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Chlorogenic Acid Moderately Decreases the Quality of Whey Proteins in Rats

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During processing and storage, phenolic compounds (PCs) may react with food protein bound amino acids (AAs). Such reactions have been reported to change physicochemical and to decrease in vitro digestion properties of proteins. A rat growth and nitrogen (N) balance study was conducted to prove whether derivatization with chlorogenic acid (CA) affects the nutritional quality of β-lactoglobulin (β-LG). Test diets (10% protein level) contained nonderivatized β-LG (LG, treated under omission of CA), low derivatization level β-LG (LGL), high derivatization level β-LG (LGH), or casein supplemented with L-methionine (0.3% of diet; C+met) as an internal standard. An additional group received untreated β-LG supplemented with pure CA (1.03% of diet; LG+CA). The AA composition of test proteins, plasma AAs, and liver glutathione (GSH) concentrations were determined. Protein digestibility-corrected amino acid score (PDCAAS) was calculated using human or rat AA requirement patterns and rat fecal digestibility values. N excretion was significantly higher in feces and lower in urine of rats fed with LGH as compared to LG and LGL. Consequently, true N digestibility (TND) was significantly lower with LGH as compared to LG and LGL. The lower content of methionine, cysteine, lysine, and tryptophan in LGH corresponded to a reduced TND. Net protein utilization (NPU) was not different between treated β-LG fed diet groups but was lower than in LG+CA and C+met fed groups. Only at a relatively high level of derivatization with CA, the otherwise good nutritional quality of β-LG is affected so that TND is reduced, while NPU still remains unaffected. Derivatization of β-LG with CA does not seem to lead to an additional deficiency in a specific indispensable AA in growing rats fed with 10% protein.

KEYWORDS: Chlorogenic acid; lactoglobulin isolate derivatization; protein quality; nitrogen balance; rats

INTRODUCTION

Phenolic compounds (PCs) as food components represent, with more than 6000 identified substances, the largest group of secondary metabolites in plant foods (*1*, *2*). The daily intake of these substances varies depending on the dietary behavior. Chlorogenic acid (CA), the ester of caffeic and quinic acid, is one of the most abundant PCs found in plant foods. According to recent estimates, dietary intake of CA in humans ranges from 25 mg up to 1 g/day (*3*, *4*).

Dietary PCs have received much attention in recent years due to their biological activity. They have been attributed with positive properties such as having antiinflammatory, antimutagenic, and anticancerogenic effects as well as acting directly or indirectly as antioxidants contributing to protect the body from free radicals and reactive oxygen and nitrogen (N) species (*5*−*8*). Plant phenols on the other hand can also display detrimental effects, including inhibition of iron absorption and irreversible complexation of gut enzymes and dietary proteins (*4*, *9*), the consequences of which may result in low nutritional quality of polyphenol-rich foods (*10*, *11*).

PCs are reactive substances, capable of undergoing both enzymatic and nonenzymatic oxidation. Oxidation occurs via a formation of quinones, the latter being reactive electrophilic intermediates, which can readily react with nucleophiles such as lysine, methionine, cysteine, and tryptophan residues in a protein chain. The first consequence of these reactions is the derivatization of proteins. Such reactions have been well-characterized in terms of changes in physicochemical properties of selected proteins in model systems (*12*, *13*) as well as in food matrices (*14*). This derivatization produces changes in digestion in vitro (*12*). Reactions of PC also take place with enzymes of the digestive tract in vitro, as shown for α-amylase, trypsin, and α-chymotrypsin (*15*).

