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Reactions of chlorogenic acid and quercetin with a soy protein isolate – Influence on the *in vivo* food protein quality in rats

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Plant phenolic compounds are known to interact with proteins producing changes in the food (*e.g.*, biological value (BV), color, taste). Therefore, the *in vivo* relevance, especially, of covalent phenol-protein reactions on protein quality was studied in a rat bioassay. The rats were fed protein derivatives at a 10% protein level. Soy proteins were derivatized with chlorogenic acid and quercetin (derivatization levels: 0.056 and 0.28 mmol phenolic compound/gram protein). Analysis of nitrogen in diets, urine, and fecal samples as well as the distribution of amino acids were determined. Depending on the degree of derivatization, the rats fed with soy–protein derivatives showed an increased excretion of fecal and urinary nitrogen. As a result, true nitrogen digestibility, BV, and net protein utilization were adversely affected. Protein digestibility corrected amino acid score was decreased for lysine, tryptophan, and sulfur containing amino acids.

Keywords: Amino acid score / Plant phenolic compounds / Protein derivatization / Protein digestibility / Soy protein

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1 Introduction

Phenolic compounds are the largest class of the so-called secondary plant metabolites and research on their role in nutrition has developed fast over the last few years. Many of the phenolic compounds consumed with a fruit- and vegetable-rich diet are considered to be antithrombotic, immunomodulating, antimutagenic, and even anticarcinogenic, but these health promoting effects are mostly determined in model experiments on the basis of isolated (pure) substances. Such approaches do not consider any changes of the phenolic compounds, although these alterations cannot be excluded in harvesting, storage, and in particular during food processing. Interactions with other constituents of the food, primarily the main food compounds (proteins, lipids, and carbohydrates) may lead to altered technofunctional and physiological properties. Especially, proteins have been shown to interact in diverse mechanisms with plant phenolic compounds. Beside noncovalent interactions like $\pi-\pi$-bonding (aromatic interaction), hydrogen bonding, hydrophobic or ionic interactions [1, 2], it has been assumed that covalent bonding is likely to play an important role in protein-phenol interactions [3, 4]. In model systems using different food proteins in combination with simple phenolic and related compounds, it was proved that covalent bonding take place [5]. Even flavonoid glycosides of the phenolic compounds show reactivity against proteins [6]. The basis of the phenol-protein reaction is the oxidation of the phenolic compounds to their respective quinones which are due to their electrophilic character capable of reacting with nucleophilic substances (in this case: selected protein side chains). A quinoid moiety is not only a necessary condition, but the most important step for the reactions with proteins/enzymes [4]. There are several possibilities for the formation of quinoid intermediates: First of all is the catalyzation by enzymes, which is also the basis for enzymatic browning reactions (formation of phenolic polymers) in food systems [3]. Second is heat treatment during food processing, where

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Abbreviations: AND, apparent nitrogen digestibility; **BV**, biological value; **CA**, chlorogenic acid; **NPU**, net protein utilization; **PDCAAS**, protein digestibility corrected amino acid score; **SP**, soy-protein isolate; **SPCAL**, chlorgenic acid-soy protein derivative (low derivatization level); **SPCAH**, chlorogenic acid-soy protein derivative (high derivatization level); **SPQL**, quercetin-soy protein derivative (low derivatization level); **SPQH**, quercetin-soy protein derivative (high derivatization level); **TND**, true nitrogen digestibility

a thermal-induced oxidation of the phenolic compounds takes place, leading *via* the formation of semiquinones and quinones either to dark colored phenolic polymers or to a degradation of the flavonol structure (Buchner, N., Krumbein, A., Rohn, S., Kroh, L. W.; submitted). The last, also important from a food technological point of view, is the pH value. It has been shown that reaction products (phenol-protein derivatives) occur over a broad pH range (pH 4 – 10) [5], whereas with increasing pH the formation of quinone intermediates is more marked. It was observed that the use of alkaline conditions during the isolation of proteins from different plant sources (*e. g.*, sunflower, which is a prerequisite example of a protein-rich source with high amounts of phenolic compounds), leads to the formation of dark-colored hardly soluble protein fractions [7]. These sunflower protein fractions became more useful after the removal of high amounts of chlorogenic acid (CA) present [7, 8]. The same is the case for potato proteins: After the manufacturing of starch, the proteins remain and exposition to air (even under mild pH conditions) and/or enzymatic action of polyphenoloxidases does lead to the described interactions [3].

