid) all the derivatives were prepared with corresponding 0.28 mmol of the phenolic compounds per gram protein (additional amounts applied for α-amlyase were 0.07, 0.14, and 0.42 mmol/g protein at pH 9) as follows: (A) 27 mL of every enzyme solution plus 3 mL of distilled water/ethanol (v:v 1:1) were the unmodified control samples. (B) 27 mL of α-amylase solution plus 0.28 mmol of phenolic and related compounds in 3 mL of distilled water/ethanol (v:v 1:1). Similarly, derivatives with 0.07, 0.14, and 0.42 mmol phenolic and related compounds were also prepared. The substances applied were chlorogenic, caffeic, ferulic, gallic, and quinic acids and *p*-benzoquinone. These were the α-amylase derivatives. (C) 27 mL of trypsin solution plus 0.28 mmol of phenolic and related compounds in 3 mL of distilled water/ethanol (v:v 1:1). The phenols applied were chlorogenic, caffeic, ferulic, gallic, and quinic acids, *m*-, *o*-, and *p*-dihydroxybenzene, and *p*-benzoquinone. These were the trypsin derivatives. (D) 27 mL of lysozyme solution plus 0.14 mmol (respectively 0.42 mmol) of phenolic compound in 3 mL of distilled water/ethanol (v:v 1:1). The phenol applied was chlorogenic acid. These were the lysozyme derivatives.

Derivatization of the Enzymes. In each case, after addition of the respective phenolic and related compounds (all from Fluka Chemie AG), the pH was re-adjusted to the required value. After 24 h reaction time under continuous stirring at 4 °C with free exposure to air, the samples were dialyzed for 24 h against distilled water (for trypsin 0.001 M hydrochloric acid) at 4 °C and finally lyophilized. The nonderivatized enzymes (control) were prepared under the same conditions but without addition of phenolic or related compound. The substrates applied for testing the activity were as follows: starch from potatoes (Fluka Chemie AG); N_α-benzoyl-L-arginine-ethyl-ester, BAEE (Sigma); myoglobin from horse heart (Fluka Chemie AG); Hammarsten grade casein (Merck, Darmstadt); whey proteins DSE 1591 (New Zealand Milk Products); legumin from broad beans (extracted and lyophilized from *Vicia faba* L. according to (*14*)); and cells from *Micrococcus lysodeikticus*, ATCC 4698 (Sigma).

Characterization of the Activity. The hydrolytic activity of α-amylase was investigated by degradation of starch as a substrate and determination of the splitting products (the reducing groups calculated as maltose) in a color reaction using 3,5-dinitrosalicylic acid (*15*).

The tryptic hydrolysis of a standard substrate N_α-benzoyl-L-arginine-ethyl-ester (BAEE) was determined according to a spectrophotometric method following the addition of trypsin or its derivatives (*16*). In addition to these methods, the enzymatic degradation (100 μL of enzyme, 1 mg/mL) of different protein substrates (casein, whey proteins, legumin, and myoglobin; each 6 mg/1.5 mL; 2 M urea, 0.1 M Tris−HCl pH 8, 0.02 M $CaCl_2$) with the trypsin derivatives was investigated by incubating them at 35 °C (enzyme/substrate ratio (E:S) = 1:60). After 5, 15, 30, 45, and 60 min, 300 μL was removed from the incubation mixture and the reaction was stopped by addition of 300 μL of 20% trichloroacetic acid (TCA). The reaction mixtures were allowed to stand for 10 min, and were then centrifuged at 9088*g* for 10 min (Megafuge 2.0R, Heraeus, Hanau, Germany). The TCA soluble peptides were analyzed by RPHPLC, which 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 Shimadzu (Duisburg, Germany) chromatographic system. A distilled water/acetonitrile (water acidified with 0.1% trifluoroacetic acid; v:v) gradient was applied under the following conditions: 100% water for 2 min; 0−70% acetonitrile in 10 min; 70% acetonitrile for 4 min; 70% acetonitrile −100% water in 4 min; 100% water for 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 (*17*). The lytic activity of the lysozyme derivatives against cells of *Micrococcus lysodeikticus* (ATCC 4698) was carried out with a turbidometric method in which the decrease of the absorption of a cell suspension was determined (*10*, *18*).