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Table 1. Composition of Experimental Diets Used in the Protein Quality Assay Fed to Rats (g/kg Diet)

diet	LG	LGL	LGH	LG+CA	C+met	PF[a]
whey protein,[b] treated but underivatized (control)	111					
whey protein,[b] low derivatization level with CA[c]		114				
whey protein,[b] high derivatization level with CA[c]			121			
whey protein,[b] untreated, underivatized				107		
CA[c]				1.03		
casein[d] (internal control)					114	
L-methionine[e]					3.0	
wheat starch[f]	569	566	559	572	563	680
saccharose[g]	100	100	100	100	100	100
sunflower seed oil[h]	100	100	100	100	100	100
cellulose[i]	50	50	50	50	50	50
mineral mixture[j]	50	50	50	50	50	50
vitamin mixture[k]	20	20	20	20	20	20
energy (MJ/kg)	17.8	17.9	17.9	17.9	18.0	17.1
protein (%)[l]	10	10	10	10	10	0
crude protein content of the purified protein components (%) (N × 6.38)	90.0	87.8	82.7	90.0	85.2	

[a] Protein free diet to determine metabolic and endogenous N losses. [b] β-LG isolate DSE 1591, New Zealand Milk Products GmbH. [c] CA, 97%, Fluka Chemie AG. [d] Bayerische Milchindustrie eG (Landshut, Germany). [e] Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Supplementation was calculated based on methionine + cysteine requirements of growing rats [6.5 g/100 g protein (*25*) minus content in casein (3.5 g methionine + cysteine/100g (*26*)]. [f] Kröner GmbH (Ibbenbüren, Germany). [g] Nordzucker GmbH (Uelzen, Germany). [h] Thomy GmbH (Karlsruhe, Germany). [i] Rettenmaier und Soehne GmbH (Rosenberg, Germany). [j] Mineral mixture, mineral content per 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; and Co, 0.13 mg (Altromin GmbH). [k] Vitamin mixture, vitamin content per kg diet: A, 15000 IU; D3, 500 IU; K3, 10 mg; B1, 20 mg; B2, 20 mg; B6, 15 mg; B12, 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; and C, 20 mg (C 1000 without DL-methionine, Altromin GmbH). [l] The protein quantity used for experimental diets (target protein content of 10%) was calculated based on crude protein content (N × 6.38) of purified protein components.

Moreover, it has been shown in vivo for several animal species fed with proteins of sources relatively rich in PC (e.g., sorghum, faba beans) that the protein quality can be impaired. Different varieties of sorghum, for example, with varying concentrations of polyphenols (tannins), were found to slow the growth of weanling rats in proportion to the amount of PC contained (*16*). Furthermore, it was shown with casein diets supplemented with tannins from different sources that the fecal N (FN) excretion was increased (*17*). An enhanced fecal excretion of both endogenous as well as of dietary protein sources correlated with an increased amount of condensed tannins present in food containing faba bean hulls in a study with young pigs (*18*). The fact that the increased FN in the presence of dietary PC can originate from endogenous sources was concluded from a rat study using ^{15}N-labeled glycine (*19*). N digestibility, egg weight, and laying rate correlated negatively with the intake of PC of horse bean in poultry (*20*). In general, it was concluded that the reduced nutritional value can be a result of interactions of polyphenols with dietary and endogenous proteins. However, such interactions have not yet been emphasized enough for their role played in reducing the quality of proteins such as altering digestibility, organoleptic properties, prolonging or shortening storage life and stability, and adversely affecting the functional properties (*21*).

Therefore, in this study reported here, we have determined the consequences of covalently bound CA to proteins on the biological value (BV) in vivo in rats. Commercial whey protein isolate was chosen as the test protein because it is of high nutritional value, showed a good reactivity against PC (*14*), and is widely used in human nutrition. Because CA is one of the most abundant PCs found in plant foods (*3*), it was applied as the reaction partner.

MATERIALS AND METHODS

Derivatization of Whey Proteins. β-Lactoglobulin (β-LG) isolate DSE 1591 (New Zealand Milk Products GmbH, Rellingen, Germany) was derivatized using different amounts of CA (Fluka Chemie AG, Buchs, Switzerland). CA was added on the basis of an assumed average dietary intake of PCs in humans of 100 mg/day, which is equivalent to 0.28 mmol of CA (*3*). Therefore, derivatives of β-LG isolate were prepared in the presence of 0.28 mmol of CA/g protein (high derivatization level) and also with 0.056 mmol CA/g protein (low derivatization level). Nonderivatized β-LG isolate (control) was prepared under the same conditions but without the addition of CA. Details of derivatization conditions are given in the Supporting Information. The characterization and analysis of physicochemical protein properties of the derivatization products were performed as described in Rawel et al. (*10*). The results of these preliminary investigations giving insight on the nature of the derivatized β-LG are summarized in the Supporting Information.