The dietary combination of protein-rich foods with ingredients rich in plant phenolic compounds is quite common (coffee/tea with milk, milk chocolate or soy milk mixed with fruit juice). Experiments implementing apple together with milk proteins as found in yoghurt showed that reactions took place influencing the protein properties [9]. The reaction with proteins influences not only the physicochemical properties (*e. g.*, color, solubility), but also, in the case of enzymes, the enzymatic activity [10]. Beside high concentrations of phenolic compounds, supplementary food products, recently flooding the commercial food markets, often contain other health promoting ingredients, such as enzymes of plant origin, in order to improve the digestibility of the main food components (proteins, carbohydrates, and lipids). In preparations containing high amounts of quercetin beside bromelain (a proteolytic enzyme from *Ananas comosus* var.), it was shown that no enzyme activity was left, resulting from a reaction between these two major compounds [11].

After consumption of such food-borne protein derivatives, the *in vivo* behavior (degradation and/or metabolism) might also be influenced. As recently shown in a nitrogen-balance study using rats, the physiological properties of whey proteins were influenced moderately, resulting from a reaction with CA. It was further observed that the nitrogen digestibility of the CA-whey protein derivatives was reduced. For the net protein utilization (NPU), the biological value (BV) and the protein digestibility corrected amino acid score (PDCAAS), the observed effects were not so distinct due to the high nutritional quality of the whey proteins. In that study, it was proposed that a comparatively low quality plant protein is likely to show more pronounced effects [12].

Therefore, the main aim of the present study was to prove if a plant protein, soy protein – one of the most commonly consumed proteins – shows more pronounced changes in nutritional quality, resulting from the (covalent) reaction with selected plant phenolic compounds. It is thereby intended to confirm the observations made *in vitro* with regard to digestibility and reactions at specific amino acids (lysine, cysteine, and tryptophan) [13]. The latter should also visualize the stability of the covalent reactions to withstand the digestion conditions.

2 Materials and methods

2.1 Derivatization of the soy protein isolate (SP)

SP S90 (Gewürzmüller, Stuttgart, Germany) was derivatized with different amounts of CA (Fluka Chemie AG, Buchs, Switzerland) and quercetin (Q) (Riedel de Haen Laborchemikalien, Seelze, Germany): One gram of SP isolate was dissolved in 45 mL of distilled water. The respective phenolic compound was dissolved in 5 mL of ethanol and added. Reaction was performed under continuous stirring at room temperature with free exposure to air after the pH was readjusted to 9.0 using 0.5 mol/L NaOH, in order to get a high yield of protein derivative in a short period. A buffer system was not applied since the use of buffer causes the ionic strength to increase and certain ion-pairs may effect not only the structure of proteins but may also interact with phenolic compounds causing them to change their reactivity. After 24 h, samples were dialyzed for 24 h against distilled water and finally lyophilized. On the basis of an assumed intake of phenolic substances of 100 mg/d (equivalent to 0.28 mmol CA), the derivatives were prepared according to [14] with corresponding 0.28 mmol of the phenolic compounds/gram protein (high derivatization level). In addition, derivatization was performed with only 0.056 mmol phenolic compound/gram protein (low derivatization level). Nonderivatized SP isolate (control) was prepared under the same conditions but without addition of the phenolic compounds. For the characterization and analysis of physicochemical protein properties of the derivatization products (including free amino and thiol groups, tryptophan residues, molecular weights using SDS-PAGE and MALDI-TOF-MS, electrophoretic and chromatographic behavior, solubility, *in vitro* protein digestibility using trypsin, chymotrypsin and pepsin) the reader is referred to the methods of Rawel *et al.* [15].

2.2 Animals, diets, and feeding experiment

The protein quality assay was performed according to the UNU/WHO guidelines [16]. The experimental protocol has been evaluated and approved by the Ethical Committee on

Table 1. Composition of experimental diets used in the protein quality assay fed to rats (g/kg diet)

Diet	SP	SPCAL	SPCAH	SPQL	SPQH	PF[a)]
Soy protein[b)], underivatized (control)	126	–	–	–	–	–
Soy protein[b)], low derivatization level with CA[c)]	–	130	–	–	–	–
Soy protein[b)], high derivatization level with CA[c)]	–	–	136	–	–	–
Soy protein[b)], low derivatization level with Q[d)]	–	–	–	128	–	–
Soy protein[b)], high derivatization level with Q[d)]	–	–	–	–	136	–
Wheat starch[e)]	554	550	544	552	544	680
Saccharose[f)]	100	100	100	100	100	100
Sunflower seed oil[g)]	100	100	100	100	100	100
Cellulose[h)]	50	50	50	50	50	50
Mineral mixture[i)]	50	50	50	50	50	50
Vitamin mixture[j)]	20	20	20	20	20	20
Protein (%)[k)]	10	10	10	10	10	0
Crude protein content of the purified protein components (%) (N × 5.71)	79.6	76.7	73.6	77.9	73.6	–