Determination of the Extent of Blocking of Selected Reactive Sites in the Enzymes. The protein content in the solutions was determined according to a modified Lowry method (*19*). Changes in the content of free amino groups were determined using trinitrobenzenesulfonic acid (TNBS) in a 1% sodium dodecyl sulfate (SDS) solution of the samples (*20*). Tryptophan fluorescence determination in 8 M urea was measured using a Jasco fluorescence detector FP 920 (Gross-Umstadt, Germany; Tokyo, Japan) (*21*). The sample containing 0.5 mg substance per mL of urea solution was excited at 290 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 320 to 380 nm was used to quantify the tryptophan content.

The free thiol groups were also determined by a fluorescence method (*22*), using monobrombimane as a derivatization reagent. The following GPC of the labeled samples was performed with a Jasco (Gross-Umstadt, Germany; Tokyo, Japan) chromatographic system, a TSKgel Octadecyl-4PW column (150 × 4.6 mm, 3 μm, flow rate 0.5 mL/min) at a column temperature of 25 °C with following fluorescence detection by a Jasco fluorescence detector FP 920 , with excitation at 380 nm and emission of the fluorescence peak at 480 nm, as well as UV detection at 220 nm.

Statistical Analysis. The determination of the free amino groups and the activity of the standard substrates (starch, BAEE, and *Micrococcus lysodeikticus*) were repeated thrice and analyzed by standard deviation. Analyses of the tryptophan fluorescence and the free thiol groups were also performed three times. A maximum of ±5% standard deviation from the averaged values was generally obtained. The averaged values are documented in the respective figures.

RESULTS AND DISCUSSION

Effect of the Derivatization on Amylytic Properties. The specific aim of the experiments with α-amylase was to show the different reactivity of this enzyme with the following phenolic and related substances: ferulic acid, caffeic acid, chlorogenic acid, gallic acid, quinic acid, and *p*-benzoquinone. The experiments were performed under derivatization conditions of pH 9 and a concentration of the phenolic substances of 0.28 mmol/g protein. The influence of different concentrations (0.07, 0.14, 0.28, and 0.42 mmol/g protein) of chlorogenic acid on the reaction is demonstrated. Similarly, the effect of different pH conditions (5, 7, and 9) on the reaction with caffeic acid is illustrated.

The α-amylase reacts with phenolic and related substances causing a decrease in the amounts of free amino groups and tryptophan as illustrated in **Table 1**. The strength of reaction is different depending on the structures of the substances applied. The effect of the reaction on α-amylase with quinic acid and ferulic acid was comparatively lower than that obtained with the other investigated phenolic compounds (**Table 1**). The highest reactivity was obtained with *p*-benzoquinone and chlorogenic acid (**Table 1**). Because the tryptophan fluorescence (fluorescence of the indole structure), as an indirect expression of the content of tryptophan, was measured in the presence of 8 M urea and the amount of free amino groups was determined in the presence of 1% SDS (both well-known agents which

Table 1. Activity and Content of Free Amino Groups and Tryptophan of α-Amylase Derivatives, Depending on the Derivatization Parameters (Different Phenolic and Related Substances; 0.28 mmol/g Protein; pH 9)

derivatives	activity [units/mg solid]	free amino groups [nmol/mg protein]	tryptophan [nmol/mg solid]
control	114 ± 1	723 ± 3	57 ± 3
+ quinic acid	103 ± 2	710 ± 6	54 ± 1
+ ferulic acid	97 ± 1	655 ± 4	46 ± 1
+ caffeic acid	80 ± 1	570 ± 3	9 ± 1
+ chlorogenic acid	70 ± 1	481 ± 6	4 ± 1
+ gallic acid	76 ± 2	565 ± 4	8 ± 1
+ *p*-benzoquinone	63 ± 1	538 ± 2	7 ± 1

destroy noncovalent protein interactions like hydrogen bonding, and hydrophobic and ionic interactions), we can assume that the interaction has occurred through covalent binding. Generally, phenolic substances may be readily oxidized in alkaline solutions or in the presence of polyphenoloxidase to the respective quinones, which in turn represent a reactive species capable of undergoing interactions with the free amino groups of proteins. The stepwise addition of protein-bound amino groups to an oxidized *o*-diphenol has already been reported (*6*). The rate of the oxidation depends on the pH as documented for caffeic acid (*23*), and as a result, the further reaction with nucleophilic amino groups is also dependent on the pH value. This was already observed for lysozyme derivatized with chlorogenic acid (*11*). As a consequence, these reactions of phenolic compounds with the reactive side chains of the enzymes result in changes of physicochemical properties such as solubility, electrophoretical behavior, hydrophobicity, molecular weight, secondary and tertiary structure, as well as thermodynamic parameters as already shown for selected food proteins in model studies (*12*, *24*, *25*).