Animals, Diets, and Feeding Experiment. The protein quality assay was performed according to the UNU/WHO guidelines (*22*). The experimental protocol has been evaluated and approved by the Ethical Committee on 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. The housing of rats was essentially as described (*23*).

Male weanling Wistar rats (Schoe-Wist-Schoe, Tierzucht Schönwalde GmbH, Schönwalde, Germany) weighing 50−60 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, so that the mean group weights differed by no more than ±0.8 g. 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 the 10% crude protein level (**Table 1**).

Underivatized but treated (without addition of CA) β-LG isolate was included in group I (LG). Test diets for groups II (LGL) and III (LGH) contained derivatized β-LG at a low or high derivatization level, respectively. Underivatized and untreated β-LG was included in group IV (LG+CA). In addition, group IV was supplemented with pure CA (hemihydrate, Fluka) to separate a possible direct effect of CA on the protein quality assay. Group V (C+met) was maintained on a casein diet, fortified with L-methionine (0.3 g/100 g diet), which was used as an internal standard. Metabolic and endogenous N losses per gram dry matter intake were determined in a group fed a protein free diet VI (PF). Each rat receives 11 g of dry matter daily throughout the preliminary and balance periods. Dry food was mixed with water (1:

Table 2. Body Mass, Food Intake, and FE in Rats Fed Diets Containing CA-Treated Lactoglubulin and Casein Fed Controls[a−c]

diet	LG	LGL	LGH	LG+CA	C+met
body mass, initial (g)	79.4 ± 4.8	78.4 ± 3.9	78.0 ± 4.2	77.6 ± 4.1	76.0 ± 3.8
BMG (g)	26.3 ± 1.7a	26.2 ± 1.2a	27.0 ± 2.6a	27.4 ± 2.9a	30.9 ± 2.0b
food intake (g)	65.2 ± 0.2b	64.9 ± 0.2b	64.8 ± 0.1b	65.0 ± 0.1b	63.9 ± 0.2a
FE (g body mass gain/g food intake)	0.404 ± 0.26a	0.404 ± 0.019a	0.417 ±0.038a	0.422 ± 0.045a	0.484 ± 0.032b
NE (g body mass gain/g NI)	26.5 ± 1.7	26.3 ± 1.3	26.8 ± 2.4	26.8 ± 2.9	28.0 ± 1.8

[a] Values are means ± SD, $n = 8$. Means within a row not sharing a common superscript letter are significantly different, $P < 0.05$. [b] N balance collection period days 6–11. [c] For food composition, see **Table 1**.

0.5, wt/wt) except for the C+met diet (1:0.75, wt/wt) because of consistency and presented in pots.

After a preliminary feeding period of 5 days, subsequently, diets were provided during a N balance period of 6 days. Urine and feces were collected in 1.4 mol/L hydrochloric acid. Fecal samples were dried and ground. The body weight was recorded at the end of the preliminary and balance periods, after access to food and water was refused for 3 h before weighing procedures. The food intake (FI) was monitored daily, and any remaining food was taken into account for the calculations. Rats were sedated and killed after the balance period (1−2 h) to obtain liver and plasma samples (*23*).

Calculation of Results. Food (FE) and N efficiency (NE) were calculated according to equations:

$$FE = BMG/FI$$

$$NI = BMG/NI$$

where BMG is the body mass gain and NI is the N intake in grams. Apparent N digestibility (AND), true N digestibility (TND), BV, and net protein utilization (NPU) were expressed on a percentage scale and calculated using the following equations (*22*):