a) Protein-free diet to determine metabolic and endogenous nitrogen losses.
b) Soy protein isolate S90, Gewürzmüller, Stuttgart, Germany.
c) CA, 97%, Fluka Chemie AG, Buchs, Switzerland.
d) Q, 99%, Riedel de Haen Laborchemikalien, Seelze, Germany.
e) Kroener, Ibbenbüren, Germany.
f) Nordzucker, Uelzen, Germany.
g) Thomy, Karlsruhe, Germany.
h) Rettenmaier und Soehne, Rosenberg, Germany.
i) Mineral mixture, mineral content/kg diet: Ca, 9500 mg, P, 7500 mg, Mg, 750 mg, Na, 2500 mg, K, 7040 mg, S, 2810 mg, Cl, 3630 mg, Fe, 180 mg, Mn, 100 mg, Zn, 30 mg, Cu, 12 mg, J, 0.45 mg, F, 4 mg, Se, 0.31 mg, Co, 0.13 mg, (Altromin GmbH, Lage, Germany).
j) Vitamin mixture, vitamin content/kg diet: A, 15000 IU, D_3, 500 IU, K_3, 10 mg, B_1, 20 mg, B_2, 20 mg, B_6, 15 mg, B_{12}, 0.03 mg, niacin, 50 mg, pantothenate, 50 mg, folic acid, 10 mg, biotin, 0.2 mg, choline chloride, 1000 mg, *p*-aminobenzoic acid, 100 mg, inositol, 100 mg, C, 20 mg (C 1000 without DL-methionine, Altromin, Lage, Germany).
k) The protein quantity used for experimental diets (target protein content of 10%) was calculated based on crude protein content (N × 5.71) of purified protein components.

the Use of Animals as Experimental Subjects of the Ministry of Agriculture, Nutrition and Forestry (State Brandenburg, Germany), and was registered under the reference number 32/48-3560-0/3. Housing of rats was essentially as described in [17].

Male weanling Wistar rats (Charles River, Sulzfeld, Germany) weighing 70 – 80 g were fed with free access to a stock diet (Altromin, Lage, Germany; 190 g/kg of crude protein, 40 g/kg of crude fat, 11.9 MJ/kg of metabolizable energy) prior to the experiment. In the balance experiment 48 rats were divided into six groups. Rats were housed in individual metabolic cages in a climate-controlled room, and a 12 h light/dark cycle. Water was provided ad libitum. The test diets were prepared at 10% crude protein level (Table 1).

The rats of group I were fed nonderivatized SP. Test diets for groups II (chlorgenic acid-soy protein derivative (low derivatization level) SPCAL) and III (chlorogenic acid-soy protein derivative (high derivatization level) SPCAH) contained soy protein derivatized with CA at a low or high derivatization level. Group IV (quercetin-soy protein derivative (low derivatization level) SPQL) and V (quercetin-soy protein derivative (high derivatization level) SPQH) contained soy protein derivatized with quercetin, also low or high derivatized. Metabolic and endogenous nitrogen losses/gram of dry matter intake were determined in a group fed a protein free diet (group VI, PF). Each rat received 15 g of dry matter daily throughout the preliminary and balance periods. Dry food was mixed with water (1 : 0.5 w/w) and presented in pots.

After a preliminary feeding period of 5 days, subsequently, diets were provided during an N-balance period of 6 days. Urine and feces were collected in 1.4 mol/L hydrochloric acid. Fecal samples were dried and ground. Body weight was recorded at the end of the preliminary and balance period, after access to food and water being refused 3 h before weighing procedures. The food intake was monitored daily and any remaining food was taken into account for the calculations [17].