The reactions of α-amylase with the different phenolic and related substances affect the enzyme activity, which was significantly reduced (**Table 1**). As a result, the reactivity and the effect on the amylytic activity follows the order *p*-benzoquinone > chlorogenic acid > gallic acid > caffeic acid > ferulic acid > quinic acid. A good correlation was obtained between the decrease in the amount of free amino groups caused by the different phenolic and related compounds given in **Table 1** and the corresponding decrease in α-amylase activity (R^2 = 0.90). Similarly, a high correlation was also obtained for the decreased tryptophan content and the corresponding lowered α-amylase activity (R^2=0.92).

The observed order of reactivity shown above is also valid for all four applied concentrations (0.07, 0.14, 0.28, and 0.42 mmol/g protein) of phenolic and related substances. With increasing concentration the reactions are stronger, and the activity decreases correspondingly (**Figure 1**, exemplar for chlorogenic acid). The investigations at different pH values showed that with increasing pH the reactivity of the phenolic compounds is more marked and so the activity is decreased again (**Figure 2**, exemplar for caffeic acid).

Effect of the Derivatization on Proteolytic Properties. The specific aim of the investigations with trypsin was to show the reactivity of this enzyme with the following phenolic and related substances: ferulic acid, caffeic acid, chlorogenic acid, gallic acid, quinic acid, and *p*-benzoquinone. Here the derivatization was also performed at pH 9 and 0.28 mmol/g protein. Additionally, the effect of number and position of hydroxyl groups present in phenolic substances was demonstrated by applying *m*-, *o*-, and *p*-dihydroxybenzene (*m*-, *o*-, *p*-DHB) in comparison to the oxidized form of *p*-DHB, namely *p*-benzoquinone. Further

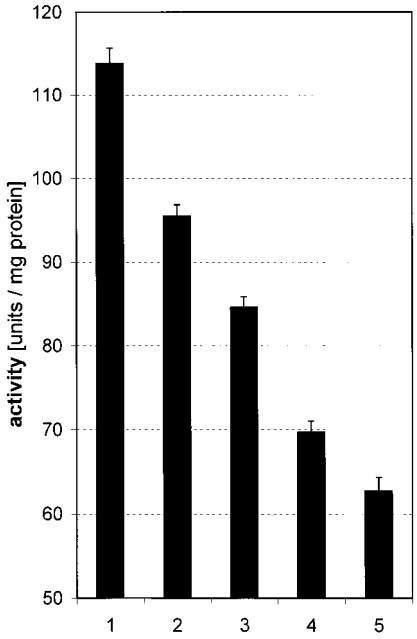

Figure 1. Activity of α-amylase derivatives against starch, depending on the derivatization parameters (different concentrations of chlorogenic acid; pH 9): 1 = unmodified control α-amylase; 2–5 = chlorogenic acid derivatives (0.07, 0.14, 0.28, and 0.42 mmol chlorogenic acid/g protein).

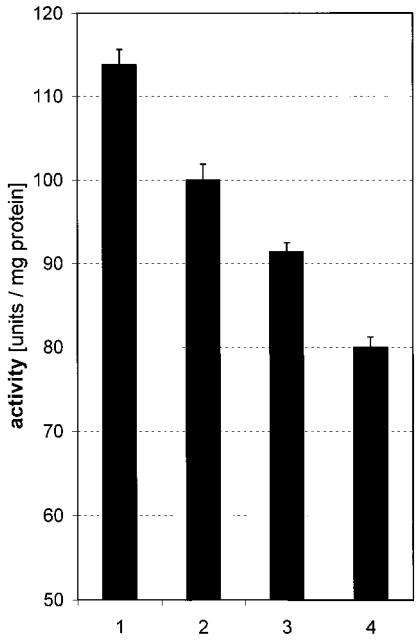

Figure 2. Activity of α-amylase derivatives against starch, depending on the derivatization parameters (different pH values; 0.28 mmol caffeic acid/g protein): 1 = unmodified control α-amylase; 2–4 = caffeic acid derivatives (0.28 mmol caffeic acid/g protein; pH 5, 7, and 9).

the influence of different types of substrates for the determination of trypsin activity was elucidated.