$$AND = (NI - FN)/NI$$

$$TND = [NI - (FN - EFN)]/NI$$

$$BV = [NI - (FN - EFN) - (UN - EUN)]/[NI - (FN - EFN)]$$

$$NPU = BV \times TND$$

where FN is the fecal N and UN is the urinary N. The estimation of the endogenous fecal N (EFN) and endogenous urinary N (EUN) was performed using values of the PF group during the N balance collection period [EFN = 0.71 ± 0.07 mg/g dry food intake (DFI); EUN = 1.37 ± 0.29 mg/g DFI]. Two sets of protein digestibility-corrected amino acid scoring patterns (PDCAAS) were calculated (expressed on a percentage scale) based on average AA requirements for children from 1 year onward and all older age groups ($PDCAAS_{human}$) (*24*) and on AA requirements for laboratory rats ($PDCAAS_{rat}$) (*25*), respectively, and on the fecal protein digestibility value that was determined in this study. The following equation was used

$$PDCAAS_{human\ or\ rat}\ (\%) = (AAC \times TD)/AAP_{human\ or\ rat}$$

where AAC is the AA content in food protein (mg/g crude protein) and AAP is the AA content (mg/g crude protein) in respective requirement pattern for man or for laboratory rats, respectively. The calculated PDCAAS values were not cut at the 100% level to demonstrate surplus of individual indispensable AAs.

Analytical Procedures. The AA composition of protein samples was determined based on the recommendations made in the Report of the Joint FAO/WHO Expert Consultation (*26*). Proteins were hydrolyzed with 6 mol/L hydrochloric acid (5 mg crude protein/75 mL of hydrochloric acid) in 24 h at 110 °C under reflux and by a continuous flow of N. After they were dried (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 to acid-stable derivatives (cysteinic acid and methionine-sulfone, respectively) by performic acid oxidation as described elsewhere (*27*). Thirty milligrams 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 GmbH, 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. (*28*). Proteins were hydrolyzed using 4.3 mol/L NaOH (20 mg crude protein/10 mL NaOH) and addition of 50 mg of wheat starch to reduce tryptophan degradation (*29*) in Teflon containers, which were flushed with N_2 and placed in an oven (T 6030, Heraeus Instruments, Hanau, Germany), and maintained at 110 °C for 20 h. 5-Methyltryptophan was used as an internal standard. After they were cooled to room temperature, the 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.

AA concentrations in hydrolysates and plasma were determined by ion exchange chromatography (high efficiency column, 3 mm × 150 mm, Pickering Laboratories Inc., Mountain View, CA) with a step-change elution method using lithium citrate buffers (Onken Laborservice GmbH) and postcolumn ninhydrin detection (TRIONE Ninhydrin Reagent, Pickering Laboratories Inc.) and high-performance liquid chromatography units (System Gold, Beckman Instruments GmbH, Munich, Germany) as reported in ref *23*. AAs were detected at a wavelength of 540 nm. For calculation of the AA concentration, norleucine was used as an internal standard added prior to hydrolysis. The reduced glutathione (GSH) concentration in liver was determined as described elsewhere (*23*).

N contents in feces (dried at 110 °C until weight constancy) and urine were measured by a Kjeldahl method (Kjeldatherm-Turbosog-Vapodest 45, C. Gerhardt GmbH & Co. KG, Bonn, Germany), and the gross energy content of diets was determined by means of an adiabatic bomb calorimeter (IKA-Calorimeter C 5000, IKA-Werke GmbH & Co. KG, Staufen, Germany). The crude protein content was calculated by using the factors 6.38 for milk protein-based diets.

All determinations were duplicated with chemicals purchased from several suppliers (Fluka Chemie AG; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany; Merck KGaA, Darmstadt, Germany) and of analytical grade.

Statistical Analysis. Data are reported as means ± standard deviation (SD). Differences between mean values were determined by analysis of variance 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.

RESULTS

N Balance. The food intake, body weight of rats, and FE of the diets are shown in **Table 2**. A rise in the degree of derivatization of β-LG with CA is accompanied by a significantly higher FN excretion (**Table 3**). The mean value of FN