2.3 Calculation of results

Food and nitrogen efficiency were calculated according to the equations

$$\text{Food efficiency} = \text{body mass gain/food intake} \quad (1)$$

Nitrogen efficiency = body mass gain/nitrogen intake (2)
(body mass gain and food intake in grams)

Apparent nitrogen digestibility (AND), true nitrogen digestibility (TND), BV, and NPU were expressed on a percentage scale and calculated using the following equations [16]:

AND = (nitrogen intake – fecal nitrogen)/
nitrogen intake (3)

TND = [nitrogen intake – (fecal nitrogen –
endogenous fecal nitrogen)]/nitrogen intake (4)

BV = [nitrogen intake – (fecal nitrogen – endogenous
fecal nitrogen) – (urinary nitrogen – endogenous
urinary nitrogen)]/[nitrogen intake – (fecal nitrogen –
endogenous fecal nitrogen)] (5)

NPU = BV × TND (6)

Estimation of the endogenous fecal nitrogen (EFN) and endogenous urinary nitrogen (EUN) was performed using values of the protein free group during nitrogen-balance collection period (EFN = 0.91 ± 0.14 mg/g dry food intake; EUN = 5.56 ± 0.5 mg/g dry food intake).

PDCAAS was calculated based on average amino acid requirements for children from 1 year onward [18] and on the fecal protein digestibility value as determined in this study:

PDCAAS (%) = (AAC × TND)/AAP (7)

where AAC is the amino acid content in food protein (mg/g crude protein) and AAP is the amino acid content (mg/g crude protein) in the respective requirement pattern for human. The calculated PDCAAS values were not cut at the 100% level to demonstrate surplus of individual indispensable amino acids.

2.4 Analytical procedures

Amino acid composition of protein samples was determined based on the recommendations made in the report of the Joint FAO/WHO Expert Consultation [19]. Proteins were hydrolyzed with 6 mol/L hydrochloric acid for 24 h under reflux by a continuous flow of nitrogen. After drying (40°C), hydrolysates were washed twice with distilled water to remove residual hydrochloric acid and dried again. Norleucine served as an internal standard. Cysteine and methionine, which can be destroyed during the acid hydrolysis, were converted in to acid-stable derivatives (cysteinic acid and methioninesulphone, respectively) by performic acid oxidation [20]: 30 mg of crude protein was used for performic acid oxidation. The oxidized samples were then hydrolyzed with 6 mol/L hydrochloric acid as described above. The hydrolyzed samples were dissolved in a lithium citrate buffer at pH 2.2 (Onken Laborservice, Gründau, Germany) and after filtration (0.45 µm) stored at –20°C prior to analysis. For tryptophan determination, alkaline hydrolysis was performed according to Rowan *et al.* [21]. Proteins were hydrolyzed using 4.3 mol/L NaOH [22] in Teflon containers which were flushed with nitrogen and placed in an oven (T 6030, Heraeus Instruments, Hanau, Germany) maintained at 110°C for 20 h. 5-Methyltryptophan was used as an internal standard. After cooling to room temperature, pH was adjusted to 5–6 using 6 mol/L hydrochloric acid and filtered samples (0.45 µm) were stored frozen (–80°C) until analysis. Amino acid concentrations in hydrolysates were determined by ion exchange chromatography (high efficiency column, 3×150 mm^2, Pickering Laboratories, Mountain View, CA) with a step-change elution method using lithium citrate buffers (Onken Laborservice) and postcolumn ninhydrin detection (TRIONE® Ninhydrin Reagent, Pickering Laboratories) and HPLC units (System Gold, Beckman Instruments, Munich, Germany) as reported [17]. Amino acids were detected at a wavelength of 540 nm. For calculation of the amino acid concentration norleucine was used as an internal standard added prior to hydrolysis.

Nitrogen contents in feces (dried at 102°C) and urine were measured by a Kjeldahl method (Kjeldatherm-Turbosog-Vapodest 45, C. Gerhardt, Bonn, Germany). The crude protein content was calculated by using the factor 5.71 for soy protein-based diets.

2.5 Statistical analysis

Kjeldahl analysis was performed twice. The determination of the amino acid composition was repeated four times. Data are presented as means ± SD. Differences between mean values were determined by ANOVA followed by comparisons using the Newman-Keuls multiple range test (WinSTAT®, vers. 1999.2, R. Fitch software, Staufen, Germany). Pearson correlation coefficients were calculated to determine the relationship between selected parameters. Differences with $P < 0.05$ were considered statistically significant if not stated otherwise.

3 Results

3.1 Nitrogen-balance

The food intake, body weight of the rats and food efficiency of the diets are shown in Table 2. In comparison to the control group, body mass gain and food efficiency were significantly lower for the groups fed with derivatized soy protein,

Table 2. Body mass, food intake, and food efficiency in rats fed diets containing soy protein derivatized with CA or Q.