Trypsin as a typical representative of proteolytic enzymes reacts with the phenolic and related substances applied in the same way as α-amylase. Depending on the structure of these phenolic substances the reactions at the free amino and free thiol groups, as well as with tryptophan side chains, take place causing a decrease in the amount of the reactive nucleophilic groups of the enzymes, as shown in **Table 2**. These results agree with experiments done for α-chymotrypsin (*13*).

Table 2. Activity and Content of Free Amino and Thiol Groups, as well as Tryptophan, of Trypsin Derivatives, Depending on the Derivatization Parameters (Different Phenolic and Related Substances; 0.28 mmol/g Protein; pH 9)

derivatives	activity [units/mg solid]	free amino groups [nmol/mg protein]	tryptophan [nmol/mg solid]	free thiol groups [nmol/mg solid]
control	11178 ± 232	329 ± 5	71 ± 1	587 ± 3
+ quinic acid	11384 ± 334	321 ± 5	70 ± 1	571 ± 6
+ ferulic acid	10265 ± 175	304 ± 3	64 ± 1	542 ± 2
+ caffeic acid	6250 ± 170	269 ± 2	20 ± 1	468 ± 6
+ chlorogenic acid	4401 ± 197	247 ± 3	7 ± 1	417 ± 7
+ gallic acid	6514 ± 261	260 ± 4	16 ± 1	n.d.
+ meta-dihydroxybenzen (*m*-DHB)	11449 ± 243	305 ± 4	67 ± 1	552 ± 4
+ ortho-dihydroxybenzen (*o*-DHB)	7450 ± 268	284 ± 5	40 ± 1	525 ± 8
+ para-dihydroxybenzen (*p*-DHB)	5708 ± 293	264 ± 3	30 ± 1	448 ± 2
+ *p*-benzoquinone	4177 ± 147	251 ± 4	13 ± 1	426 ± 2

The effect of derivatization of trypsin with phenolic compounds on its in vitro activity was investigated by two different approaches. In one of the approaches the proteolytic hydrolysis of a standard substrate, BAEE, a small peptide was determined; the results are illustrated in **Table 2**. Because of the covalent attachment of the phenolic substances, the proteolytic activity was significantly reduced, with the exception of the derivatives formed with quinic acid and *m*-DHB. The reactivity and the effect on the proteolytic activity follows for trypsin, with reference to the above-mentioned small substrate, the order *p*-benzoquinone > chlorogenic acid > *p*-DHB > caffeic acid > gallic acid > *o*-DHB > ferulic acid.

As already shown for α-amylase, also in this case a good correlation was found between the decrease in the tryptic activity and in the diminishing amount of free amino groups ($R^2 = 0.95$) as well as in the decrease of tryptophan ($R^2 = 0.95$). Further, the graphical plot between the decrease in trypsin activity and the reduction of thiol groups also gave a high correlation factor ($R^2 = 0.97$). A similar effect on the proteolytic activity was also reported for α-chymotrypsin (*13*).

To support the investigations with the standard substrate, we tested in a second approach the enzymatic hydrolysis of other substrates like food proteins (whey proteins, casein, myoglobin, and legumin) to show the influences of the proteolytic hydrolysis on larger substrates in comparison to the smaller standard substrate. For this purpose it was necessary to partly denature the substrates with urea because of their compact globular structure. Generally, proteins taken with the diet are also partly denatured as a result of processing and cooking. In comparison to the unmodified control samples, the influence of derivatization causes the hydrolysis to become slower (**Figure 3 A**,**B**,**C** shows the tryptic degradation of casein as a substrate). There was a corresponding lower amount of trichloroacetic acid soluble peptides (compared to the results of the unmodified tryptic hydrolysis) liberated during the digestion. The hydrolysis with trypsin derivatized with quinic acid and *m*-DHB liberated in each case amounts of trichloroacetic acid soluble peptides similar to that of the unmodified control samples. This confirms the observations made for the small standard substrate described above. The derivatization with *p*-benzoquinone showed again the strongest influence on the activity. With ferulic acid there is a small effect, but significantly different from that of the control samples (**Figure 3A**). Under the considerations of the oxidation mechanism, ferulic acid cannot be oxidized to the corresponding quinone. The results show that the significance *t*-test allows the statement of a possible reaction of ferulic acid with nucleophilic amino groups. One possible explanation can be given by considering the formation of a semiquinone radical, which in turn can attack a nucleophilic reaction partner as proposed in the reaction steps reported formerly (*10*).