Table 3. Protein Quality Evaluation by N Balance in Rats Fed Diets Containing CA-Treated Lactoglobulin and Casein Fed Controls[a−c]

diet	LG	LGL[d]	LGH	LG+CA	C+met
NI (g)	993 ± 2a	998 ± 3a	1009 ± 10b	1023 ± 2c	1103 ± 4d
FN (mg)	70 ± 6a	76 ± 6b	109 ± 2c	66 ± 3a	79 ± 5b
UN (mg)	166 ± 23cd	163 ± 26c	130 ± 20b	142 ± 18bd	94 ± 11a
FN/UN	0.43 ± 0.07a	0.48 ± 0.12a	0.86 ± 0.14b	0.47 ± 0.07a	0.85 ± 0.12b
N balance (mg)	757 ± 25a	759 ± 21a	770 ± 23a	815 ± 19b	930 ± 13c
apparent digestibility (%)	93.0 ± 0.6c	92.4 ± 0.6b	89.2 ± 0.2a	93.5 ± 0.3d	92.8 ± 0.4bc
true digestibility[d] (%)	95.5 ± 0.6c	94.9 ± 0.6b	91.7 ± 0.2a	96.0 ± 0.3c	95.1 ± 0.4b
BV (%)	87.5 ± 2.4a	87.8 ± 2.7a	91.1 ± 2.2b	90.4 ± 1.9b	95.6 ± 1.1c
NPU (%)	83.6 ± 2.5a	83.4 ± 2.3a	83.5 ± 2.0a	86.7 ± 1.8b	90.9 ± 1.1c

[a] Values are means ± SD, $n = 8$. Means within a row not sharing a common superscript letter are significantly different, $P < 0.05$. [b] N balance collection period days 6–11. [c] For food composition, see **Table 1**. [d] On the basis of a group fed with protein free diet ($n = 8$).

excretion in the balance period is 6 or 39 mg higher in the groups fed with, respectively, LGL or with LGH than in the LG fed control group.

At the same time, the UN excretion is lowered by 36 mg during the balance period in the LGH group but not significantly lowered in the LGL group as compared to LG controls. The ratio between fecal to UN is twice as high in the LGH group as compared to LG controls (and LGL, LG+CA) but equal to C+met controls. It should be noted that this higher ratio results either from a relatively higher FN excretion in LGH or from a lower UN excretion in C+met groups, respectively. The UN excretion is generally higher (30−60 mg during balance period) in all β-LG diet fed groups as compared to C+met fed controls.

The apparent and TND of β-LG decreased with increasing derivatization degree as compared to the underivatized β-LG and was about 4% lower for diet LGH than for diet LG. As a consequence, BV was significantly higher for diet LGH as compared to diet LG. The NPU did not differ for diets LGL and LGH as compared to LG but was significantly lower than diets LG+CA and C+met control. Additionally, rats fed with diet LG+CA showed a significantly lower UN excretion and a higher N balance as compared to rats fed the LG diet. At the same time, values for TND did not differ between diets LG+CA and LG. Furthermore, BV and NPU were significantly higher by about 3% for diet LG+CA than for diet LG.

AA Composition of Dietary Proteins and PDCAAS. Comparing the AA composition of the differently treated β-LGs reveals that only at a high derivatization level in LGH, a meaningful decrease in lysine, methionine, tryptophan, and cysteine could be detected relative to LG (**Table 4**). On the other hand, the concentrations are higher in untreated and underivatized β-LG fed in the LG+CA group for most of the indispensable AAs except methionine and tryptophan as compared to treated β-LG with or without derivatization. As a consequence, the PDCAAS values are lower with high derivatization level for those AAs based on human AA requirement patterns but clearly remain above 100% for all indispensable AAs (**Table 5**). However, relating the AA composition to growing rats indispensable AA requirement pattern (**Table 6**), lower $PDCAAS_{rat}$ values than 100% were calculated for sulfur containing AAs in addition to arginine and histidine for LGH. The $PDCAAS_{rat}$ values for arginine and histidine in β-LG were only about 50 or 80%, respectively, irrespective of derivatization level, and are based on the low concentrations of these AAs in β-LG. The decrease in lysine and tryptophan concentrations during derivatization was not significant enough to drop the $PDCAAS_{rat}$ values below 100%.