Diet	Soy protein, underivatized control(SP)	Soy protein, low derivatization level with CA(SPCAL)	Soy protein, high derivatization level with CA(SPCAH)	Soy protein, low derivatization level with Q(SPQL)	Soy protein, high derivatization level with Q(SPQH)
Body mass, initial (g)	78.1 ± 2.5[a)]	79.0 ± 3.1[a)]	83.8 ± 3.6[a)]	83.2 ± 2.2[b)]	86.2 ± 2.0[b)]
Body mass gain (g)	11.7 ± 1.3[a)]	9.9 ± 1.3[b)]	9.1 ± 1.4[b)]	8.1 ± 2.3[c)]	6.7 ± 1.1[d)]
Food intake (g)	64.4 ± 2.0[a)]	65.5 ± 0.5[a)]	64.5 ± 2.5[a)]	65.1 ± 1.4[a)]	65.9 ± 0.7[a)]
Food efficiency (g body mass gain/g food intake)	0.156 ± 0.023[a)]	0.151 ± 0.020[a)]	0.140 ± 0.023[a)]	0.124 ± 0.036[a)]	0.101 ± 0.017[b)]
N efficiency (g body mass gain/g N intake)	8.68 ± 1.26[a)]	7.93 ± 1.03[a)]	7.53 ± 1.22[a)]	6.81 ± 0.97[a)]	5.94 ± 0.99[b)]

N-balance collection period 6–11 days; for food composition see Table 1; CA, chlorogenic acid, Q, quercetin, N, nitrogen; values are means ± SD, $n = 8$.
[a–d)] Means within a row sharing a common superscript letter are not significantly different, $P < 0.5$.

Table 3. Protein quality evaluation by N-balance in rats fed diets containing soy protein derivatized with CA or Q.

Diet	Soy protein, underivatized control (SP)	Soy protein, low derivatization level with CA (SPCAL)	Soy protein, high derivatization level with CA (SPCAH)	Soy protein, low derivatization level with Q (SPQL)	Soy protein, high derivatization level with Q (SPQH)
N intake (mg)	1115 ± 35[a)]	1248 ± 54[a)]	1204 ± 46[a)]	1183 ± 25[a)]	1120 ± 12[a)]
Fecal N (mg)	123 ± 7[a)]	140 ± 8[b)]	156 ± 8[c)]	141 ± 8[b)]	176 ± 7[d)]
Urinary N (mg)	579 ± 23[a)]	603 ± 35[b)]	624 ± 22[b)]	632 ± 32[b)]	645 ± 14[d)]
Fecal N/urinary N	0.21 ± 0.01[a)]	0.23 ± 0.02[a)]	0.25 ± 0.01[a)]	0.22 ± 0.02[a)]	0.24 ± 0.01[a)]
Total N excretion (mg)	702 ± 29[a)]	744 ± 36[b)]	780 ± 26[c)]	773 ± 34[c)]	822 ± 18[d)]

Values are means ± SD, $n = 8$; N-balance collection period 6–11 days; for food composition see Table 1; CA, Q, quercetin, N, nitrogen.
[a–d)] Means within a row sharing a common superscript letter are not significantly different, $P < 0.5$.

whereas food intake was on an equal level in all groups. Unfortunately, initial body weight differed significantly for SPQL and SPQH (Table 2), but according to Pellett and Young [16], the expected slightly higher muscle mass does not contribute to the effects that should be observed in such an assay. During the feeding experiment, all rats were in the stage of adolescence (linear body mass gain and equal food intake for all groups). Differences in protein turnover were corrected by taking excretion of endogenous protein into account, according to the UNU/WHO guidelines [16, 19].

Fecal nitrogen excretion was significantly higher, depending on the degree of derivatization (Table 3). The mean value of fecal N excretion is nearly 17 mg (15%) higher for both groups fed with low derivatized soy protein (SPCAL and SPQL) than in the control group. The increasing degree of derivatization with quercetin becomes even more pronounced due to a significant rise in the excretion of fecal nitrogen compared not only to the corresponding control diet, but also to the high CA derivatization level (Table 3).

For the urinary nitrogen excretion, a significant increase was observed for all groups fed derivatized soy protein when compared to the control group (Table 3). The group fed the high derivatized quercetin derivative showed an elevated urinary N excretion amounting to nearly 66 mg than in the control group. Compared to both chlorogenic acid-soy protein derivatives, the feeding of diets containing quercetin derivatives led to a higher excretion of nitrogen with the urine following the order: SPCAL < SPCAH < SPQL < SPQH. As a result, the ratio of fecal to urinary nitrogen remained unchanged for all groups (Table 3).