The semiquinones thus formed may react further with the ϵ-amino group of lysine, with the tryptophan side chains and the free thiol group of cysteine.

As a result, the influence of the phenolic derivatization on the digestion behavior for trypsin against the proteins applied (whey proteins, casein, myoglobin, and legumin) was in following order: *p*-benzoquinone > caffeic acid > *p*-DHB > chlorogenic acid > gallic acid > *o*-DHB > ferulic acid > quinic acid > *m*-DHB (**Figure 3 A**,**B**,**C** shows the tryptic degradation of casein as a substrate; for the other substrates results not shown). These results show differences from the order obtained with the synthetic substrate described above, especially with regard to the derivatization of the enzyme with caffeic acid and its position in the two rankings. Such influences may result from the structure and the size of the reacting molecules.

Effect of the Derivatization on Lytic Hydrolysis. The physicochemical characterization of derivatives obtained with lysozyme and chlorogenic acid has been already reported (*11*).

Therefore, the specific aim of these investigations was to document the effect of such a derivatization on the lytic activity. Since chlorogenic acid proved to be highly reactive as shown above, the effect on the lytic activity under different derivatization parameters (pH 4, 7, and 10; 0.28 and 0.42 mmol/g protein) has been discussed here.

As observed for the other enzymes described above, the activity of lysozyme was again significantly reduced (**Table 3**) depending on the amount of chlorogenic acid applied and the pH value used for the derivatization. These dependences were already shown for α-amylase. With regard to the decrease in the lytic activity a good correlation was obtained with the amount of free amino groups blocked (*11*) ($R^2 = 0.94$), the corresponding decrease of tryptophan content (*11*) ($R^2 = 0.81$), and the decrease of the free thiol groups ($R^2 = 0.98$).

CONCLUSIONS

These and our preliminary results (*10*−*13*, *24*, *25*) show that plant phenolic substances react with proteins/enzymes influencing their physicochemical properties and as a consequence their in vitro enzymatic activity. The reaction occurs at lysine side chains, at the indole ring of the tryptophan residues, and at the free thiol groups of cystein side chains of the enzymes. As illustrated, the activity of α-amylase, trypsin, lysozyme, and α-chymotrypsin (*13*) decreased depending on the concentration and on the number and position of hydroxyl groups of the phenolic compounds applied. *p*-Benzoquinone showed the strongest reactivity, and the phenolic substances which are able to form quinones (caffeic acid, chlorogenic acid, gallic acid, *o*- and *p*-DHB) are much more reactive than those phenolic substances which do not have the ability for quinone formation.