Postabsorptive Plasma Indispensable AA and Liver GSH Concentrations. The postabsorptive indispensable free plasma AA concentrations (**Table 7**) were mostly not influenced significantly ($P > 0.05$) by feeding β-LG derivatized with CA as compared to LG controls. However, the branched chain AA concentrations were significantly higher in the LGL and LGH diet groups as compared to LG controls. Furthermore, the plasma concentrations of histidine, phenylalanine, and branched chain AAs were also significantly higher in the LG+CA group as compared to the LG control group. Comparison of plasma free AA concentrations of rats fed diets containing β-LG (with or

Table 4. AA Composition of Treated or Derivatized Lactoglobulin and of Casein Used in the Protein Quality Assay[a]

	mg/g protein				
dietary protein (for diet)	LG	LGL	LGH	LG+CA	C+met[b]
	indispensable AAs				
histidine	20.9	21.4	21.1	23.3	32.1
isoleucine	60.8	61.1	62.0	70.3	57.0
leucine	147.7	150.7	149.8	169.7	108.8
lysine	115.1	118.4	106.4	133.0	84.4
methionine	22.7	22.9	20.6	23.1	26.6
phenylalanine	39.8	39.5	39.8	44.0	58.1
threonine	51.9	53.8	53.6	61.5	49.1
tryptophan	31.4	32.8	29.8	30.6	14.4
valine	63.7	65.0	65.2	73.3	83.5
	dispensable AAs				
alanine	55.9	56.9	57.8	64.6	32.9
arginine	25.6	26.6	25.9	28.8	39.4
aspartic acid + asparagine	124.7	128.2	127.3	142.0	77.8
cystin (2 × cysteine)	34.6	33.8	28.7	31.8	3.9
glutamic acid + glutamine	183.1	189.0	187.5	210.3	250.5
glycine	19.0	19.2	19.2	21.5	20.4
proline	44.6	47.9	47.6	53.1	121.9
serine	37.2	39.2	38.9	43.1	58.0
tyrosine	40.1	40.8	40.6	45.7	61.9

[a] Values are means of three hydrolyzations. [b] Casein without supplementation with L-methionine.

Table 5. $PDCAAS_{human}$ of Treated or Derivatized Lactoglobulins and of Casein Used in the Protein Quality Assay Based on Human AA Requirement Pattern (*24*)[a]

dietary protein (for diet)	LG	LGL	LGH	LG+CA	C+met[b]
histidine	111	113	109	125	170
isoleucine	233	232	228	270	217
leucine	258	260	251	296	188
lysine	217	220	192	250	157
methionine + cysteine	220	215	182	211	116
phenylalanine + tyrosine	163	162	158	183	243
threonine	184	189	183	219	173
tryptophan	430	445	392	420	195
valine	191	193	188	220	248

[a] For details of lactoglobulin treatment and calculation of PDCAAS values, see the Materials and Methods. [b] Without consideration of supplementation with L-methionine.

Table 6. $PDCAAS_{rat}$ of Treated or Derivatized Lactoglobulins and of Casein Used in the Protein Quality Assay Based on AA Requirement Pattern of Growing Rats (*25*)[a]

dietary protein (for diet)	LG	LGL	LGH	LG+CA	C+met[b]
arginine[c]	49	50	48	55	75
histidine	80	81	78	90	122
isoleucine	139	138	136	161	129
leucine	229	231	223	263	167
lysine	190	194	169	220	138
methionine + cysteine	110	108	91	106	58
phenylalanine + tyrosine	116	116	112	131	173
threonine	119	122	118	141	111
tryptophan	241	249	220	235	110
valine	122	123	120	141	159

[a] For details of lactoglobulin treatment and calculation of PDCAAS values, see the Materials and Methods. [b] Without consideration of supplementation with L-methionine. [c] Arginine was included as an indispensable AA for growing rats (*25*).

without derivatization) and C+met reveals lower values for arginine, histidine, and valine and higher values for lysine, methionine, and threonine in the β-LG fed groups. Plasma threonine concentrations were found to be about four times higher in all β-LG fed groups than in the C+met group.