3.2 Protein quality parameters

Resulting from the nitrogen-balance values, the effect on the following protein quality parameters was assessed: AND, TND, BV, and NPU.

In comparison to the control group, the AND and TND parameters of all other groups decreased due to the derivatization of the soy proteins with the phenolic compounds (Fig. 1A and B). Significantly diminished values were recorded not only for both groups fed high derivatized protein (SPCAH and SPQH), but also for the group receiving soy protein derivatized with a lower level of quercetin (SPQL). Consequently, the BV is also significantly lowered for the respective diets (Fig. 2A). The highest decrease is more than 10% points, from 63.6% in the control diet to 53.4% for SPQH (Fig. 2A). The NPU, which reflects the BV after a correction with TND, also follows the same

trend, showing a significant decline in the order: SPCAH >SPQL > SPQH with a minimum value of 47% for the SPQH (Fig. 2B). Interestingly, although a significant higher excretion in fecal and urinary nitrogen was observed for the low derivatized SPCAL, no comparable change was monitored for the four protein quality parameters.

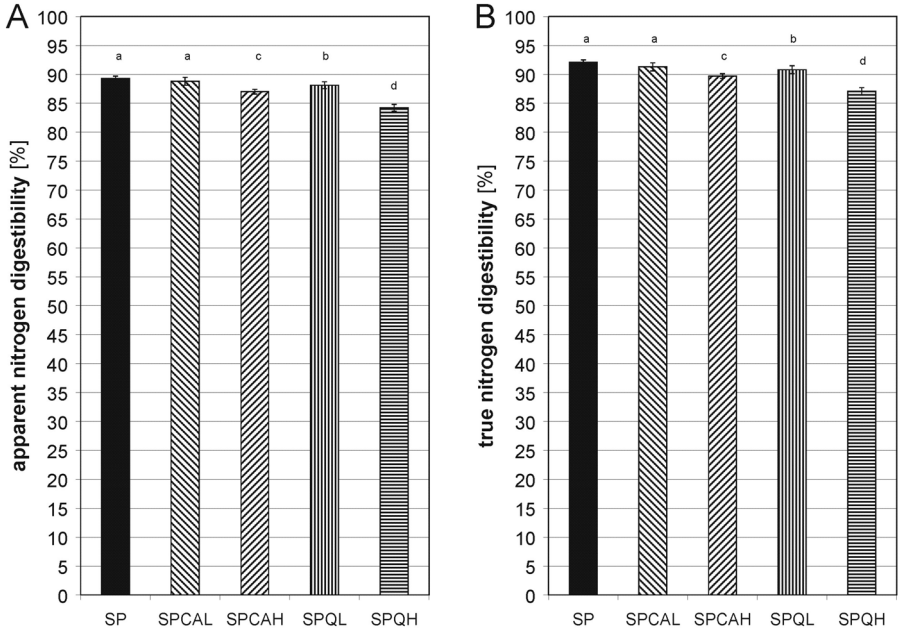

Figure 1. Apparent AND and TND of the phenol-soy protein derivatives. Code: SP = soy protein control, SPCAL = chlorogenic acid-soy protein derivative, low derivatized, SPCAH = CA-soy protein derivative, high derivatized, SPQL = quercetin-soy protein derivative, low derivatized, SPQH = quercetin-soy protein derivative, high derivatized. Means sharing a common superscript letter are not significantly different, $P < 0.5$.