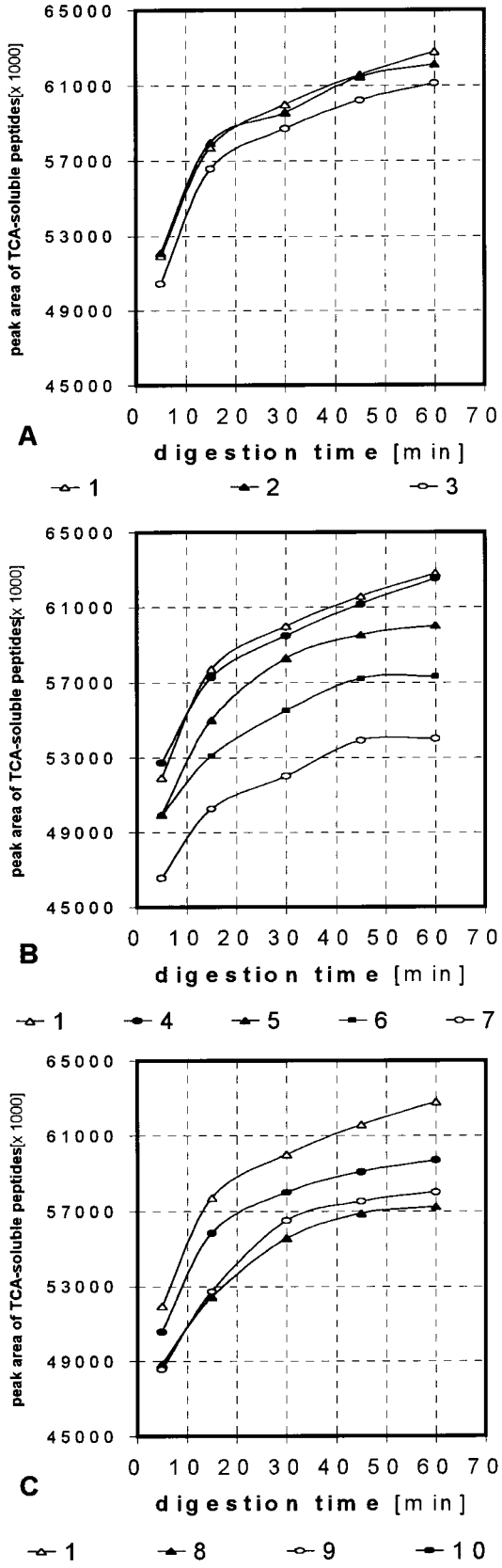

Figure 3. Proteolytic activity of trypsin derivatives against casein: 1 = unmodified control trypsin; 2 = quinic acid derivative; 3 = ferulic acid derivative; 4 = *m*-DHB derivative; 5 = *o*-DHB derivative; 6 = *p*-DHB derivative; 7 = *p*-benzoquinone derivative; 8 = caffeic acid derivative; 9 = chlorogenic acid derivative; 10 = gallic acid derivative.

These results agree with the described reactions, demonstrating that for the reactions of phenolic substances with proteins/enzymes the formation of quinones is the most important step (*6*) as well as for nonenzymatic and enzymatic browning reactions (formation of phenolic polymers) (*8*). The results show further that phenolic substances which are not able to form quinones (ferulic acid and *m*-DHB) also react with enzymes but to a comparable lower extent. In agreement with results already reported (*10*, *23*) the reaction is based on the formation of semiquinone intermediates. The reaction mechanism with quinic acid is not yet clear. The reactions of phenolic substances with proteins/enzymes are also influenced by pH value. With increasing pH the reactivity of the phenolic compounds is more marked and so the enzyme activity decreased.

As a main consequence the enzyme activity decreases depending on the described reaction conditions. A good correlation was found between the changes of physicochemical properties and the enzyme inhibition.

The reactions of enzymes with phenolic substances can have technological uses (applications in food industry, e.g., macerating enzymes in production of juice) and physiological consequences (consumption of phenol rich diets effecting the gastrointestinal enzymes). These results are significant with regard to further experiments planned to investigate physiological effects of enzymes derivatized with phenolic compounds. In this respect, the application of further in vivo and in vitro techniques to characterize the reactions at physiological conditions are also planned.

Table 3. Activity, Content of Free Amino and Thiol Groups, as Well as Tryptophan, of Lysozyme Derivatives, Depending on the Derivatization Parameters (Different Concentrations of Chlorogenic Acid; Different pH Values)

derivatives	activity [units/mg solid]	free amino groups [nmol/mg protein]	tryptophan [nmol/mg solid]	free thiol groups [nmol/mg solid]
control	68012 ± 730	454 ± 3	23 ± 3	527 ± 3
+ chlorogenic acid 0.14 mmol/g protein; pH 4	56000 ± 690	461 ± 8	17 ± 1	509 ± 4
+ chlorogenic acid 0.14 mmol/g protein; pH 7	54938 ± 1226	437 ± 4	14 ± 1	494 ± 3
+ chlorogenic acid 0.14 mmol/g protein; pH 10	21416 ± 604	373 ± 3	6 ± 1	310 ± 5
+ chlorogenic acid 0.42 mmol/g protein; pH 4	50576 ± 1186	441 ± 7	10 ± 1	472 ± 3
+ chlorogenic acid 0.42 mmol/g protein; pH 7	46524 ± 1842	421 ± 3	7 ± 1	461 ± 4
+ chlorogenic acid 0.42 mmol/g protein; pH 10	13438 ± 1144	343 ± 4	3 ± 1	273 ± 8

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