Interestingly, the liver GSH concentration was significantly ($P < 0.05$) lower in rats fed with LGH diet and tended to be lower ($0.1 < P < 0.05$) in LG+CA groups as compared to LG controls (values were not determined in LGL and C+met) and were 1.63 ± 0.31, 1.85 ± 0.46, and 2.27 ± 0.46 μmol/g, respectively. The liver weight was also significantly lower in LGH group as compared to LG (4.82 ± 0.50, 4.36 ± 0.19, and 4.67 ± 0.28 g for LG, LGH, and LG+CA groups, respectively). Consequently, the mean total liver GSH content was likewise significantly lower by 35% in LGH rats than in LG rats and tended to be lower (by 31%) in the LG+CA group. This rather indicates an influence of CA on liver GSH status independent of including CA in the diet derivatized with a food protein or as a pure supplement.

DISCUSSION

We treated β-LG with CA and tested the effects on protein quality by a rat bioassay. Overall, we expected more clear effects of a reaction with PC on protein quality based on the high reaction potential of β-LG due to its AA composition (*10*, *14*, *30*). However, the effects on digestibility remain either more moderate or appeared only when using a relatively high CA concentration during derivatization. Furthermore, derivatization of β-LG has no significant influence on NPU values. A significantly higher BV for the LGH diet as compared to LG reflects a more efficient retention of the absorbed N resulting in lower UN excretion. This effect can be explained on the basis of preferential binding of CA to dispensable AAs, and if indispensable AA residues were derivatized, then their initial concentration in β-LG is too high to be dropped to a limiting level by the applied derivatization conditions. Furthermore, binding of CA with AA residues cannot be excluded to be partly reversed during digestion and/or metabolism especially if bindings occur noncovalently (*31*). The true protein digestibility of the high level derivatized β-LG (LGH) revealed only about 4% reduction in comparison to underivatized β-LG (LG). However, about 92% of this true digested derivatized β-LG was retained in the organism in contrast to the value for the LG control diet, for which a significantly lower amount of true digested protein was computed to be retained.

A higher FN excretion correlated negatively ($P < 0.000$) with the digestibility of the derivatized proteins. This reflects the results obtained during in vitro degradation of the same protein derivatives with trypsin, chymotrypsin, and pepsin (Supporting Information). Comparable observations on the basis of in vitro experiments have also been reported (*12*, *14*, *30*). In general, protein digestibility is dependent on protein structure (*13*). Furthermore, lysine−quinone complexes formed during protein−CA interactions are not absorbed and cause a drop in lysine availability (*32*). These structural changes depend on the concentration of the reactants (*10*). Therefore, we suggest that the increase in FN excretion by feeding the diets LGL or LGH as compared to diet LG is a result of a concentration-dependent binding of CA to β-LG. However, the possibility of an increased excretion of endogenous N cannot be excluded. Several studies have shown that an increased FN excretion in the presence of dietary polyphenols originates from endogenous sources (*18*, *19*). However, this presupposes that the CA bound to derivatized β-LG would react with endogenous proteins after splitting during digestion in the digestive tract. The results of physicochemical characterization show that some of the covalent bonds formed are liable to be destroyed during hydrolysis (Supporting Information). Further studies are required to estimate the exact contribution of CA on mechanisms of endogenous N excretion when derivatized proteins were fed.

The nutritional value of a protein is related both to its digestibility and to the metabolism of the absorbed AAs (*33*). On the basis of our results, we cannot assess the influence of derivatization on absorption kinetics. However, with higher pH value, the reaction of the PC with proteins is more pronounced (*30*), but alkaline conditions may also lead to significant changes in proteins responsible for changed absorption kinetics and

Table 7. Postabsorptive Indispensable Free AA Concentration in Plasma of Rats Fed Diets Containing Treated or Derivatized Lactoglobulins and of Casein Used in the Protein Quality Assay[a]