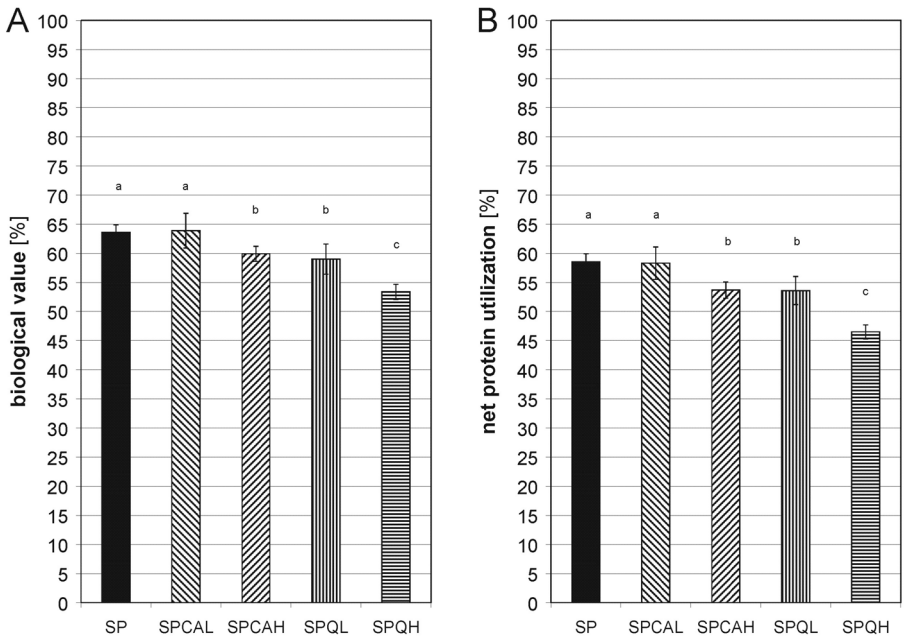

Figure 2. BV and NPU of the phenol-soy protein derivatives. Code: See legend to Fig. 1.

3.3 Amino acid composition of phenol protein derivatives and PDCAAS

Amino acid distribution of the derivatives applied in the rat feeding assay reveals that the amino acids, especially, lysine and tryptophan as well as the sulfur-containing amino acids (cystein, methionine) are markedly influenced. The content of these amino acids decreases depending on the level of derivatization (Fig. 3). In comparison to the control, the lysine content of the high derivatized quercetin derivative was 40 mg/g protein, reflecting a decrease of 35%. The maximum decrease for the tryptophan is again observed in SPQH with nearly 14%. As a consequence, the PDCAAS values are lowered for lysine, tryptophan and the sulfur-containing amino acids with increasing degree of derivatization. With regard to the requirement pattern for humans from 1 year onward [18], the values for tryptophan clearly remain above 100%, the PDCAAS values of lysine drop below this 100% limit for both groups fed high derivatized protein (SPCAH and SPQH) and for the group receiving the SPQL (Fig. 3). The limiting amino acid(s) in soy protein are naturally the sulfur-containing ones. The amino acid distribution of the soy protein control confirms this fact (Fig. 3). It only has 24 mg/g protein of sulfur-containing amino acids which results respectively in a PDCAAS value of only 89%. As the derivatization is also leading to a decrease in sulfur-containing amino acids, the PDCAAS drops to a value of 21 mg/g protein (78%) for SPQH.

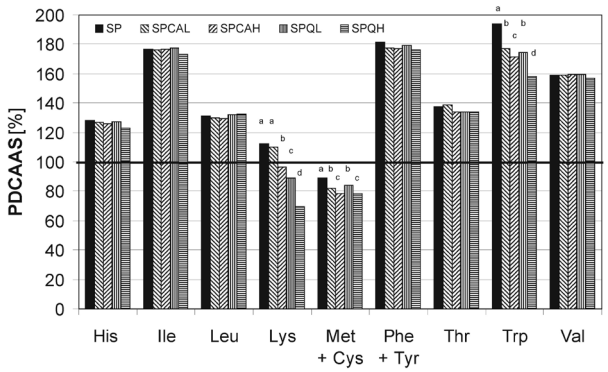

Figure 3. PDCAAS of the phenol-soy protein derivatives. Code: See legend to Fig. 1.

4 Discussion

The aim of this study was to investigate the effects of a reaction of plant phenolic compounds with food proteins. To compare the results with a recent animal experiment [12], CA was again included in this study as a reaction partner. A second reason was that soy proteins are structurally similar to sunflower proteins and soy does not contain CA. This combination may therefore highlight the interactions found by Sabir *et al.* [7] and Sastry *et al.* [8]. The ratio in sunflower seeds between CA and protein fraction (sunflower: 11S protein fraction 15–30% [8]; CA up to 1% [23]) is comparable to the conditions chosen for this study.

Further, quercetin, one of the most abundant flavonoids, was applied in the present study, due to its comparatively high reactivity against proteins [13]. Surely, one thing which raises questions is the use of quercetin aglycone, when it is very well known that quercetin mostly occurs as a glycoside in plant foods. The glycosidic form reacts similarly as compared to its aglycone form as reported in [6]. With regard to the costs, the application of the glycosidc form in a feeding assay is very expensive. Further, it is known that during food processing the aglycone is set free to some extent (Buchner, N., Krumbein, A., Rohn, S., Kroh, L. W.; submitted). The use of the aglycone in combination with a protein or an enzyme in supplementary food products is also frequently observed in practice [11].