diet	LG	LGL[b]	LGH	LG+CA	C+met
arginine[b]	68.1 ± 17.1^{a}	66.8 ± 4.6^{a}	70.1 ± 22.4^{a}	81.0 ± 13.3^{a}	102.2 ± 22.1^{b}
histidine	41.9 ± 8.7^{a}	49.8 ± 11.2^{ab}	41.2 ± 7.5^{a}	53.2 ± 6.7^{b}	98.5 ± 7.4^{c}
isoleucine	31.7 ± 8.1^{a}	45.8 ± 16.4^{b}	46.4 ± 6.8^{b}	$55.3 \pm 11.9b^{c}$	57.1 ± 5.5^{c}
leucine	58.9 ± 14.9^{a}	86.7 ± 30.7^{b}	89.2 ± 13.0^{b}	100.8 ± 22.7^{b}	103.7 ± 9.9^{b}
lysine	629 ± 61^{b}	699 ± 89^{b}	659 ± 54^{b}	699 ± 113^{b}	544 ± 85^{a}
methionine	44.3 ± 9.1^{b}	46.0 ± 6.3^{b}	41.7 ± 2.1^{ab}	49.8 ± 6.9^{b}	36.5 ± 4.9^{a}
phenylalanine	40.6 ± 7.2^{a}	47.1 ± 11.4^{ab}	48.3 ± 5.2^{ab}	52.3 ± 9.2^{b}	50.4 ± 2.6^{ab}
threonine	428 ± 81^{b}	384 ± 44^{b}	415 ± 105^{b}	402 ± 32^{b}	101 ± 39^{a}
tryptophan	32.3 ± 8.3	24.8 ± 5.8	24.8 ± 5.7	24.8 ± 4.4	31.6 ± 12.0
valine	76.0 ± 11.4^{a}	93.8 ± 24.7^{b}	99.0 ± 13.2^{b}	110.2 ± 21.8^{b}	120.4 ± 7.3^{c}

[a] Values are means ± SD, $n = 8$. Within a row, values without a common superscript differ, $P < 0.05$. [b] Arginine was included as an indispensable AA for growing rats (*25*).

render some of the AAs nutritionally unavailable (*34*). However, a significantly higher N balance, BV and NPU for untreated (LG+CA) as compared to treated β-LG (LG), suggests an impaired protein value due to alkali treatment. In contrast to derivatized β-LG (LGL, LGH), the addition of pure CA to a diet does not impair its utilization, which corresponds to observations made earlier (*35*). Although CA is poorly absorbed through the small intestinal barrier, it can be metabolized by the gut microflora providing several metabolites (*2*), which may not only effect the endogenous proteins but are also suggested to potentially affect GSH status. We found a significant decrease of liver GSH concentrations of rats fed with protein bound CA (LGH) as compared to the LG diet. In addition, the liver GSH concentration was of borderline significance lower ($P < 0.1$) when pure CA was fed (LG+CA) as compared to the LG diet. Additional studies are required to examine the influence of microbial metabolites of CA on the physiological status.

In conclusion, the results of the in vivo experiment show that the digestibility of whey proteins is diminished as a consequence of the reaction with CA. Furthermore, it was noted that the observed effects were not so distinct due to the high nutritional quality of whey proteins. This leads us to the conclusion that, for example, a comparatively low quality plant protein is likely to show more distinct effects. To 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. In this connection, the interactions with PC described here are likely to play an important role. The different aspects of the nutritional and physiological consequences of such interactions in food and in the organism, especially considering their significance to food science and technology, need further investigations. In this context, additional animal experiments are planned to evaluate these aspects by applying, e.g., soy protein isolate derivatized with CA and quercetin.

ABBREVIATIONS USED

AAs, amino acids; β-LG, β-lactoglobulin; BV, biological value; CA, chlorogenic acid; C+met, casein supplemented with L-methionine; GSH, reduced glutathione; LG, nonderivatized lactoglobulin, treated under omission of CA; LGH, high level derivatized lactoglobulin; LGL, low level derivatized lactoglobulin; LG+CA, untreated lactoglobulin supplemented with pure CA; N, nitrogen; NPU, net protein utilization; PC, phenolic compounds; PDCAAS, protein digestibility-corrected amino acid score.

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Supporting Information Available: Derivatization of whey proteins, AA analysis of the derivatized samples, estimation of CA bound covalently to β-LG, reaction mechanisms of adduct formation of CA with β-LG, confirmation of the covalent binding of CA, proof of intermolecular cross-linking, and effect of derivatization on some functional properties of β-LG. This material is available free of charge via the Internet at http://pubs.acs.org.

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