The SP was derivatized with the two most common plant phenolic compounds (CA and quercetin). The *in vivo* protein quality of the resulting derivatives was tested on the basis of a rat bioassay. Since a previous feeding assay applying whey protein-CA derivatives illustrated a change in the protein quality parameters only at a comparatively high derivatization level [12], it was expected that a lower quality protein of plant origin would give more clear effects. Due to the high reaction potential of soy proteins against phenolic compounds [13, 24], SP was chosen to prove this assumption.

The results confirm that the effects of such a derivatization on the digestibility of the soy proteins are strongly pronounced. The NPU was adversely affected even for a reaction with a low amount of quercetin (SPQL), resulting in a significantly lower BV for SPCAH, SPQL, and SPQH compared to the unmodified control. This is further reflected in a declined retention of the absorbed nitrogen, resulting in a higher urinary nitrogen excretion. Such an effect may be explained on the basis of the preferential binding of phenolic compounds of nitrogen-containing indispensable amino acids. Former studies document that the ε-amino group of lysine and the indole ring of tryptophan are capable of undergoing covalent reactions because of their nucleophilic character (reviewed in [24]). As a consequence, the contents of lysine and tryptophan decreased dramatically following derivatization. Beside covalent reaction products, a noncovalent binding cannot be excluded [25], but the latter may be partly reversed during digestion and/or metabolism [26].

In vitro degradation of the same protein derivatives with the main proteolytic gastronintestinal enzymes (results not shown) underlined the higher fecal nitrogen excretion. Comparable observations on the basis of *in vitro* experiments were made using whey proteins and myoglobin deri-

vatized with selected phenolic and related compounds [24]. In general, protein digestibility depends on the changes inflicted on protein structure [13] and therefore may explain the observed digestion behavior. As mentioned above, the attack of the phenolic compounds occurs at the nucleophilic side chains of proteins such as lysine, tryptophan, and cysteine, depending on the concentration of the reactants [24]. So the increase in fecal nitrogen excretion after feeding the phenol-soy protein derivatives is a result of a concentration-dependent covalent binding of the respective phenolic compounds. However, the possibility of a more pronounced excretion of endogenous nitrogen cannot be excluded. Several studies showed that an increased fecal nitrogen excretion in the presence of dietary plant phenolic compounds originates from endogenous sources [27, 28]. In this context, two different effects can be discussed: 1) The phenolic compounds are able to react even further with the endogenous proteins after their release from the consumed phenol-protein derivatives during digestion and/or metabolism; 2) The phenolic compounds may affect the mechanisms of the endogenous nitrogen formation.

As soy protein possesses a natural limitation in sulfur-containing amino acids, it does not provide enough of these amino acids with the diet when used as a single source of protein. This fact is even worse after the reaction with the phenolic compounds. But interestingly, the reaction at the ε-amino groups of lysine is so distinct that in the case of the high derivatized SPQH the limiting amino acid is in the end lysine and not anymore the sulfur-containing amino acids.

In conclusion, results of this *in vivo* experiment confirm the results of model experiments (reviewed in [24]). There it was shown that a reaction of phenolic compounds with various food proteins leads to decrease in the amount of free amino and thiol groups as well as tryptophan resulting in a change of the digestion behavior *in vitro* [24]. Compared to a previous feeding assay where CA-whey protein derivatives were applied [12], in the present study more marked effects of a reaction of the phenolic compounds on a protein of plant origin were shown. The comparatively lower protein quality of soy proteins is dropped even further. The value of the amino acid score for the indispensable amino acid lysine decreased below the requirement patterns.

As the indispensable amino acids are often limited in many proteins, a reaction with secondary plant metabolites might be assessed as critical from physiological point of view. This is very important for the regions of the world where nutrition is mainly characterized by an intake of lower quality plant proteins accompanied by the intake of high amounts of secondary plant metabolites resulting from a predominantly plant-food based diet (*e.g.*, sorghum-based diets). Compared to the reactions of glucosinolate breakdown products with proteins leading to adducts, which have been shown to have several negative aspects in underprivileged countries [29 – 31], phenolic compounds may also contribute to such adverse effects. To further evaluate the biological utilization of proteins, especially of low quality proteins in developing countries, a better understanding is needed of the various interrelated parameters that influence their nutritional value. The problem might appear quite easy to solve by increasing the intake of the involved plant protein or mixing it with other plant proteins, which is also an obvious way when a subject consumes vegetarian diet or by supplementing indispensable amino acids. But, in this context the different aspects of the metabolic consequences of these reactions still need further investigations. Recently, *e.g.*, the quercetin adduct formation with the thiol group of glutathione was indicated to be reversible, leading to possibilities of further electrophilic reactions and release of the quercetin quinone methide at different sites [32].

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